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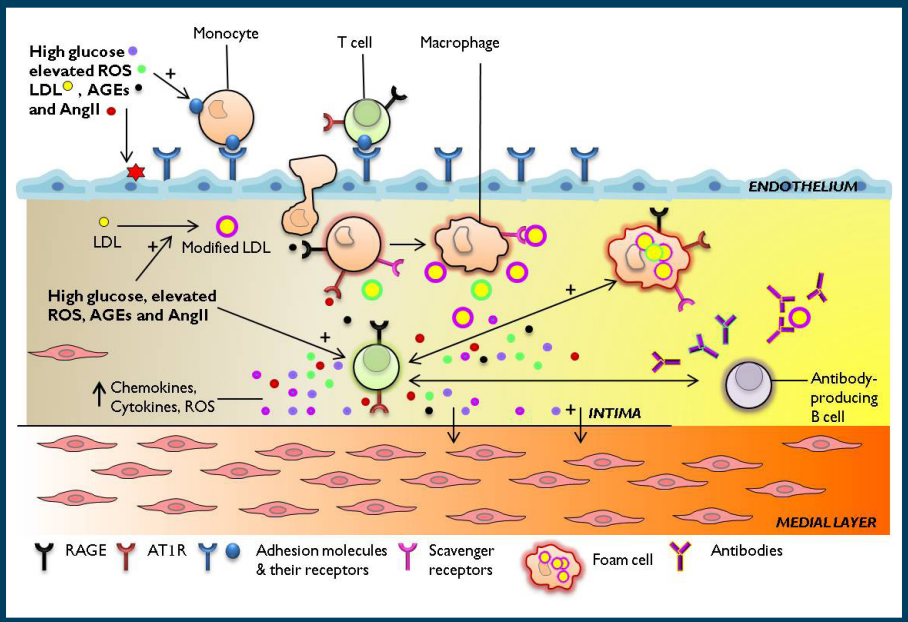

THE ROLE OF THE IMMUNE SYSTEM IN THE PATHOGENESIS OF DIABETIC COMPLICATIONS

Topic Editors
Gabriel Virella and Maria Lopes-Virella

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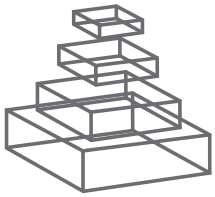

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THE ROLE OF THE IMMUNE SYSTEM IN THE PATHOGENESIS OF DIABETIC COMPLICATIONS

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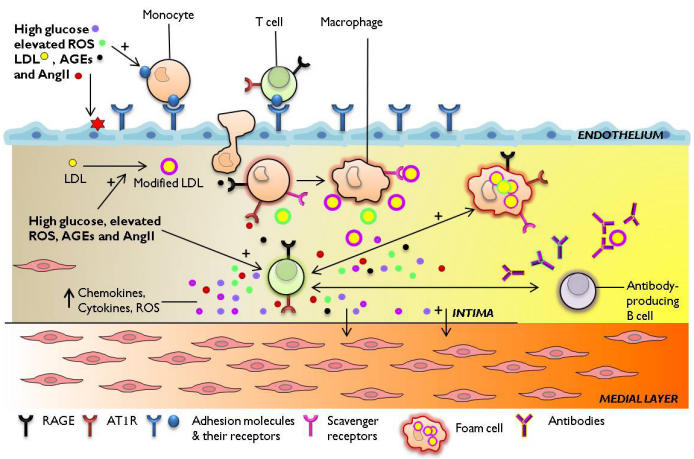

Immune mechanisms engaged in diabetes- accelerated atherosclerosis. Diabetes- associated hyperglycaemia, hyperlipidaemia and oxidative stress render the endothelium dysfunctional, leading to the retention and oxidation of LDL molecules in the intimal space. The increased expression of adhesion molecules E-selectin, ICAM-1, VCAM-1 at the endothelial membrane and upregulation of chemotactic molecules such as MCP-1 facilitate the continuous infiltration of immune cells to the inflamed aorta. Resident and monocyte-derived macrophages engulf LDL to form foam cells which release a host of pro-inflammatory cytokines, protease and ROS. Activated T cells recruited from the circulation to the lesion also secrete cytokines which amplify pro-inflammatory cellular immune responses in the diabetic plaque. In addition, B cells activated in the surrounding lymphoid tissue produce antibodies (and auto-antibodies) against a number of plaque-derived antigens including glycated and oxidised LDL. The diabetes- mediated increase in vascular inflammation drives the development and progression of atherosclerosis. AGEs, advanced glycation end- products; AT1R, angiotensin II type 1 receptor; ICAM-1, intercellular adhesion molecule-1; LDL, low density lipoprotein; MCP-1, monocyte chemotactic protein-1; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule-1

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The main causes of morbidity and mortality in diabetes are macrovasular and microvascular complications, including atherosclerosis, nephropathy, and retinopathy. As the definition of atherosclerosis as a chronic, smoldering, inflammatory disease has gained general acceptance, the attention of researchers has focused on the triggers of chronic vascular inflammation. The oxidation and other forms of modification of lipids and lipoproteins have emerged as a major pathogenic factor in atherosclerosis, with a significant interaction with the immune system. Modified lipoproteins by themselves are proinflammatory through the activation of the innate immune system as a consequence of the interaction with scavenger receptors and/or toll-like receptors expressed by a variety of cell types, including phagocytic cells and dendritic cells. A variety of modified forms of LDL (mLDL), including oxidized, malondialdehyde-modified, and Advanced Glycation End-product-modified LDL induce autoimmune responses in humans. Those modifications seem enhanced in diabetes, and the progression of atherosclerosis is accelerated in diabetic patients. The immune response to all forms of mLDL results in both activation of T cells in the arterial wall and in an autoimmune response characterized by the formation of IgG antibodies. Both arms of the immune response are believed to play a role in vascular inflammation. While the cell response is likely to activate resident macrophages, the humoral immune response results in the production of IgG antibodies that bind to specific epitopes in modified forms of LDL, generate immune complexes both intra- and extravascularly, and those complexes are able to activate the classical pathway of the complement system as well as phagocytic cells via Fcγ receptors. In vitro studies suggest that the pro-inflammatory activity of immune complexes containing mLDL is several-fold higher than that of the modified LDL molecules by themselves. Clinical studies have provided significant support to the pathogenic role of immune complexes containing modified LDL in the development of atherosclerotic complications in patients with both type 1 and type 2 diabetes. At the same time, there is increasing evidence that the formation of immune complexes containing modified forms of LDL may also be involved in the pathogenesis of diabetic nephropathy and retinopathy. These are areas in which more research is needed to fully understand the pathogenic mechanisms activated by those immune complexes. Of interest is the fact that animal models have suggested the possibility of modifying the adaptive humoral immune response in ways that would result in slowing down, and perhaps prevent, the atherosclerotic process. This possibility is sufficiently alluring as to justify increased research efforts, both in animal models (including diabetic animals) and translational clinical studies. The manipulation of the T regulatory population is another area of potential translational impact, which has hardly been explored. Indeed at this point of time, what seems to be a high priority is an increased and open interchange of information among investigators, trying to reach a better general understanding and integration of knowledge generated from a variety of approaches and perspectives. This Research Topic provided an optimal platform for this open interchange of information. We encouraged interested scientists to submit mini-reviews, methods papers, review articles, perspectives and original research articles covering this topic in all its diversity to facilitate the communication of perspectives and new information between scientists interested in understanding the multiple implications of the involvement of the immune system in the pathogenesis of diabetic complications.

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The role of the immune system in the pathogenesis of diabetic complications

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The main causes of morbidity and mortality in diabetes are macrovascular and microvascular complications. The pathogenesis of these complications is multifactorial, but there is strong evidence implicating chronic, smoldering, and inflammation as a main pathogenic event in the development of diabetic complications (1). Although the mechanisms responsible for vascular inflammation in diabetes are similar to those involved in vascular disease in non-diabetics (2), chronic hyperglycemia and dysregulated immune responses in diabetes are responsible for the activation of inflammatory circuits, inducing oxidative stress and promoting insulin resistance (1, 3), thus creating conditions that lead to the development of diabetes and diabetic complications. Changes in gene expression associated with diabetes like increased ICAM-1 expression (4) may also play a role in inducing inflammation and the development of diabetic complications.

Increased expression of adhesion molecules is associated with the development of diabetic complications. As discussed by Gu et al. (5), over-expression of ICAM-1, may be one of the key events in the development of nephropathy, as reflected by significant correlations between ICAM-1 levels and the development of proteinuria. Although the increased expression of ICAM-1 is not exclusive of diabetes, a linkage of the ICAM-1 gene to diabetes and diabetic nephropathy (5) suggests a selective involvement of this particular inflammatory event in both type 1 and type 2 diabetes. Clinically, ICAM-1 and other adhesion molecules like E-selectin can predict development of diabetic nephropathy and perhaps other complications in type 1 diabetes (5). Therapeutically, experimental data suggest that inhibiting ICAM-1 gene expression may prevent or slow down the development of diabetic nephropathy (5). As uncontrolled chronic inflammation progresses, kidney fibrosis develops, leading to end-stage kidney disease. Recruitment and activation of macrophages and of $CD4^+$ T cells after initial tissue injury precede and have a critical role in the development of fibrosis, thus linking inflammation to the development of renal fibrosis (6). Several drugs used in the treatment of renal insufficiency have anti-inflammatory properties. However, the use of anti-inflammatory agents has not been effective in the treatment of diabetic nephropathy (6), likely because, once the fibrotic process becomes irreversible, the value of such interventions is limited.

Oxidation and other forms of modification of lipids and lipoproteins have emerged as a major pathogenic factor in atherosclerosis. Modified lipoproteins deliver pro-inflammatory signals that activate innate and adaptive immune responses and disturb the integrity of the microvasculature (3). In diabetes, the combination of hyperglycemia and increased oxidative stress results in enhanced LDL modification. Advanced glycation end-products (AGE)-modified LDL plays an important pathogenic role through its interactions with RAGE and angiotensin receptors (3). Oxidized LDL activates T cells, leading to enhanced inflammation through the release of macrophage-activating mediators (2). The adaptive humoral autoimmune response to modified forms of LDL is well characterized and strong evidence exists linking the formation of immune complexes (IC) involving modified forms of LDL and the corresponding autoantibodies with the development of diabetic complications. High levels of oxidized LDL in IC strongly predict the progression of atherosclerosis in patients with type 1 diabetes, while high levels of malondialdehyde-modified LDL in IC indicate strong risk for acute cardiovascular events in patients with type 2 diabetes (7).

Modified LDL molecules express a variety of immunogenic epitopes. The most immunogenic and better characterized are modified lysine epitopes, but oxidized phospholipids are also exposed to the immune system (2). Phosphorylcholine is a particularly interesting epitope because the resulting antibodies appear to protect against the development of atherosclerosis (2). Because phospholipid autoantibodies are usually of the IgM isotype, their protective effect could be a result of the reduced inflammatory potential of IgM IC, which cannot activate phagocytic cells through Fc receptors. Therefore, the end result of the humoral immune response to modified LDL could depend on which antibodies predominate: the strongly pro-inflammatory IgG antibodies or the non-inflammatory IgM antibodies.

There is a large diversity of autoantibodies, besides those directed to modified LDL and phospholipids that are believed to play a pathogenic role in diabetes. To that long list, Zimmering et al. added anti-neurotrophic antibodies, which seem to be involved in the pathogenesis of open-angle glaucoma and/or dementia in adult diabetics (4).

There is great interest in defining biomarkers predictive of diabetic complications. Among the many biomarkers that have been proposed, the blood levels of ICAM-1 (5), the levels of modified LDL in circulating IC (7), and the levels of fibroblast growth factor (8) are discussed in this e-book. While ICAM-1 levels and its polymorphisms appear linked to the development of nephropathy, the levels of modified LDL in IC and the levels of fibroblast growth factor have predictive value for cardiovascular

disease in type 1 and type 2 diabetes. The results are quite strong since they were based on data obtained on a large number of patients.

As evidence supporting the pathogenic role of the adaptive immune response in diabetes accumulates, there has been a surge in the investigation of down-regulatory mechanisms that could be therapeutically exploited. The role of T regulatory (Treg) cells has been the object of considerable attention. Data generated in animal models suggest that Treg cells play a critical role in controlling the development of diabetes, both in type 1 and type 2 models (9). Also in animal models, there is data suggesting that administration of CD3 monoclonal antibodies permanently reverses diabetes in NOD mice. In humans, the results have not been so spectacular, but in type 1 diabetic patients CD3 antibody administration seems to preserve islet cell function for 1–5 years (9). Although human trials have never replicated the animal model data, the data remain very appealing.

A second alternative, also suggested by data in animal models, is to enhance the regulatory effect on incretin hormones, particularly glucagon-like peptide-1 (GLP-1) whose levels or effects can be enhanced by the administration of GLP-1 receptor agonists, or by inhibitors of dipeptidyl peptidase (DDP)-4, and enzyme that degrades GLP-1 (10). Data obtained in mice suggest that GLP-1 has modulatory effects, promoting the survival of Treg cells, and treatment with incretins suppresses the progression of atherosclerosis (10). Whether the administration of GLP-1R agonists or DDP-4 inhibitors may have similar effects in humans is a very interesting concept and worth investigating.

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The pathogenic role of the adaptive immune response to modified LDL in diabetes

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The main causes of morbidity and mortality in diabetes are macro and microvascular complications, including atherosclerosis, nephropathy, and retinopathy. As the definition of atherosclerosis as a chronic inflammatory disease became widely accepted, it became important to define the triggers of vascular inflammation. Oxidative and other modifications of lipids and lipoproteins emerged as major pathogenic factors in atherosclerosis. Modified forms of LDL (mLDL) are pro-inflammatory by themselves, but, in addition, mLDLs including oxidized, malondialdehyde (MDA)-modified, and advanced glycation end (AGE)-product-modified LDL induce autoimmune responses in humans. The autoimmune response involves T cells in the arterial wall and synthesis of IgG antibodies. The IgG auto-antibodies that react with mLDLs generate immune complexes (IC) both intra and extravascularly, and those IC activate the complement system as well as phagocytic cells via the ligation of Fcγ receptors. *In vitro* studies proved that the pro-inflammatory activity of IC containing mLDL (mLDL-IC) is several-fold higher than that of the modified LDL molecules. Clinical studies support the pathogenic role of mLDL-IC in the development of macrovascular disease patients with diabetes. In type 1 diabetes, high levels of oxidized and AGE-LDL in IC were associated with internal carotid intima-media thickening and coronary calcification. In type 2 diabetes, high levels of MDA-LDL in IC predicted the occurrence of myocardial infarction. There is also evidence that mLDL-IC are involved in the pathogenesis of diabetic nephropathy and retinopathy. The pathogenic role of mLDL-IC is not unique to diabetic patients, because those IC are also detected in non-diabetic individuals. But mLDL-IC are likely to reach higher concentrations and have a more prominent pathogenic role in diabetes due to increased antigenic load secondary to high oxidative stress and to enhanced autoimmune responses in type 1 diabetes.

Keywords: LDL modification in diabetes, immune complexes, autoimmune response to modified LDL, diabetic complications, oxidized LDL, oxidized LDL antibodies, atherosclerosis, nephropathy

INTRODUCTION: LDL MODIFICATION

Oxidative stress is believed to be a critical factor in the initiation of pathogenic pathways that lead to the development of complications in diabetes (Giacco and Brownlee, 2010). Hyperglycemia plays a key role by inducing mitochondrial overproduction of reactive oxygen species (ROS), which, in turn, will cause oxidative modification of proteins, enzymes, and other substrates, including the formation of advanced glycation end (AGE) products (Giacco and Brownlee, 2010; Miller et al., 2010).

Lipoproteins are among the proteins that are modified as a consequence of oxidation and glycation. Endothelial cells (EC), monocytes/macrophages, lymphocytes, and smooth muscle cells (SMC) are all able to enhance the rate of oxidation of LDL. ROS and sulfur-centered radicals initiate metal ion-dependent lipid peroxidation leading to the generation of aldehydes that interact with lysine residues in ApoB-100, resulting in oxidation of LDL. Alternatively, endothelial injury secondary to oxidative stress results in increased prostaglandin synthesis and platelet activation. These processes also cause the formation of aldehydes such as malondialdehyde (MDA) that interact with the lysine residues of ApoB-100 (Holvoet, 1999). *In vitro*, MDA-lysine (as well as carboxymethyl lysine, carboxyethyl lysine, and other unidentified modifications) are generated by copper oxidation of LDL. Direct treatment of LDL with MDA, on the other hand, results in the formation of highly modified LDL with a 10-fold excess of MDA-lysine over copper-oxidized LDL (oxLDL) and no other detectable modifications (Virella et al., 2004).

PATHOGENIC ROLE OF MODIFIED LDL

The pathogenic role of modified lipoproteins in the progression of atherosclerosis is well established. It has been investigated from two different angles: the direct pro-atherogenic effect of modified forms of LDL (mLDL; Lopes-Virella and Virella, 2003; Miller et al., 2010) and the consequences of the immune response directed against neoepitopes resulting from lipoprotein modification (Lopes-Virella and Virella, 2010). Both types of effects have been extensively characterized in the case of oxLDL. OxLDL is taken up by macrophages via receptor-mediated pathways other than the classic LDL receptor (Henriksen et al., 1983; Arai et al., 1989; Sparrow et al., 1989; Endemann et al., 1993; Penn and Chisolm,

1994) and it induces accumulation of cholesteryl esters and the transformation of macrophages into foam cells (Fogelman et al., 1980; Hoff et al., 1989). It has also been reported that high concentrations of oxLDL are cytotoxic and experimental data suggests that oxLDL can injure vascular cells, both endothelial and SMC (Henriksen et al., 1979; Hessler et al., 1983). Furthermore, oxLDL induces enhanced synthesis of growth factors, including PDGF-AA, and PDGF receptor in SMC, as well as of granulocyte-monocyte colony stimulating factor, macrophage colony stimulating factor, and granulocyte-colony stimulating factor in aortic EC from humans and rabbits (Rajavashisth et al., 1990). In addition, oxLDL may affect fibrinolysis, by inhibiting the secretion of tissue plasminogen activator (tPA) by human EC (Kugiyama et al., 1993) and stimulating the secretion of plasminogen activator inhibitor (PAI)-1 (Kugiyama et al., 1993). Thus, oxLDL inhibits the endothelium-dependent activation of fibrinolysis, possibly promoting a chronic prothrombotic state.

Oxidized LDL has also been found to have pro-inflammatory effects relevant to the atherosclerotic process. It has chemotactic effects on monocytes (Quinn et al., 1987), enhances monocyte adhesion to EC in culture (Berliner et al., 1990; Kume et al., 1992), as well as the expression of VCAM-1 and ICAM-1 by human aortic EC induced by TNFα (Kahn et al., 1995) and of ICAM-1 in resting human endothelial vein cells (Takei et al., 2001). These pro-inflammatory effects are the result of the activation of a variety of functional pathways intimately related to innate immunity processes (Shalhoub et al., 2011). Finally, oxLDL has been shown to activate a variety of cell types expressing CD36 and other scavenger receptors and contribute to the generation of ROS (Li et al., 2010).

Advanced glycation end-product-modified LDL, as well as other AGE-modified proteins, are also pro-inflammatory (Vlassara et al., 2002; Wendt et al., 2002). AGE-modified proteins will impact EC eliciting increased permeability and pro-coagulant activity (Vlassara et al., 1994) and inducing the overexpression of VCAM-1 (Schmidt et al., 1995). AGE also contributes to fibroblast proliferation and T cell activation (Vlassara et al., 1994), and activated T cells in the atheromatous lesions release interferon-γ (De Boer et al., 1999), which in turn will prime macrophages in the lesion enhancing the release of pro-inflammatory cytokines and chemotactic factors in response to the recognition of AGE-LDL and other mLDL by the corresponding receptors. The impact of AGE in the atherosclerotic process associated with diabetes was confirmed in streptomycin-induced diabetic ApoE$^{-/-}$ mice. Administration of soluble forms of AGE receptors (RAGE) resulted in reduction of vascular permeability and slowed down the progression of atheromatous lesions (Bucciarelli et al., 2002).

THE IMMUNOGENICITY OF MODIFIED LDL

The pro-inflammatory properties of modified LDL appear to be considerably enhanced as a consequence of their immunogenicity. The immunogenicity of modified LDL was first reported by Steinbrecher et al. (1984) based on the immunization of laboratory animals with several types of modified LDL. Of all the mLDL, oxLDL has been studied in greatest detail from the immunological point of view. Steinbrecher (1987) as well as Palinski et al. (1990) characterized its immunogenic epitopes. Furthermore, human auto-antibodies to oxLDL were the first to be purified and characterized (Yla-Herttuala et al., 1994; Mironova et al., 1996; Virella et al., 2000). The cell-mediated immune system is also activated by antigen-presenting cells presenting modified LDL oligopeptides together with co-stimulatory signals to Th-1 cells, resulting in a chronic inflammatory reaction in which interferon-γ released by Th-1 cells enhances the pro-inflammatory response of macrophages, including the release of chemokines that attract more T cells to the area and the process becomes self-perpetuating (De Boer et al., 1999; Andersson et al., 2010).

CELLULAR RESPONSE TO ANTIGEN-ANTIBODY COMPLEXES (IMMUNE COMPLEXES) CONTAINING DIFFERENT MODIFIED FORMS OF LDL

It has been established that atherosclerotic plaque rupture is a critical event triggering thrombus formation and subsequent acute coronary events (Libby and Theroux, 2005). Plaques that are prone to rupture consist of a larger intimal lesion with abundant macrophages and foam cells and a thinned fibrous cap (Shah, 2002). Necropsy studies have demonstrated that atherosclerosis in diabetic patients is more diffuse and accelerated than in non-diabetic patients (Jarrett, 1981). Furthermore, studies have also shown that atherosclerotic lesions in diabetic patients were more vulnerable as they had larger intimal lesions and more macrophage infiltration as compared to those in non-diabetic patients (Moreno et al., 2000). Analysis of gene expression in atherosclerotic plaques showed that when compared to stable plaques, vulnerable plaques have higher expression of matrix metalloproteinases (MMPs) with collagenase activity, which contribute to the thinning of the fibrous cap, causing plaque instability and rupture (Galis et al., 1994). Among the MMPs, MMP-9 has been the object of considerable interest in recent years, and according to some studies is an independent risk factor for atherothrombotic events (Loftus et al., 2001; Blankenberg et al., 2003). MMP-9 synthesis and release can be induced through TLR-4 stimulation, usually involving bacterial endotoxins (Lundberg and Hansson, 2010), but also by minimally modified LDL (Choi et al., 2009) and likely by other types of modified LDL.

Besides overexpression of MMPs, vulnerable plaques are characterized by the accumulation of apoptotic macrophages around the necrotic core (Seimon and Tabas, 2009). A variety of pro-apoptotic insults has been proposed to play a significant role in the evolution of atheromas, including oxidative stress, endoplasmic reticulum (ER) stress, accumulation of non-esterified (free) cholesterol, and effects of pro-inflammatory cytokines released by activated macrophages (Seimon and Tabas, 2009). Most likely these factors play additive or synergistic effects in the induction of apoptosis. For example, intracellular accumulation of free cholesterol is a known inducer of ER stress, but low levels of ER stress usually protect against apoptosis (Seimon and Tabas, 2009). On the other hand, accumulation of free cholesterol in macrophages in combination with signals delivered through scavenger receptors or with interferon-γ, known to be released by activated T cells in atheromas (De Boer et al., 1999; de Boer et al., 2000), leads to serine phosphorylation of STAT-1 which is a critical element in the induction of apoptosis secondary to ER stress (Lim et al., 2008). The apoptotic macrophages in atheromas are ingested

by functional macrophages (efferocytosis). Efferocytosis in early lesions seems to result in suppression of inflammation, while in advanced lesions is associated with enhanced inflammation (Seimon and Tabas, 2009). This evolution appears to be the result of defective efferocytosis in advanced lesions, allowing the apoptotic cells to undergo necrosis, resulting in the accumulation of cell fragments that promote inflammation and plaque instability (Seimon and Tabas, 2009).

The activation of functional pathways by oxLDL and immune complex (IC) containing oxLDL has been studied in detail. oxLDL has been shown to activate a variety of cell types expressing CD36 and other scavenger receptors and contribute to the generation of ROS (Li et al., 2010). On macrophages, the interaction of oxLDL with CD36 (mediated by oxidized phospholipids) results in activation of the src family members Fyn/Lyn, and of several components of the MAP kinase pathway, including MKKK, MKK, FAK, and mitogen-activated protein kinase (MAPK; c-Jun N-terminal kinase, c-JNK; Silverstein et al., 2010). The activation of these kinases and associated proteins such as Vav is associated with foam cell formation as well as with unregulated actin polymerization and loss of cell polarity causing a migration defect and the trapping of activated cells in the atheromatous lesions (Silverstein et al., 2010). In platelets the same signaling events lead to enhanced platelet reactivity and enhanced formation of thrombi (Silverstein, 2009). Recently it has been reported that ligation of CD36 by oxLDL leads to the formation of a TLR-4-TLR-6 heterodimer that, in turn, will activate MyD88 and NFkB, a critical step in the induction of the synthesis and release of pro-inflammatory cytokines (Stewart et al., 2010).

OxLDL-IC have been demonstrated to be more potent activators of human macrophages than oxLDL (Saad et al., 2006). The uptake of IC prepared with native or oxLDL by human macrophages is primarily mediated by Fcγ receptors, primarily FcγRI (Lopes-Virella et al., 1991, 1997; Oksjoki et al., 2006). It has been shown that binding of IgG antibody to oxLDL blocks the interaction of oxLDL with CD36 (Nagarajan, 2007), so CD36 is not involved in the process. For MDA-LDL-IC and AGE-LDL-IC FcγRI is also involved, but the possible interaction of these mLDL with scavenger receptors or receptors for AGE-modified proteins has not been proven or excluded.

One fundamental property of modified LDL-IC is their ability to deliver large concentrations of free and esterified cholesterol to macrophages (Virella et al., 2002). The intracellular accumulation of cholesterol by itself may not induce apoptosis (Seimon and Tabas, 2009). In fact, both oxLDL-IC and oxLDL (at concentrations not exceeding 75 μg/mL) have the opposite effect and prevent macrophage apoptosis (Hundal et al., 2003; Oksjoki et al., 2006). *In vitro* data suggests that oxLDL-IC have a predominantly anti-apoptotic effect, more pronounced than that of oxLDL (Hammad et al., 2006; Oksjoki et al., 2006) but not unique to oxLDL-IC, because it has also been reproduced with keyhole limpet hemocyanin (KLH)-anti-KLH-IC (Oksjoki et al., 2006). However, there are significant differences between oxLDL-IC and other IgG-containing IC. Only oxLDL-IC can both engage FcγRI and deliver cholesterol to the cells and the magnitude of the pro-inflammatory response induced in human macrophages is greater with oxLDL-IC than with KLH-IC, for example Saad et al. (2006).

While oxLDL cell signaling is mediated by scavenger receptors, oxLDL-IC deliver activating signals via Fcγ receptors. The cross-linking of Fcγ receptors by IC induces phosphorylation of ITAMs by kinases of the Src family, and consequent activation of Syk (Crowley et al., 1997; Tohyama and Yamamura, 2009). Activation of Syk triggers a variety of pathways, including the MAPK signaling cascade, which includes ERK1/2, p38 MAPK, and c-JNK (Luo et al., 2010), responsible for NFkB activation and the expression of pro-inflammatory gene products, and the PI3K and AKT pathway secondary to phospholipase C activation (Oksjoki et al., 2006), which promotes cell survival by at least four different mechanisms: (1) phosphorylating the Bad component of the Bad/Bcl-X_L complex which results in its dissociation and cell survival, (2) caspase 9 inactivation, (3) regulation of the expression of transcription factors, and (4) activation of IKK kinases which phosphorylate IκB and, as a consequence, release the active form of NFkB which upregulates the expression of genes favoring cell survival (Datta et al., 1999).

Furthermore, the anti-apoptotic effect of oxLDL-IC seems to involve additional pathways, including activation of sphingosine kinase 1, which causes the levels of anti-apoptotic sphingosine-1-phosphate (S1P) to increase. S1P activates phospholipase C (PLC) and, through the generation of diacylglycerol, the Ras/ERK, and phosphokinase C are activated. PLC also activates the P13K-dependent pathway, which results in Akt activation (Hundal et al., 2003; Hammad et al., 2006; Chen et al., 2010; **Figure 1**).

Not surprisingly, the repertoire of oxLDL-IC-induced pro-survival genes is much wider than that induced by oxLDL alone (Hammad et al., 2009). Also, oxLDL-IC induce HSP70B expression in macrophages. This protein binds to the internalized lipid moiety of oxLDL-IC and prevents its degradation, while at the same time inducing sphingokinase-1 (Al Gadban et al., 2010; Smith et al., 2010).

In contrast to oxLDL, there is no published information concerning pathways of cell activation triggered by MDA-LDL or MDA-LDL-IC. The association of MDA-LDL with acute coronary syndromes (Holvoet et al., 1998; Holvoet, 1999) and the association of high levels of MDA-LDL in the circulating IC isolated from patients with type 2 diabetes who had acute CVD events, mainly MI (discussed later in this review), strongly suggest that MDA-LDL and MDA-LDL-IC have pro-apoptotic activity, although the precise pathways involved can only be suggested (**Figure 1**). Preliminary results obtained in our laboratory in experiments exposing human monocyte-derived macrophages to MDA-LDL-IC have shown increased expression of caspase 3, implying that, in contrast to oxLDL-IC, MDA-LDL-IC do not activate survival pathways. This difference between oxLDL and MDA-LDL could be a result of the large excess of MDA-modified lysine molecules in MDA-LDL relative to oxLDL (Virella et al., 2005). Also, while copper oxidation predominantly results in ApoB fragmentation, MDA modification is associated with ApoB aggregation (Viita et al., 1999). Obviously, physico-chemical differences in ApoB could determine different biological properties of the two forms of modified LDL.

Modified LDL isolated from circulating IC reacts with antibodies to oxidized, MDA, and AGE-modified LDL. The content of these modifications in IC-associated LDL is variable from patient to patient, but overall it reflects the predominance of a given type of

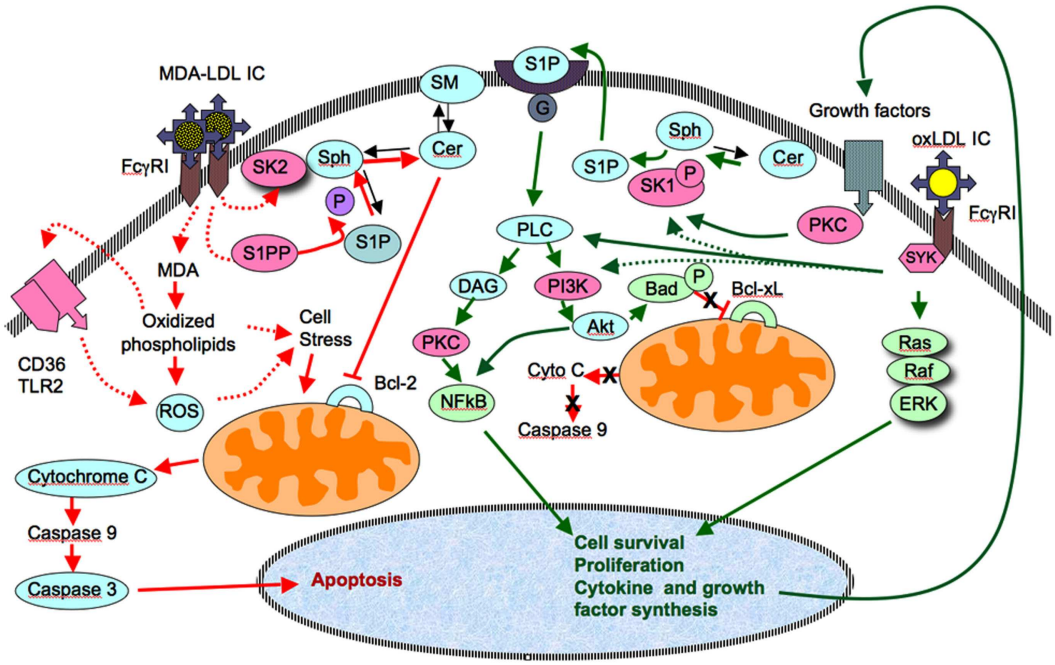

FIGURE 1 | Comparison of the pathways responsible for the anti-apoptotic and pro-apoptotic effects of immune complexes containing oxidized LDL (oxLDL-IC) and malondialdehyde-modified LDL (MDA-LDL-IC). OxLDL-IC activate cell proliferation pathways through Syk, a pathway that leads to activation of Akt and NFkB. The activation of Akt leads prevents the inactivation of anti-apototic gene products (Bcl-xL in the diagram). S1P-mediated activation of Akt and proliferation genes has been suggested by previously published data from our group (Hammad et al., 2006). This could result from the direct activation of SK1 by Syk, or as a consequence of the release of growth factors, upon ligation of the corresponding receptor, which activate S1k via PKC. As for the pro-apoptotic properties of MDA-LDL-IC, two possible pathways could be involved. One would result from the simultaneous activation of SK2 (whose phosphorylation is less stable than that of SK1) and S1PP. This would result in a reduced generation of S1P, and accumulation of ceramides, which in turn would inhibit anti-apoptotic genes (Bcl-2 in the diagram) and allow the activation of the pro-apoptotic intrinsic pathway. An alternative (and not exclusive) pathway to reach the same effect would involve the degradation of internalized MDA and release of highly charged phospholipids whose interaction with a CD36-TLR2 complex would activate the generation of ROS and increased cellular stress.

epitope. The enrichment in MDA-modified lysine could explain the differences in clinical associations that emerged in the data obtained in the EDIC/DCCT cohort and the VADT patient cohort. In the EDIC/DCCT cohort, high levels of oxLDL (which *in vitro* experiments show that is associated with macrophage survival) (Oksjoki et al., 2006; Hammad et al., 2009) in isolated IC are strong predictors of progression of CAD, as assessed by longitudinal measurements of carotid intima-media thickness (IMT; Lopes-Virella et al., 2011a). In contrast, the levels of MDA-LDL (which *in vitro* data show that is associated with macrophage apoptosis) in isolated IC, although associated with CAD progression, are a weaker predictor, comparable to high levels of LDL-cholesterol (Lopes-Virella et al., 2011a). This could be a reflection of the fact that at the time of admission into the DCCT/EDIC cohort the patients were young and basically CAD-free. Therefore, at that stage, the pro-apoptotic effect of MDA-LDL-IC would be associated with effective efferocytosis and the inhibition of the expansion of atherosclerotic lesions (Seimon and Tabas, 2009) However, in older patients with more advanced lesions, like those included in the VADT cohort, chronic and extensive ER stress is present leading to impaired processing of heavily oxidized and aggregated LDL by macrophages as well as to defective efferocytosis (Hoff et al., 1993). Defective efferocytosis favors macrophage necrosis and the release of pro-inflammatory mediators and MMPs leading to plaque destabilization. Release of cholesterol and oxidized phospholipids also takes place when macrophages undergo necrosis and the oxidized phospholipids can be transported to the extracellular compartment and then react with scavenger receptors and/or TLRs, delivering signals that favor the activation of pro-apoptotic pathways, increase cellular stress, and block the anti-apoptotic pathways. Recent data from our laboratory, still unpublished, supports the pro-apoptotic effect of MDA-LDL-IC in human macrophages and data obtained in the VADT cohort supports the role of MDA-LDL-IC in inducing plaque destabilization and acute CVD events (Lopes-Virella et al., 2012b).

MODIFIED LDL CONCENTRATIONS AS RISK FACTORS FOR DIABETIC COMPLICATIONS

Our group has studied extensively the pathogenic role of modified LDL antibodies (Virella et al., 2002, 2008; Saad et al., 2006; Lopes-Virella et al., 2007, 2011a; Lopes-Virella and Virella, 2010), and has developed methodology for the measurement of circulating antibodies to mLDL (Virella et al., 1993) and for the measurement of modified form of LDL and the corresponding antibodies involved in IC formation through the isolation and fractionation of circulating IC (Atchley et al., 2002; Virella et al., 2004, 2005,

2008). Several groups reported studies concerning the possible association between modified LDL (particularly oxLDL) or the corresponding antibodies with cardiovascular disease with conflicting results (Virella and Lopes-Virella, 2003; Lopes-Virella and Virella, 2010). The proposed assays for modified LDL are mostly enzymoimmunoassays and are affected by fact that 95% of circulating modified LDL exists as part of IC (Virella et al., 2004), a well-known cause of error in both antigen and antibody assays. The interference of IC was never clearly addressed in the assays used by different groups, and there has been no effort to develop a standard assay that could accurately measure circulating forms of modified LDL. Orekhov et al. (1991, 1995) first called attention to the use of circulating IC containing LDL as markers indicative of the severity of the atherosclerotic process. Their methodology consisted of precipitating IC from serum samples and measuring the cholesterol content in the precipitates as a surrogate marker for LDL. We used a similar approach in our initial attempts to measure LDL-IC levels (Lopes-Virella et al., 2007), but we decided that a direct measurement of modified LDL in precipitated IC would be more specific and informative. Our current approach, as previously noted, involves isolation and fractionation of circulating IC and allows to measure the levels of different forms of modified LDL involved in IC formation without interference of the high affinity IgG auto-antibodies (Virella et al., 2005). The composition of those IC should reflect that of the complexes deposited in the arterial wall, given that both LDL and IgG antibodies can diffuse across the endothelial barrier (Langer et al., 1972; Virella, 2007).

Data generated in clinical studies carried out on a type 1 diabetes cohort (the DCCT/EDIC cohort) have shown that high levels of oxLDL and AGE-LDL in circulating IC are associated with increased odds to develop diabetic nephropathy (Lopes-Virella et al., 2012a) and progression of retinopathy (Lopes-Virella et al., 2012c). Also in nephropathy, predominance of IgG antibodies (particularly those with higher avidity) over IgM antibodies in oxLDL-IC was associated with parameters indicative of deteriorating renal function in same cohort (Atchley et al., 2002; Virella et al., 2008). Using coronary artery calcification (CAC) indices and carotid IMT as end-points indicative of cardiovascular disease progression we also found that increased levels of oxLDL and of AGE-LDL in circulating IC are associated in the DCCT/EDIC cohort with the development of coronary calcification and with increased levels and progression of carotid IMT. The levels of MDA-LDL in isolated IC show a significant but weaker correlation with increased carotid IMT (Lopes-Virella et al., 2011a,b). In contrast, in patients with type 2 diabetes (VADT cohort), the levels of oxLDL and AGE-LDL in circulating IC are not significantly associated with the occurrence of acute events but high concentrations of MDA-LDL in IC are strong predictors of acute events, especially myocardial infarction (Lopes-Virella et al., 2012b). It must be noted that Holvoet et al. (1995, 1998) reported in two separate studies a link between high levels of oxLDL and established CAD and between elevated plasma MDA-LDL levels and plaque instability. As previously discussed, the association of circulating MDA-LDL and IC-associated MDA-LDL specifically with plaque instability/acute CV events raises interesting questions such as whether differences in the predominant species of modified LDL involved in IC formation may induce distinct cell signaling patterns and, in the case of IC carrying predominantly MDA-LDL, lead to plaque instability by inducing macrophage apoptosis and/or increased synthesis of MMPs, such as MMP-9 (Koenig and Khuseyinova, 2007), known to break down collagen and thus contribute to plaque thinning and rupture. This is very novel concept, proposing that the effects of the interaction of IC with phagocytic and antigen-presenting cells depend not only of the engagement of Fcγ receptors but also of the physico-chemical characteristics of the antigen.

One significant question that has not yet been directly answered is whether the formation and pathogenic role of IC containing modified LDL is unique to patients with diabetes. The fact that antibodies to modified LDL have also been detected and isolated from non-diabetic patients with coronary heart disease and healthy volunteers (Virella et al., 1993, 2002) and that IC complexes prepared with human oxLDL and human oxLDL antibodies isolated from patients with diabetes and healthy volunteers have similar pro-atherogenic and pro-inflammatory properties (Virella et al., 2002) argues in favor of a general role of LDL-IC in the pathogenesis of atherosclerosis. On the other hand, IC isolated from patients with type 2 diabetes and macrovascular disease are enriched in cholesterol and apolipoprotein B and induce significantly higher accumulation of cholesterol than IC isolated from non-diabetic patients with coronary artery disease or from healthy volunteers (Mironova et al., 2000). This could reflect an enhanced generation of modified LDL in diabetes, due to the increased oxidative stress, and to a lower content of antioxidants in the LDL isolated from diabetic patients (Mironova et al., 2000). An increased antigenic load would lead to a more vigorous immune response and to higher levels of IC containing modified LDL, thus resulting in a more prominent pathogenic role in diabetes.

MODIFIED FORMS OF LDL AS BIOMARKERS OF PLAQUE INSTABILITY

There is considerable interest in identifying biomarkers indicative of plaque instability. A variety of proteins and enzymes have been proposed as candidates, as reviewed recently by Koenig and Khuseyinova (2007). Besides MMPs, C-reactive proteins (CRP), cytokines (IL-6, IL-18), enzymes [glutathione peroxidase, lipoprotein-associated phospholipase A2 (Lp-PLA2), type II secretory phospholipase A2, myeloperoxidase, MMPs, particularly MMP-9, and pregnancy-associated plasma protein A], chemotactic proteins (monocyte chemotactic protein-1), placental growth factor, soluble CD40 ligand, soluble Fas ligand, and oxLDL have been proposed as indicators of plaque instability (Holvoet et al., 1995, 1998; Holvoet, 1999; Loftus et al., 2001; Pelisek et al., 2009; Colley et al., 2011). CRP has been extensively studied and several studies support the correlation between CRP levels and risk to suffer an acute cardiovascular event, but its predictive power beyond the traditional risk factors remains unproven (Koenig and Khuseyinova, 2007). Similar uncertainties, often compounded by lack of precise measuring methods, apply to most other proposed indicators of plaque instability (Koenig and Khuseyinova, 2007).

Of these markers, MDA-LDL and MDA-LDL-IC, oxLDL-IC, Lp-PLA2, TIMPs, and MMPs appear the most likely to have clinical

relevance, given the results obtained in preliminary clinical studies and/or their biological properties that are directly related plaque instability (Holvoet et al., 1995, 1998; Holvoet, 1999; Loftus et al., 2001; Koenig and Khuseyinova, 2007; Lopes-Virella et al., 2007, 2011a,b, 2012b; Pelisek et al., 2009; Colley et al., 2011). However, most studies have been carried out in small patient populations, and the results are not consistent. Our studies in the DCCT/EDIC and VADT cohorts of patients with type 1 and type 2 diabetes are exceptions to this rule, and have demonstrated significant associations not only with cardiovascular disease (Lopes-Virella et al., 2007, 2011a,b), but also with nephropathy (Lopes-Virella et al., 2012a) and retinopathy (Lopes-Virella et al., 2012c). This high predictive power of the measurement of mLDL in IC reflects the fact that our assay allows the accurate measurement of the levels of different modifications of the LDL molecules contained in IC (Virella and Lopes-Virella, 2003; Virella et al., 2005). This is extremely significant, because, as previously mentioned, 95% or more of the modified LDL in circulation is associated with the corresponding antibodies forming IC (Virella et al., 2004), and because IC containing modified LDL are considerably more pathogenic than modified LDL by itself as consequence of their ability to engage Fcγ receptors and activate phagocytic cells to a much greater extent (Virella et al., 2002; Saad et al., 2006). Ongoing studies will further define the predictive value of the analysis of the content of mLDL in circulating IC, which at the present time appears to have the potential to become the best predictor of progression and complications of cardiovascular disease in diabetic patients. Also in the planning stages are studies aimed at proving that the same predictive value will extend to non-diabetic populations.

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Inflammation in the pathogenesis of microvascular complications in diabetes

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Diabetes and hyperglycemia create a proinflammatory microenvironment that progresses to microvascular complications such as nephropathy, retinopathy, and neuropathy. Diet-induced insulin resistance is a potential initiator of this change in type 2 diabetes which can increase adipokines and generate a chronic low-grade inflammatory state. Advanced glycation end-products and its receptor, glycation end-products AGE receptor axis, reactive oxygen species, and hypoxia can also interact to worsen complications. Numerous efforts have gained way to understanding the mechanisms of these modulators and attenuation of the inflammatory response, however, effective treatments have still not emerged. The complexity of inflammatory signaling may suggest a need for multi-targeted therapy. This review presents recent findings aimed at new treatment strategies.

Keywords: inflammation, diabetes mellitus, microvascular complications, oxidative stress, advanced glycation end-products, inflammatory cytokines

INTRODUCTION

Inflammation plays an essential role in the progression of diabetic microvascular complications. Proinflammatory cytokines C-reactive protein, tumor necrosis factor (TNF)-α, and interleukin (IL)-6 all demonstrate increased expression in diabetes (Peters et al., 1986; Ford, 1999; Festa et al., 2000; Müller et al., 2002; Temelkova-Kurktschiev et al., 2002). In chronic hyperglycemia, cytokines infiltrate vascular tissues and inhibit function and repair. Obesity is a major risk factor for diabetes and can induce inflammation by Toll-like receptor (TLR) activation to recruit proinflammatory cytokines and chemokines (Kwon et al., 2012). With the onset of diabetes, adipokines such as TNF-α and IL-6 may contribute to insulin resistance (Rajala and Scherer, 2003; Suganami et al., 2005). Adiponectin is initially upregulated to increase glucose uptake, and nitric oxide (NO) production; however, continued obesity may reduce adiponectin leading to complications observed in type 2 diabetes (T2D; Berg et al., 2001; Matsuzawa, 2005). Obesity is also associated with hyperlipidemia with elevated levels of cholesterol and triglycerides which may contribute to inflammation and diabetic retinopathy (DR; Dodson et al., 1981). The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study found no relationship between serum lipid levels and DR (Keech et al., 2007; Chew et al., 2010). Fenofibrate is known to lower lipid levels, but it can also activate peroxisome proliferator-activated receptors (PPARs) and suppress inflammation by inhibiting nuclear factor kappa B (NF-κB; Tomizawa et al., 2011). As metabolic syndrome and inflammation persist, oxidative stress, hypoxia, and advanced glycation end-products (AGEs)/AGE receptor (RAGE) converge to exacerbate the problem (Brownlee, 2005; Vincent et al., 2011). A schematic summarizing the pathogenesis of diabetic microvascular complications is presented (**Figure 1**). The focus of this review is to overview the most recent findings relevant to treating nephropathy, retinopathy, and neuropathy.

DIABETIC NEPHROPATHY

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (Nilsson et al., 2008). DN results in basement membrane thickening, expansion of the mesangium, reduced filtration, albuminuria, and ultimately renal failure (Graves and Kayal, 2008). Inflammatory cells can accumulate in glomeruli and interstitium to worsen DN (Lim and Tesch, 2012). Recent findings have identified a few key receptors involved in renal protection. Studies targeting these pathways along with other known mediators of inflammation have revealed the importance of inflammation in worsening DN.

Peroxisome proliferator-activated receptors are activated in response to fatty acids and regulate lipid and glucose homeostasis (Wahli and Michalik, 2012). In the kidney, PPARγ expression has been found in medullary collecting ducts, pelvic urothelium, and isolated glomeruli and cultured mesangial cells (Iwashima et al., 1999; Yang et al., 1999; Asano et al., 2000; Kume et al., 2008). Pioglitazone, a PPARγ agonist, increased anti-oxidant activity and reduced inflammation in hyperoxaluric rats (Taguchi et al., 2012). This suggests activation of PPARγ may have renoprotective functions. Similarly, the same agonist treated in T2D diabetic rats showed improved insulin resistance, glycemic control, and lipid profile while reducing inflammation by reducing macrophage infiltration and NF-κB expression (Ko et al., 2008).

Resveratrol (*trans*-3,4′,5-trihydroxyestilbene, RSV) is a polyphenolic compound found in grapes and other plants providing anti-oxidant effects (Chang et al., 2011). RSV improved renal function and reduced oxidative stress in type 1 diabetic (T1D) rats (Sharma et al., 2006; Dhaunsi and Bitar, 2012). Similarly, RSV treatment showed significant decreases in superoxide anion and protein carbonyl oxidative stress markers (Chang et al., 2011). RSV was shown to reduce renal lipotoxicity and mesangial cell glucotoxicity in diabetic mice mediated through activation of PPARγ co-activator 1α (Kim et al., 2012). In another study,

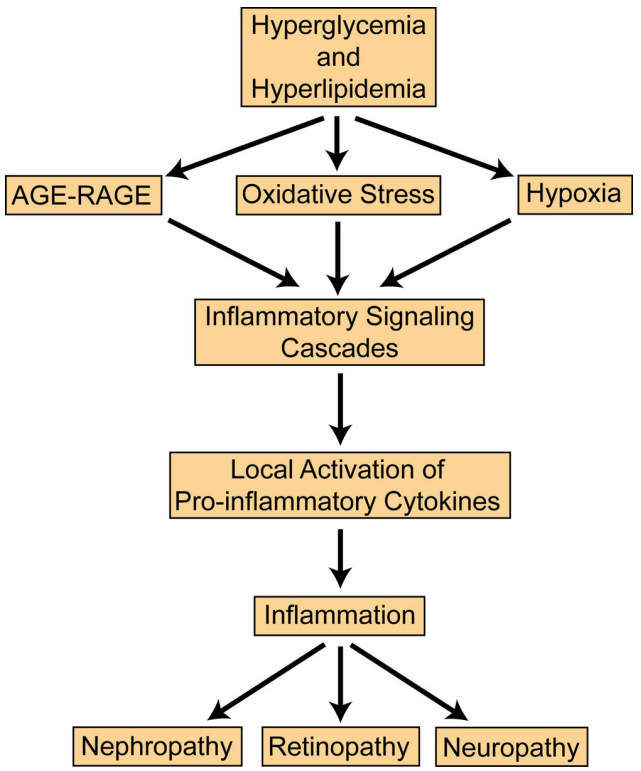

FIGURE 1 | General pathway in the progression of diabetic microvascular complications.

RSV reduced IL-1β in streptozotocin (STZ)-diabetic rat kidneys, but there was a significant increase in TNF-α and IL-6 levels independent of NF-κB activation, suggesting RSV has both stimulatory and inhibitory effects on cytokines simultaneously and achieving the optimal dose may be critical to establishing efficacy (Chang et al., 2011).

Fcγ receptors (FcγR) are present in leukocytes, glomerular, and mesangial cells (Gómez-Guerrero et al., 1994, 2002; Radeke et al., 2002). FcγR can bind to immunoglobulin G (IgG). Circulating oxidized LDL-containing immune complexes (oxLDL-IC) are increased in diabetes stimulate synthesis of IgGs in addition to other proinflammatory cytokines such as IL-1β, IL-6, IL-18, and TNF-α in Mono Mac 6 cells and primary human macrophages (Saad et al., 2006; Abdelsamie et al., 2011). Increased oxLDL-ICs also increases matrix production in mesenchymal mesangial cells through activation of FcγRI and FcγRIII to increase collagen IV production in nephropathy (Abdelsamie et al., 2011). Attenuating FcγR activity may reduce the development of a proinflammatory environment and enhanced matrix production. A genetic defect in FcγR attenuated diabetic renal injury based on histological analyses and reduced leukocyte accumulation in glomeruli and interstitium (Lopez-Parra et al., 2012). There was also a reduction in intracellular superoxide generation *in vivo* and oxidative response to oxLDL-ICs *in vitro* (Lopez-Parra et al., 2012).

Dietary lipids preceding diabetes have been shown to upregulate proinflammatory cytokines and TLR transcriptional levels along with downregulation of transcripts involved in glucose metabolism in epididymal and mesenteric white adipose tissue (Kwon et al., 2012). TLR are innate immune receptors that have been implicated in T1D, T2D, and its associated complications (de Kleijn and Pasterkamp, 2003; Park et al., 2004; Rudofsky et al., 2004; Wen et al., 2004; Lang et al., 2005). In DN, TLR4 expression was increased in T2D and uremic patients and in mouse mesangial cells, suggesting its role in monocyte recruitment (Kaur et al., 2012; Yang et al., 2012). Studies confirmed increased TLR4 activation when cells were incubated with high glucose (Kaur et al., 2012). Monocytes displaying $CD14^+CD16^+$ surface markers in the kidney can associate with TLR and activate NF-κB, and STAT expression to further promote a proinflammatory microenvironment (Yang et al., 2012). Therapeutic targets correcting dysregulated TLR signaling may therefore be an important target against inflammation and complications within the kidney.

Advanced glycation end-product production is widely associated with diabetic microvascular complications. Recent studies showed little benefit using benfotiamine, a lipophilic thiamine-derivative that activates transketolase to reduce AGE precursors (Babaei-Jadidi et al., 2003; Karachalias et al., 2010). Benfotiamine had no effect in decreasing existing plasma AGE or increasing AGE excretion (Alkhalaf et al., 2012). Similarly, evaluation of benfotiamine in cerebral cortex of STZ-induced diabetic rats showed little effect on reducing AGEs and TNF-α, however, it slightly attenuated oxidative stress (Wu and Ren, 2006). Despite the outcome, this approach remains active and a recent proposal has aimed at modifying the delivery to have dual targets instead of singular targeting. Using a nanoparticle shell, both AGE and RAGE inhibitors will be encased within the shell to suppress both axes and redundancy not addressed with a single therapy (Zhou et al., 2012). The exterior of the shell will contain RAGE analogs, which can also provide specificity to AGEs and delivery of therapeutics (Zhou et al., 2012). This dual therapy approach is still in its infancy, but it may have potential benefits if pursued to target both receptors and its ligands.

Current standard treatment of DN targets the renin–angiotensin system (RAS) through usage of angiotensin converting enzyme (ACE) inhibitors to limit systemic blood pressure to control intraglomerular pressure (Bonegio and Susztak, 2012). Upstream targeting may further decrease RAS activity. Aliskiren, a direct renin inhibitor, has been recently evaluated in DN. Treatment using aliskiren showed a significant reduction in TNF-α and transforming growth factor (TGF)-β (Gandhi et al., 2012). Some studies have shown that TGF-β may have a role in influencing renal growth and inflammation as well as fibrosis and renal dysfunction (Ziyadeh et al., 2000; Phillips and Steadman, 2002).

DIABETIC RETINOPATHY

Diabetic retinopathy is one of the leading causes of blindness in adults of working age adults. Background DR is characterized by ischemic injury which creates a hypoxic environment in ocular tissues. Hypoxia has been shown to induce microglia activation and recruitment to ischemic sites in retinas (Kielczewski et al., 2011). Vascular injury in background DR and proliferative DR (PDR) increases proinflammatory cytokines which

can promote leukostasis and vascular endothelial growth factor (VEGF) mediated permeability in the retinal vasculature (Chistiakov, 2011).

The retinal pigment epithelium (RPE) provides functional barriers for the exchange of nutrients to photoreceptor cells. Under hyperglycemia, microglia and macrophages accumulate in the RPE in Goto Kakizaki rats (Omri et al., 2011). Increases in transepithelial pores compromise tight junction integrity and allow materials to enter the choroidal space (Omri et al., 2011). Presence of inflammation can reduce transepithelial resistance (TER) and impact ion gradient generation between membrane transporters and tight junctions (Rizzolo et al., 2011). TNF-α exposure to human RPE cells showed decreased TER (Peng et al., 2012). GPR109A is a G protein-coupled receptor (GPCR) present in RPE that is upregulated in diabetic mouse and human retina (Gambhir et al., 2012). GPR109A has immunomodulatory effects in adipose tissue and progression of atherosclerosis (Digby et al., 2010; Montecucco et al., 2010; Lukasova et al., 2011). Two ligands of GPR109A, niacin and β-hydroxybutyrate, was shown to suppress IL-6 and chemokine ligand-2 (CCL2) induced by TNF-α (Gambhir et al., 2012). Additional studies should explore potential value of modulating GPR109A activity with its ligands to suppress inflammation in the retina of those discussed as well as other proinflammatory cytokines (Gambhir et al., 2012).

β-catenin is a downstream effector of the Wnt pathway and is found to be increased in several diabetic rodent models and in humans (Chen et al., 2009). Increased β-catenin may be due to sustained Wnt signaling where it can also activate NF-κB to induce inflammation (Dale, 1998; Yamashina et al., 2006; Yan et al., 2008). DR is characterized by hypoxia and oxidative stress, which contribute to Wnt activation. Blockage of Wnt led to reduced inflammation through decreased ICAM-1 in the retina (Chen et al., 2009). Mab2F1, a monoclonal antibody targeting Wnt co-receptor LDL receptor-related protein 6 resulted in reduced retinal vascular leakage, inflammation, and attenuation of leukostasis (Lee et al., 2012).

Comparing cytokine levels of peripheral blood in diabetic patients revealed that levels of IL-22 expressed by T-helper (Th) 22 was significantly increased compared to controls, however, the differences were not significant between NPDR, PDR, and in diabetic patients without DR (Chen et al., 2012). IL-22 levels were also positively correlated with duration of diabetes (Chen et al., 2012). TNF-α has been shown to be increased in serum of diabetic patients. The results from this study suggest a potential role of Th22 expressing IL-22 levels in the pathogenesis of diabetic complications.

Increased RAGE levels and its ligand S100B are found in rat diabetic retinas and also found in cultured Müller glial cells exposed to high glucose (Limb et al., 2002; Zong et al., 2010). S100B has been shown induce inflammatory cytokines such as TNF-α and vascular CAM (VCAM)-1 in human microvascular endothelial cells (Valencia et al., 2004). Similarly, Müller glial cells treated with exogenous S100B showed increased levels of TNF-α, IL-6, IL-8, VEGF, and CCL2 (Zong et al., 2010). Treatment of S100B in cells showed a dose-dependent activation of mitogen-activated protein kinase pathway (MAPK) (Zong et al., 2010). *In vivo* studies should assess the relevant concentrations of S100B in pathogenesis of DR.

The RAS plays a vital role in regulating many physiological processes of the vascular system. Elevated levels of renin, prorenin, and Angiotensin II (Ang II) are found in patients with DR (Wilkinson-Berka, 2008). In PDR, prorenin and its receptor [(P)RR] are upregulated in retinal endothelial cells (Kanda et al., 2012). Increased (P)RR, prorenin, and activated prorenin were found in human vitreous fluid which can promote inflammatory angiogenesis in the eye (Satofuka et al., 2008; Kanda et al., 2012). (P)RR can activate extracellular signal-regulated kinases (ERK) and induce inflammatory responses in the eye (Kanda et al., 2012). Blockage of (P)RR reduced ERK activity and decreased diabetes-induced retinal inflammation (Satofuka et al., 2012).

Downstream effectors also have important functions in DR. Ang II, a product of ACE, activates the AT_1 receptor to induce vasoconstriction, proliferation, fibrosis, and inflammation. The protective arm of the RAS involves ACE2, which produces Ang-(1-7). As a vasodilator peptide with anti-hypertensive, anti-hypertrophic, anti-fibrotic, and anti-thrombotic functions (3), Ang-(1-7) stimulates NO production by activating endothelial NO synthase (eNOS) in an Akt-dependent manner and decreases ROS production by attenuating NADPH oxidase. Ang-(1-7) mediates its effects by activating the GPCR, the Mas receptor (Sampaio et al., 2007; Benter et al., 2008). Chronic Ang-(1-7) treatment preserves endothelial function in rat models of myocardial ischemia and in-stent restenosis (Loot et al., 2002; Langeveld et al., 2005). Treatment with ACE2 or Ang-(1-7) corrected diabetic defects in therapeutic angiogenesis (Oudit et al., 2010; **Figure 2**). Intraocular administration of adeno-associated virus expressing ACE2/Ang-(1-7) significantly reduced $CD45^+$ macrophages, $CD11b^+$ microglial cells, and oxidative damage in mice (Verma et al., 2012). Targeting both upstream and downstream components of the RAS axis may provide synergistic effects in treating microvascular complications.

DIABETIC NEUROPATHY

Diabetic neuropathy (DNO) is the most common complication of diabetes, where population-based studies have indicated more than half of the patients with either T1D or T2D develop DNO, and as much as 30% of those manifestations are painful (Harati, 2007; Ramos et al., 2007; Farmer et al., 2012). Recent reviews have emphasized the importance of targeting oxidative stress and inflammation in the treatment of DNO (Vincent et al., 2011; Farmer et al., 2012).

Tumor necrosis factor-α has been implicated in contributing to insulin resistance in obesity due to its increased expression in adipose tissue. Obese mice with a TNF-$\alpha^{-/-}$ mutation displayed improved insulin sensitivity and lowered circulating fatty acids, improving obesity-induced glucose tolerance (Uysal et al., 1997). Increased plasma TNF-α and macrophages are also associated with the progression of DNO, suggesting continued expression of these cytokines contribute to diabetic microvascular complications (Purwata, 2011). Similar experiments evaluating TNF-α null mice showed that they are less susceptible to developing diabetic complications (Gao et al., 2007). Targeting TNF-α through pharmacological means can potentially reverse the deleterious effects in DNO. Infliximab, a monoclonal anti-TNF-α antibody approved for treatment of autoimmune diseases such as rheumatoid arthritis

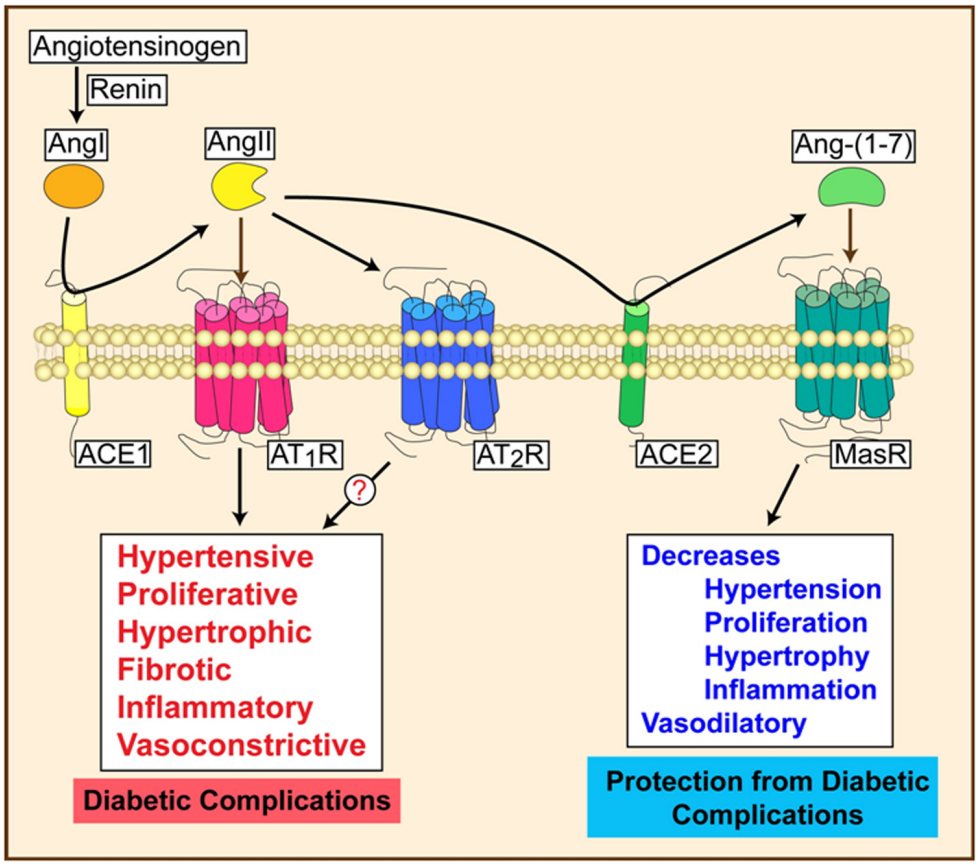

FIGURE 2 | Activation of RAS can lead to progressive or protective effects depending on the signaling mechanisms.

and psoriasis has been explored (Lin et al., 2008). Administration of infliximab into T1D mice showed significant improvement in neural function comparable to non-diabetic controls (Yamakawa et al., 2011).

Tumor necrosis factor-α can also influence AGE/RAGE activity making it a relevant target in DNO. In the progression of DNO, RAGE expression was increased in diabetic peripheral nerves and dorsal root ganglia (DRG; Toth et al., 2008). Mice models deficient in RAGE attenuated the structural and electrophysiological changes in peripheral nerves and DRG after prolonged diabetes of 5 months and also reduced NF-κB and protein kinase C activation (Toth et al., 2008). NF-κB can induce apoptosis, cell cycle, and plasticity, neurogenesis, and differentiation in the central nervous system (Foehr et al., 2000; Kumar et al., 2004; Fraser, 2006). RSV has been shown to inhibit NF-κB activity and TNF-α, IL-6, and cyclooxygenase-2 levels (Kumar and Sharma, 2010). BAY 11-7082, an inhibitor of kappa B (IκB) phosphorylation, down-regulated NF-κB and led to improved sensory response, motor nerve conduction velocity, and nerve blood flow (Kumar et al., 2012). Similarly, there was a significant reduction in the oxidative stress marker, malondialdehyde, IL-6, and TNF-α levels (Kumar et al., 2012). While IL-6 is generally regarded as proinflammatory, its role in DNO is still unclear since IL-6 administration may have neurotrophic effects (Cotter et al., 2010).

Bradykinin B1 receptor (B1R) of the kallikrein–kinin system has been shown to be upregulated in response to increases of oxidative stress in diabetes (Dias et al., 2010). In another study, minocycline has been shown to exhibit anti-inflammatory and anti-oxidant effects by inhibiting microglia activation (Pabreja et al., 2011). Inhibition of microglia activation in STZ-diabetic rats using either fluorocitrate or minocycline reduced B1R expression along with IL-1β and TNF-α proinflammatory cytokines in spinal dorsal horn (Talbot et al., 2010). Microglia inhibitors may have an effect on thermal hyperalgesia and allodynia which support a role of B1R in pain neuropathy (Talbot et al., 2010). Antagonists to B1R showed a reversal of allodynia in STZ-diabetic rats, suggesting the mediation of early DNO due to inflammation (Talbot et al., 2010). However, in Akita mice, loss of B1R and bradykinin B2 receptor (B2R) appears to exacerbate nephropathy and neuropathy, suggesting that its activation in this diabetes model may be protective (Kakoki et al., 2010). Further studies should assess the role of B1R in different animal models of diabetes.

Angiopoietin-1 (Ang-1) has been demonstrated to have benefits against vascular leakage and endothelial cell survival (Cho et al., 2004). Variants have been developed to improve on solubility and potency (Cho et al., 2004). Matrilin-1-Ang-1 (MAT-Ang-1) has been demonstrated to have anti-inflammatory protection against cytokines IL-1α, IL-1β, IL-6, and TNF-α

in sepsis (Alfieri et al., 2012). Another variant, cartilage oligomeric matrix protein (COMP)-Ang-1, has been hypothesized to improve regeneration of nerve fibers and endoneural microvessels in leptin-deficient obese (ob/ob) mice, a model for T2D (Kosacka et al., 2012). COMP-Ang-1 treatment was capable of reducing macrophage infiltration and T-cell number in sciatic nerves of ob/ob mice by 45 and 47%, respectively (Kosacka et al., 2012). Upstream effectors of Ang-1 have also recently been explored. Thymosin β4 improved diabetes-induced vascular dysfunction in sciatic nerve, nerve function and can mediate this through upregulation of Ang-1 in diabetic mice (Wang et al., 2012). Regulators of Ang-1 may therefore have benefits against neural and vascular dysfunction.

CONCLUSION

The worldwide increase in prevalence of obesity and diet-induced insulin resistance increases the need to reduce chronic inflammation. Diabetic microvascular complications progress due to inflammation which originates from multiple pathways and mechanisms. This complexity warrants the need for effective therapies that target more than one signaling cascade. Inhibition of both inflammatory cytokines and their activators/regulators may provide additional coverage to treating nephropathy, retinopathy, and neuropathy. Similarly, this can be combined and optimized with anti-oxidant and AGE/RAGE therapies to mitigate compensatory mechanisms. As further studies emerge to address current limitations, improved therapies targeting diabetic microvascular complications may ultimately transition from treating the pathology to prevention.

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Immune mechanisms in atherosclerosis, especially in diabetes type 2

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Atherosclerosis and ensuing cardiovascular disease (CVD) are major complications of diabetes type 2. Atherosclerosis is a chronic inflammatory condition involving immunocompetent cells of different types present in the lesions. Even though inflammation and immune activation may be more pronounced in atherosclerosis in diabetes type 2, there does not appear to be any major differences between diabetics and non-diabetics. Similar factors are thus implicated in atherosclerosis-associated immune activation in both groups. The cause of immune activation is not known and different mutually non-exclusive possibilities exist. Oxidized and/or enzymatically modified forms of low-density lipoprotein (OxLDL) and dead cells are present in atherosclerotic plaques. OxLDL could play a role, being pro-inflammatory and immunostimulatory as it activates T-cells and is cytotoxic at higher concentrations. Inflammatory phospholipids in OxLDL are implicated, with phosphorylcholine (PC) as one of the exposed antigens. Antibodies against PC (anti-PC) are anti-atherogenic in mouse studies, and anti-PC is negatively associated with development of atherosclerosis and CVD in humans. Bacteria and virus have been discussed as potential causes of immune activation, but it has been difficult to find direct evidence supporting this hypothesis, and antibiotic trials in humans have been negative or inconclusive. Heat shock proteins (HSP) could be one major target for atherogenic immune reactions. More direct causes of plaque rupture include cytokines such as interleukin 1β (IL-1β), tumor necrosis factor (TNF), and also lipid mediators as leukotrienes. In addition, in diabetes, hyperglycemia and oxidative stress appear to accelerate the development of atherosclerosis, one mechanism could be via promotion of immune reactions. To prove that immune reactions are causative of atherosclerosis and CVD, further studies with immune-modulatory treatments are needed.

Keywords: atherosclerosis, immune system, natural antibodies, phospholipids, inflammation

BACKGROUND

Type 2 diabetes represents a major and growing problem throughout the world, not only in so-called developed countries. In addition to nephropathy and microvascular disease, cardiovascular disease (CVD), and accelerated atherosclerosis often occur in diabetes, both type 1 and 2 (1–3). The main focus of this review is immune activation in atherosclerosis, especially in type 2 diabetes.

The link between type 2 diabetes and inflammation is well established, and there are signs of chronic inflammation in both diabetes and insulin resistance (IR), a typical feature of type 2 diabetes (4). Also in atherosclerosis and CVD, chronic inflammation is a major feature, and in atherosclerosis, activated immune competent cells such as T-cells and antigen-presenting cells, are abundant in lesions (5).

Even though inflammation and size of the necrotic core may be increased in atherosclerosis in diabetes (6, 7), there was no difference in the prevalence of macrophages, lymphocytes, and overall inflammation in plaque or in the atherosclerotic cap between diabetics and non-diabetics according to the largest study in this area (8). It thus appears that there is no known fundamental difference between the immune activation and inflammation present in atherosclerosis among non-diabetics as compared to diabetics. Still macrophages and surface thrombi may persist longer after ischemic symptoms in diabetes, which could contribute to the increased risk of recurrent CVD in this condition (8) and risk factors as hyperglycemia naturally play a special role. In this review, I therefore discuss immune activation in atherosclerosis in general and in diabetes type 2 in the same context.

Acute inflammatory response developed from an evolutionary point of view most likely to protect against pathogens and to repair tissue damage, which could be caused also by trauma. The classic symptoms of acute inflammation – pain, swelling, redness, heat, and decrease of function – were described already in Hippocratic medicine. When acute inflammation is not resolved, but instead persists and becomes chronic, it can become a major problem. Indeed chronic inflammatory conditions represent a major disease burden in the western world, and increasingly, also in developing countries (9). Examples of chronic inflammatory diseases include rheumatic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE); atherosclerosis and its major consequence, CVD including myocardial infarction (MI), acute

coronary syndrome (ACS), claudication, and stroke; Alzheimer's disease; diabetes type 2; increased IR and even abdominal obesity and osteoarthritis have inflammatory components (9).

Associations between these conditions are well known. For example, type 2 diabetes is a major risk factor for atherosclerosis and CVD (together with smoking, hypertension, dyslipidemia, age, and male sex) (10). Alzheimer's disease and atherosclerosis and/or CVD have some risk factors in common (11) and smoking is a risk marker for RA in addition to well known effects in CVD (12). It has also become clear that there are associations between rheumatic diseases and atherosclerosis/CVD, especially in SLE (13). Also in RA, there is an increased risk of CVD according to many reports, and a recent meta-analysis imply that atherosclerosis *per se* is more prevalent in patients in RA (13–15). It is interesting to note that there are reports which also describe an increased risk of type 2 diabetes in RA (16).

Anti-inflammatory treatments have improved the prognoses of many patients in chronic inflammatory conditions, the most notable example being biologics such as tumor necrosis factor (TNF)-inhibitors in RA and other autoimmune conditions (9). There is therefore an apparent need to evaluate targeted anti-inflammatory and immunomodulative treatments in other chronic inflammatory conditions.

An interesting possibility would be that biologics such as TNF-inhibitors could be therapeutically effective in atherosclerosis and diabetes type 2 and their complications. However, this does not appear to be the case to any significant degree. Although systemic blockade of TNF has an anti-cachectic effect in RA patients, the data on anti-TNF effects of IR are conflicting, depending on disease severity and degree of inflammation (17–19). Still, a recent case report indicates that treatment with a novel T-cell inhibitor had a dramatic effect on IR in RA (20).

As discussed in an editorial (14), it is interesting to note that inflammatory nature of atherosclerosis was known already 180 years ago, reported by the famous Austrian pathologist K. Rokitansky. R. Virchow confirmed these findings somewhat later, and the ensuing debate between these two giants in the history of medicine is of interest also now (21, 22). Rokitansky argued that atherosclerosis is secondary to other disease processes and phenomena, while Virchow supported the view that inflammation in atherosclerosis is a primary pathogenic factor (21, 22). Both could be right, since atherosclerosis is nowadays recognized as an inflammatory process, and could be secondary to other inflammatory conditions.

A role of the immune system in atherosclerosis, with or without background of diabetes type 2, has been suggested since the 1980s, when activated T-cells were detected in human atherosclerotic lesions (23). Since then, an array of data indicate that immune activation is a major feature of and plays a role in atherosclerosis, and also that immunomodulation to ameliorate disease development could be an interesting possibility (10, 24, 25).

At an early stage of atherosclerosis, macrophages accumulate and become filled with lipids, mainly derived from modified forms of low-density lipoprotein (LDL). These lipid-filled macrophages develop into foam cells, and subsequently, these and other cells die, creating a necrotic core of cell debris. An organized apoptotic clearance, is thus not effective in advanced atherosclerotic lesions. Also lymphocytes, especially T-cells, are common at a very early stage of disease development. In the 1990s, it was demonstrated that immunomodulation can change the course of atherosclerosis development; while administration of heat shock protein 60/65 accelerated atherosclerosis development (26), immunization with oxLDL, had the opposite effect (27).

However, it should be noted, that there may be important differences between animal models and human disease in this context (28). Even though mouse models of atherosclerosis have very much increased our understanding of atherosclerosis it is still interesting to note that there may be problems with translating mouse data to humans. For example, lipid levels are strikingly much higher in mice models, and another problem is that it is difficult to mimic human CVD in animal models, including mice models (29, 30). In this review, I have therefore chosen to emphasize data on immunity and atherosclerosis which are derived from human studies, including *ex vivo* and cohort studies.

As discussed in a previous review (24), available evidence indicates that atherosclerosis *per se* is a normal part of human aging, though its complications may not be part of the normal aging process, at least not to the same extent.

A direct causative role played by T-cells is suggested by animal experiments, with transfer of beta(2)-glycoprotein I-reactive lymphocytes, which enhanced early atherosclerosis in LDL receptor-deficient mice (31). Further, CD4+ T cells reactive to modified low-density lipoprotein aggravate atherosclerosis (32). An immunomodulatory role of T-cells is suggested by experiments where regulatory T cells suppress immune activation and thereby inhibit atherosclerosis (33). Interestingly, also NK T-cells may play a role in this context since CD1d-dependent activation of NKT cells aggravates atherosclerosis (34). Relatively little is known about the role of T-cells in human atherosclerosis, but it is interesting to note that Th17/Th1 imbalances have been reported, which may be related to plaque rupture (35, 36). Interestingly, data in humans and mice provide support for the concept that TH17 cells induced upon TGF-b signaling promote the development of cap structures in atherosclerotic plaques and the role of T-cells could depend on the local activation pattern and milieu (37). Clearly, the role of T-cells in plaque rupture is complicated. Further, subsets of T-lymphocytes with pro-atherogenic and plaque-destabilizing properties are increased in diabetes type 2 and associated with a worse CVD-outcome (38).

During recent years, a more detailed picture also of other inflammatory and/or immune competent cells has emerged from *in vivo* and other experimental studies, which need to be corroborated in human disease.

One example is dendritic cells (DC) which are specialized antigen-presenting cells, which may play an important role in the initiation and progression of atherosclerosis (39). DC are present in immature forms in the arterial wall and become activated during atherogenesis. In human atherosclerosis, DC are present both at an early stage (40) and late stage, with higher numbers in vulnerable plaques (41). Similar findings have been reported in

mice, where DC may promote atherogenesis (42). Further, DC and T-cells co-localize in plaques (43).

Monocytes/macrophages are present in lesions at different stages of atherosclerosis. Macrophages play a major role in inflammatory responses and may alter their phenotype which may vary on a scale, from pro-inflammatory M1 to anti-inflammatory M2 macrophages. Interestingly, even though the whole spectra of macrophages are present in lesions, including the relatively inert and surprisingly long lived macrophage-derived foam cells, M1 cells are prevalent in the vicinity of plaque rupture (44, 45).

Another cell type common in atherosclerotic lesions with inflammatory and potentially immune-modulatory properties is mast cells (46). A significant role played by this cell type in atherosclerosis and its complications is suggested by a recent report, where it is demonstrated that intraplaque mast cell numbers associate with future cardiovascular events (47).

Even though some B-cells, neutrophils, and NK-cells are present in lesions, it is not known to what extent they play a major role (10).

The role of the immune system in human atherosclerosis with or without background of diabetes type 2 is less defined as compared to the relevant animal models. T-cell reactive to OxLDL and related lipids are present in blood and atherosclerotic plaques (48, 49), patients with autoimmune diseases have increased atherosclerosis (13), and aspects of humoral immunity as natural antibodies against phosphorylcholine (PC) and other antigens are associated with atheroprotection (50). Humoral immunity has been shown to have pathogenic consequences in type 1 diabetes, triggered by immune complexes containing oxidized forms of LDL (51).

Available data thus imply that the immune system plays a major role in atherosclerosis, which could be seen as a chronic inflammatory disease or disease process. Interestingly, also diabetes, IR abdominal obesity, and the metabolic syndrome also have important inflammatory components.

DIABETES AND CAUSES OF IMMUNE ACTIVATION IN ATHEROSCLEROSIS

The key question herein is to elucidate what is the direct cause of the immune reactions in atherosclerosis, in general and in diabetes, and also how the inflammatory disease process can be influenced. There are several major hypotheses, non-mutually exclusive.

OXIDATION AND OTHER MODIFICATIONS OF LDL AND OTHER MOIETIES

Low-density lipoprotein can be modified by oxidation and/or enzymatic modification phospholipases being one example. LDL is also normally present in tissues as the intima of arteries, where it can bind to the proteoglycan matrix especially after modification. This binding is thought to be an early event in atherogenesis according to the "response to retention" hypothesis (52, 53).

Oxidized low-density lipoprotein has pro-inflammatory and immune-activating properties, activating endothelial cells, monocytes/macrophages, and T cells (49, 54, 55). OxLDL also toxic at higher concentrations and an important feature of atherosclerotic lesions, perhaps somewhat understudied, is the abundance of dead cells. It is thus possible that OxLDL is one cause of such cell death (49, 54, 55). Enzymatically modified LDL could play a major role, and PLA2, which causes such modification, is expressed in both normal arteries and atherosclerotic lesions (56) and can induce activation of DC (57). Inflammatory phospholipids such as lysophosphatidylcholine (LPC) and/or platelet activating factor (PAF)-like lipids cause much of OxLDL:s effects which can occur through the PAF-receptor (58–61) or other mechanisms including Toll-like receptor- and scavenger receptor-interaction (62, 63).

In general oxidized phospholipids (OxPL) are implicated in immune reactivity in atherosclerosis, and could be derived from LDL-modification but also from cell membrane changes. Such oxPL include LPC, and often, a shortened sn-2 position in the fatty acid moiety serves as a danger-associated molecular patterns (DAMP). Oxidation turns OxPLs into markers of modified self, which are recognized by both soluble and cell bound receptors such as scavenger receptors, natural antibodies, and also C-reactive protein (CRP). The common theme in these different system is likely to be removal of senescent and dead cells, but also oxidized or otherwise modified lipoproteins. This has been described in several recent publications (62, 64–69).

Another important example of DAMP, in addition to PC and oxPL epitopes, is malonyl-dialdehyde (MDA) which is also generated during LDL-oxidation. MDA forms adducts on proteins, carbohydrates, and DNA (63).

Other compounds which could be implicated as atherogenic in OxLDL are modified and/or oxidized forms of apoB and cholesterol. Such modified compounds may play a role but the underlying mechanisms need to be better defined (63, 70). Clinical studies support the hypothesis of inflammatory phospholipids as causes of atherosclerosis, whereas levels of OxLDL are raised in the metabolic syndrome (71), in hypertension (72) and in established type 2 diabetes (73). Further, high levels of MDA-LDL in isolated immune complexes predict future MI and acute CVD events in patients with type 2 diabetes (74).

Many epidemiological studies demonstrate that smoking is associated with atherosclerosis and CVD (75, 76) and animal experiments demonstrate that smoking promotes atherogenesis (77–79). Somewhat surprisingly, underlying mechanisms are not fully clarified. Still, one mechanism is smoking-induced increased lipid-oxidation (80) and closely related to this, oxidative stress (81). Smoking is associated with systemic and local inflammation with raised levels of pro-inflammatory cytokines and cells, in particular in chronic obstructive pulmonary disease (82), which could play a role also in atherosclerosis.

Additionally, several factors appear to be diabetes-specific and able to further aggravate atherosclerosis (and CVD) among diabetics. One major such factor is reduction of endothelial nitric oxide (NO)-levels, leading to deterioration of endothelial function, an early sign of vascular problems and increased risk of atherosclerosis and CVD. Hyperglycemia *per se* leads to increased levels of reactive oxygen species, which in turn inactivate NO and thus impair endothelial function. One common denominator is the formation of advanced glycation end products (AGEs) which have pro-inflammatory and potentially atherogenic properties (83–88). Interestingly, atherosclerosis is suppressed by the soluble receptor

for AGEs in an animal model (89). Also susceptibility of LDL and its subfractions to glycation could play a role, in diabetes type 2 (90) AGE-products of LDL are able to generate autoantibodies, which on the other hand appear to have pro-inflammatory properties (91). Another interesting connection between AGEs and OxLDL are previous findings where AGEs initiate LDL oxidation (92). Further, increased oxidation of LDL in diabetes could promote atherogenesis (73, 93).

Other potential underlying factors are present in type 2 diabetes, but where the connection to immune reactivity in atherosclerosis is less clear, and thus not a focus herein. These include increased circulating free fatty acids in patients with abdominal obesity, which could be atherogenic by decreasing HDL-levels, increasing levels of small dense LDL and also by a negative effect on endothelial function (3, 94). Other mechanisms include effects on smooth muscle cells, such as induction of apoptosis and thus plaque instability (95) and also a hypercoagulative state by alterations in platelet function (96).

AUTOANTIBODIES AND IMMUNE COMPLEXES RELATED TO PHOSPHOLIPID EPITOPES AND OxLDL

An interesting possibility is that immune complexes containing OxLDL could contribute to vascular damage by promoting inflammation and atherosclerosis, which has been reported in type 2 diabetes (97, 98). In addition to oxLDL, also MDA-LDL and AGE-product-modified LDL induce immune responses in humans (99). The role of antibodies against these different forms of modified LDL is less clear, and varies in different studies: both negative and positive associations have been described. This could depend on different methods used, different degrees of oxidation and also on different immunoglobulin subclasses and isotypes. On the other hand, the antigenic constitution of immune complexes formed with modified forms of LDL and their corresponding antibodies may influence their pathogenicity, as suggested by the recent observation that high levels of highly oxidized forms of LDL (MDA-LDL) in isolated IC predict future MI and acute CV events in patients with type 2 diabetes (74).

It is also possible that there are differences between mouse and man which could be the basis for the conflicting views about the pathogenic or protective role of the antibody response to modified forms of LDL (100, 101).

Another type of antibodies that has attracted attention are those against PC (anti-PC), which are often considered as "natural" antibodies. We have reported in several publications that anti-PC is a protection marker for atherosclerosis development and CVD, in different populations, both healthy individuals, ACS patients, and patients with SLE or RA (50, 102–107). Animal experiments also support a protective role of anti-PC. Immunization with pneumococci containing PC induced a decrease in atherosclerosis development in a mouse model in parallel with an increase in anti-PC, among other antibodies (108). Passive and active immunization raising anti-PC levels decrease atherosclerosis in mouse models (109, 110).

Human anti-PC could be anti-atherogenic and decrease risk of CVD by anti-inflammatory effects (107), inhibition of oxLDL-uptake through scavenger receptors (111) and inhibition of LPC-induced cell (112). In mouse models, the anti-inflammatory effect of anti-PC was confirmed, and facilitating phagocytosis was reported as one mechanism. Anti-PC antibodies bind dead and dying cells, enhancing their phagocytosis and clearance (113). Further investigations are necessary to determine whether the data generated in animal models translates to human medicine.

A Western life style could play a role to influence anti-PC levels, and one underlying factor could be infections which are not prevalent in developed countries (114, 115). Gluten in the diet could also play a role being a novel component of human diet from an evolutionary point of view (116). The heritability of anti-PC is 37%, allowing also for genetic factors (117). In line with these findings, a recent report indicates that antibodies against reactive a-dicarbonyls such as methylglyoxal (MGO) are negatively associated with atherosclerosis development among patients with diabetes type 2. This finding suggests an additional role played by diabetes type 2 immune reactivity in atherosclerosis development as compared to the general population (118). Still, it is interesting to note that MGO-LDL is only weakly immunogenic in humans (119). Clearly, further research is needed to clarify the role by AGE-recognizing antibodies in diabetes type 2 and atherosclerosis.

A potential role of epigenetic changes in both atherosclerosis/CVD and type 2 diabetes is not the topic of this review, though a very interesting subject. One can not exclude that epigenetic mechanisms may influence immune mechanisms in both these conditions. In an intriguing study, it was recently demonstrated that in an atherosclerosis mouse model, maternal immunization with oxidized LDL affects *in utero* programing of both atherosclerosis and IR and type 2 diabetes, promoting protection against development of these conditions (120) Whether this can be translated to humans also remains to be demonstrated.

HEAT SHOCK PROTEINS

Antibodies against heat shock proteins (HSPs), especially HSP60/65 but also others like HSP70 and HSP90 have been described as potential causes of atherosclerosis and CVD. HSPs are immunogenic, and T-cell clones recognizing HSP60 are present in atherosclerotic plaques (121, 122). HSPs may activate immune reactions through cross-reactivity with HSP from microorganisms as bacteria. This is supported by both clinical data with associations between antibodies against HSP60/65 and atherosclerosis, and experimental data where immunization with HSP 60/65 increases atherosclerosis in an animal model (26, 123).

We hypothesized that hypertension possibly could cause and immune reaction and inflammation in arteries by induction of HSP 60/65, which are also induced by oxLDL (124, 125) and in principle, in conditions such as diabetes, where LDL-oxidation is implicated, this could further enhance the progression of atherosclerosis.

INFECTIONS

Infections have since long been hypothesized to be a cause of atherosclerosis. Many pathogenic candidates have been proposed, one not excluding another. *Chlamydophila pneumoniae* (CP); periodontal organisms including *Porphyromonas gingivalis* (PG) and *Aggregatibacter actinomycetemcomitans* (AA); *Helicobacter pylori* (HP) and Cytomegalovirus (CVM) are among the most promising

candidates, since they are present in plaques, promote atherosclerosis in animal studies, and have associations with disease in humans (126).

Early studies demonstrated presence of CP in atherosclerotic plaques (127) and that antibodies against CP were associated with CVD (128–130) but later also negative studies were published (126).

However, treatment with CP-targeting antibiotics had no effect on atherosclerosis (131–133). This clearly argues against CP as a causative agent, though there could be other explanations, for example, CP is difficult to reach within the plaque and thus it is still possible that at the earlier stages of CP-infection antibacterial treatment could be beneficial (126).

Periodontal pathogens, as PG and AA, are also interesting pathogenic candidates. The association with periodontitis and CVD/atherosclerosis has been debated since there are confounders, including social ones, which may be difficult to control for. A recent statement from the American Heart Association supports an independent association between periodontal disease and atherosclerosis, but available data do not prove causation, even though it is interesting that intervention does decrease systemic inflammation and improves endothelial function (134).

Still it is interesting that periodontitis and diabetes could be related to each other, and in principle, periodontitis could be diabetogenic and then also increase the risk of atherosclerosis and CVD, while diabetes could increase the risk of periodontitis (135–137).

An inherent problem with viral infections such as CMV from the Herpes virus group is that they are very common, thus making the interpretation of any associations with diabetes, atherosclerosis or CVD complicated. It may be that certain patients subgroups that are prone to accelerated atherosclerosis and CVD, as it is seen for example after organ transplantation, CMV infection plays an important role (138). CMV is present in atherosclerotic lesions in many but not all studies (126), but also in healthy arteries (139) so in principle CMV could be just an innocent by-stander. However, CMV induces migration of arterial smooth muscle cells *in vitro*, suggesting potential pro-atherogenic mechanisms (140).

Helicobacter pylori infection, causing gastritis and gastric ulcer, may be implicated as supported by indirect evidence, where reduction of CVD after eradication of HP was reported (126, 141). However, no viable HP had been isolated from atherosclerotic plaques, and mouse experiments in general do not support a pathogenic role of HP in atherosclerosis (142).

Other pathogens including HIV, EBV, influenza virus, *Mycoplasma pneumoniae* and *Streptococcus pneumoniae* have also been discussed, but as of yet there is no conclusive causative evidence available (126). Borreliosis caused by spirochetes, is independently associated with CVD, although little is known from atherosclerosis-related experimental studies (143).

It is not clear if infections on the diabetic background contribute more to atherosclerosis, but it is interesting to note that an increased rate of infections with *Chlamydia pneumoniae* has been reported in patients with diabetes type 2 (144).

Taken together, even though the infection hypothesis in atherosclerosis with or without diabetes type 2 is very interesting and is supported by circumstantial evidence, there is still little direct evidence of a potential causative or pathogenic role of microorganisms in CVD/atherosclerosis. However, we can not exclude a possibility that infectious agents act in concert with diabetes-specific factors to promote atherosclerosis.

Infections could also be of importance indirectly. Many pathogens are present in lesions, and could start or promote an ongoing local inflammatory process which could lead to increased atherosclerosis and, in principle, also to CVD and plaque rupture – at such sites, the local inflammation appears to be especially strong (5). Also the total infectious burden is associated with increased atherosclerosis and CVD, and the risk of infection is raised in diabetes, especially when blood glucose levels are not well controlled. Platelet aggregation and endothelial dysfunction, could also influence atherogenesis in this context (126, 145).

OTHER TYPES OF IMMUNE ACTIVATION IN DIABETES TYPE 2 AND ATHEROSCLEROSIS

Another potential link between atherosclerosis and diabetes type 2, and thus also hyperglycemia and metabolic dysfunction, is provided by a recent study, where NKG2D, an immune-activating receptor expressed by different types of immune cells was tested (146). The authors reported that blocking NKG2D in apolipoprotein E-deficient (apo $E^{-/-}$) mice led to a dramatic reduction in plaque formation, suppressed systemic and organ-specific inflammation, and improved abnormal metabolic conditions. Thus the NKG2D/ligand interaction could drive both: inflammation related to metabolic dysfunction/diabetes type 2 and atherosclerosis. Further, the molecules and pathogens discussed earlier such as OxLDL, HSPs, AGEs, and infectious agents, could upregulate NKG2D on different cell types, which in turn can activate T-cells, NK, and NKT cells and thus promote atherosclerosis; also other cell types as endothelial cells could produce pro-atherogenic factors, including cytokines (146).

It is well known that inflammation is a link between IR, obesity, and diabetes. Further abdominal obesity and IR are classic features of type 2 diabetes (147).

There are many examples of links between inflammation in other conditions and atherosclerosis. Periodontitis is also a systemic inflammatory condition, which is more frequent in patients with diabetes, could increase the risk of atherosclerosis and CVD also indirectly (137).

Raised levels of CRP is a risk marker for atherosclerosis and CVD in many studies, though it is not clear if CRP is either protective or detrimental for disease development. Cytokines as IL-6 and raised systemic levels of OxLDL are other examples of pro-inflammatory molecules that are associated with increased risk of CVD and atherosclerosis (10). Another group of inflammatory compounds that are present in advanced atherosclerotic plaques are lipid mediators, such as leukotrienes (148, 149).

Another example of associations between systemic inflammation and atherosclerosis and CVD is a number of autoimmune diseases. The evidence is strongest for SLE, where the risk of CVD is very high (13, 14, 150). Also in RA and other types of autoimmune conditions, atherosclerosis is increased according to many studies including a recent meta-analysis (13–15).

TREATMENT AGAINST INFLAMMATION AND IMMUNE ACTIVATION TO AMELIORATE ATHEROSCLEROSIS, DECREASE RISK OF CVD, ESPECIALLY IN DIABETES

OPTIMAL DIABETES TREATMENT

Since diabetes type 2 is an inflammatory condition, first and foremost, control of the disease itself is likely of major importance for amelioration of pro-atherogenic inflammation and immune reactions. The specific optimal range of glycemic control in relation to CVD and atherosclerosis is still debated (151). This is similar to the situation in rheumatic diseases, where at least for RA, the risk may be similar to that in type 2 diabetes and where disease controls appears to be of importance (13).

STATINS

Statins were developed to treat hyperlipidemia and are often used in diabetes, though there is still some controversy about their exact role in this condition (152). Interestingly, statins have additional anti-inflammatory effects rather than simply decreasing cholesterol levels, which could be of major importance in atherosclerosis and CVD, not least in diabetic patients. These properties could be seen as "side-effect" of HMG CoA-reductase inhibition. Interestingly, statins have immunomodulatory properties, such as impairment of CD1d-mediated antigen presentation through the inhibition of prenylation (153) and decreasing MHC class II interaction with antigen (154). Further, the lipid-lowering effect of statins could by itself influence the immune reactions to LDL-related antigens, since a decrease in LDL-levels is likely to decrease also exposure of the immune system to modified forms of LDL, thus reducing the intensity of the corresponding immune response.

The Jupiter study demonstrated that statin treatment may be beneficial for individuals with increased high sensitivity CRP but normal LDL and it is possible that the beneficial effects of statins to some extent are in fact caused by their anti-inflammatory and immune-modulatory properties (155, 156).

ANTI-INFLAMMATORY TREATMENT

Even though the major focus of this review is immune activation it is still interesting to discuss this in the context of inflammation in general, especially in relation to treatment. There is as yet no established anti-inflammatory treatment of atherosclerosis or CVD, with or without diabetes. There are several interesting possibilities that are currently being investigated. In RA, treatment with methotrexate weekly is a very common schedule, and a recent meta-analysis demonstrate that the risk of CVD is decreased in patients with RA treated with methotrexate (157). Animal studies show that methotrexate decreases atherogenesis (158). In the cardiovascular inflammation reduction trial (CIRT) low dose methotrexate (target dose 20 mg/week) is tested for reduction of CVD events among post-MI patients with diabetes or metabolic syndrome (156). There are different opinions about whether biologics as anti-TNF are beneficial from a cardiovascular point of view, though a recent study where decrease of CVD was reported in RA adds support to this possibility (159, 160). The Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) investigates interleukin-1β (IL-1β) inhibition and reduced risk of MI, stroke, and CVD in stable coronary artery disease patients with persistent elevations of CRP (≥2 mg/L) (156, 161).

Other interesting anti-inflammatory treatments could be inhibition of inflammatory lipid mediators as PAF (162). Annexin A5 is anti-thrombotic plasma protein, which is anti-inflammatory, inhibits atherosclerosis development and improves endothelial function in a mouse model (163). Annexin A5 could thus be a possible therapy candidate. Inhibition of phospholipases as treatment in patients after ACS are currently in trials (164).

IMMUNOMODULATORY THERAPY

Studies in mid-90s demonstrated that immunization with modified forms of LDL ameliorated atherosclerosis in animal models (27), providing initial evidence for immunomodulation as a potential treatment against atherosclerosis. One line of treatment is to target the apoB components which decreases atherosclerosis development in animal models (70, 165). However, such treatment did not show positive expected effect in humans (166), though the possibility that the end points used in this study where not optimal cannot be excluded.

Another possibility is that the phospholipid moiety on OxLDL could be the basis of immunomodulation, one example being PC. This is supported by clinical studies, animal and *in vitro* experiments (50). As discussed, mechanisms include anti-inflammatory (107, 167), inhibition of cell death (112), and decreased uptake of oxLDL in macrophages (105). Further, administration of immunoglobulins has shown promising results in animal studies (168). Another interesting possibility is to ameliorate atherosclerosis by modulating immune reactions against HSP (169, 170).

SUMMARY AND CONCLUSION

Taken together, atherosclerosis and ensuing CVD represents major health problems in the developed world and especially so in patients with diabetes, where macro-vascular complications is a common problem. It has become clear that atherosclerosis on a background of diabetes or without is a chronic inflammation characterized by presence of activated immune competent cells throughout the lesions. Atherosclerosis in diabetes, though accelerated, does not seem to be very different from atherosclerosis in individuals without diabetes type 2, although certain diabetes-specific factors could contribute to pro-atherogenic immune activation and thus aggravate atherosclerosis and risk of CVD. In diabetes, reduction of endothelial NO-levels, systemic hyperglycemia if uncontrolled, generation of reactive oxygen species, oxidative stress, and increased LDL-oxidation, formation of AGEs, and increased circulating free fatty acids are factors that add to atherosclerosis and CVD risk. Potential triggers of immune activation in atherosclerosis in the general population (as well as among diabetics) include OxLDL and other LDL modifications and corresponding antibodies, possibly infections, high levels of heat shock protein antibodies, and low levels of natural antibodies as anti-PC. Clinical trials and other studies of immune-modulatory and anti-inflammatory treatment in atherosclerosis and CVD are currently conducted but until such treatments are proven to be effective in humans, the exact role of immune reactions and inflammation in human atherosclerosis with or without type 2 diabetes remains to be clarified.

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Diabetes alters activation and repression of pro- and anti-inflammatory signaling pathways in the vasculature

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A central mechanism driving vascular disease in diabetes is immune cell-mediated inflammation. In diabetes, enhanced oxidation and glycation of macromolecules, such as lipoproteins, insults the endothelium, and activates both innate and adaptive arms of the immune system by generating new antigens for presentation to adaptive immune cells. Chronic inflammation of the endothelium in diabetes leads to continuous infiltration and accumulation of leukocytes at sites of endothelial cell injury. We will describe the central role of the macrophage as a source of signaling molecules and damaging by-products which activate infiltrating lymphocytes in the tissue and contribute to the pro-oxidant and pro-inflammatory microenvironment. An important aspect to be considered is the diabetes-associated defects in the immune system, such as fewer or dysfunctional atheroprotective leukocyte subsets in the diabetic lesion compared to non-diabetic lesions. This review will discuss the key pro-inflammatory signaling pathways responsible for leukocyte recruitment and activation in the injured vessel, with particular focus on pro- and anti-inflammatory pathways aberrantly activated or repressed in diabetes. We aim to describe the interaction between advanced glycation end products and their principle receptor RAGE, angiotensin II, and the Ang II type 1 receptor, in addition to reactive oxygen species (ROS) production by NADPH-oxidase enzymes that are relevant to vascular and immune cell function in the context of diabetic vasculopathy. Furthermore, we will touch on recent advances in epigenetic medicine that have revealed high glucose-mediated changes in the transcription of genes with known pro-inflammatory downstream targets. Finally, novel anti-atherosclerosis strategies that target the vascular immune interface will be explored; such as vaccination against modified low-density lipoprotein and pharmacological inhibition of ROS-producing enzymes.

Keywords: Nox, diabetes complications, atherosclerosis, immune cells, inflammation

INTRODUCTION

A unifying feature of diabetic complications is chronic inflammation of the vasculature. The main vascular diseases that burden diabetic patients include nephropathy, retinopathy, neuropathy, and atherosclerosis. Each of these conditions has a significant immune component. In experimental and human diabetic nephropathy, infiltrating macrophages, and T cells elaborate a host of pro-inflammatory, pro-fibrotic, and pro-angiogenic factors that contribute to disease development and progression in the kidney (Lim and Tesch, 2012). Leukocyte adherence is causally associated with endothelial cell injury and cell death in the diabetic retina (Joussen et al., 2001) while infiltration of post-capillary venules with polymorphonuclear leukocytes is an early feature of proximal diabetic neuropathy (Kelkar et al., 2000). Similar to the microvasculature, immune-mediated inflammation of the macrovasculature plays a central role in the pathogenesis of diabetes-accelerated atherosclerosis (Libby et al., 2002). In this review, we examine the role of the immune response in the pathogenesis of atherosclerosis as a prototype for diabetes-associated vasculopathies.

TRIGGERS OF INFLAMMATION ELEVATED IN DIABETES COMPARED TO THE NON-DIABETIC DISEASE STATE

Atherosclerotic lesions represent an excessive inflammatory, fibroproliferative response against different noxious stimuli (Ross, 1993, 1997). The diabetic milieu comprises of a host of potentially harmful, immunogenic products including modified forms of low-density lipoprotein (LDL), advanced glycation end products (AGEs), reactive oxygen species (ROS) as well as pro-inflammatory chemokines and cytokines. Elevated levels of LDL in patients with diabetes are subject to modification by both oxidation and glycation (Cohen et al., 2004). Furthermore, accelerated generation and vascular deposition of AGEs in addition to AGE interactions with RAGE in diabetes initiate oxidative reactions that promote the formation of oxidized LDL (oxLDL) (Basta et al., 2004). Oxidation of LDL within the sub-endothelial space (intima) enhances the pro-inflammatory properties of the endothelium (Mazière and Mazière, 2009) and activates both innate and adaptive arms of the immune system (Binder et al., 2002; Hansson et al., 2002).

RELATIONSHIP BETWEEN THE ENDOTHELIUM AND LEUKOCYTES IN EARLY ATHEROGENESIS

Chronic injury to the endothelium results in endothelial dysfunction, which can be defined as increased permeability, reduced nitric oxide (NO) dependent vasodilatation as well as enhanced pro-thrombotic and pro-inflammatory properties (Hink et al., 2001; Davignon and Ganz, 2004; Hartge et al., 2007). Endothelial dysfunction is a well established precursor of atherosclerosis, particularly in the setting of diabetes (Schalkwijk and Stehouwer, 2005). The diabetes-associated factors that impair normal endothelial function include increased synthesis of vasoconstrictors such as angiotensin II (Ang II) and endothelin-1, uncoupling of endothelial nitric oxide synthase (eNOS; leading to reduced bioavailability of NO), and increased expression and activity of ROS-producing enzymes (such as NADPH oxidases, Nox) which trigger the expression of adhesion and chemotactic molecules that promote the recruitment of inflammatory cells to the arterial wall (**Figure 1**) (Hink et al., 2001; Guzik et al., 2002; Lüscher et al., 2003; Hartge et al., 2007). Aortic lesions of diabetic *ApoE* knockout (KO) mice show increased gene expression of pro-inflammatory molecules MCP-1, VCAM-1, and NF-κB subunit p65 associated with increased pro-atherogenic cellularity [macrophages, T cells, and smooth muscle cells (SMCs)] compared to non-diabetic controls (Soro-Paavonen et al., 2008). In line with these *in vivo* findings, human endothelial cells exposed to high glucose conditions show increased leukocyte binding associated with increased expression of E-selectin, ICAM-1, VCAM-1, and MCP-1 through activation of NF-κB (Kim et al., 1994; Morigi et al., 1998; Piga et al., 2007). In addition, recent studies have revealed a striking association between glucose-induced endothelial expression of adhesion molecules VCAM-1 and P-selectin as well as the chemokines MCP-1 and fractalkine with Nox-derived ROS production (Manduteanu et al., 2010; Gray et al., 2013). These data indicate that diabetes-associated hyperglycemia and oxidative stress promote leukocyte-endothelial cell interactions required for the recruitment of leukocytes to the inflamed vessel.

ROLE OF SMOOTH MUSCLE CELLS IN DIABETES-MEDIATED VASCULAR INFLAMMATION

Vascular SMCs play a central role in the initiation and progression of atherosclerosis (Doran et al., 2008). The expression of cellular adhesion molecules and pro-inflammatory cytokines by

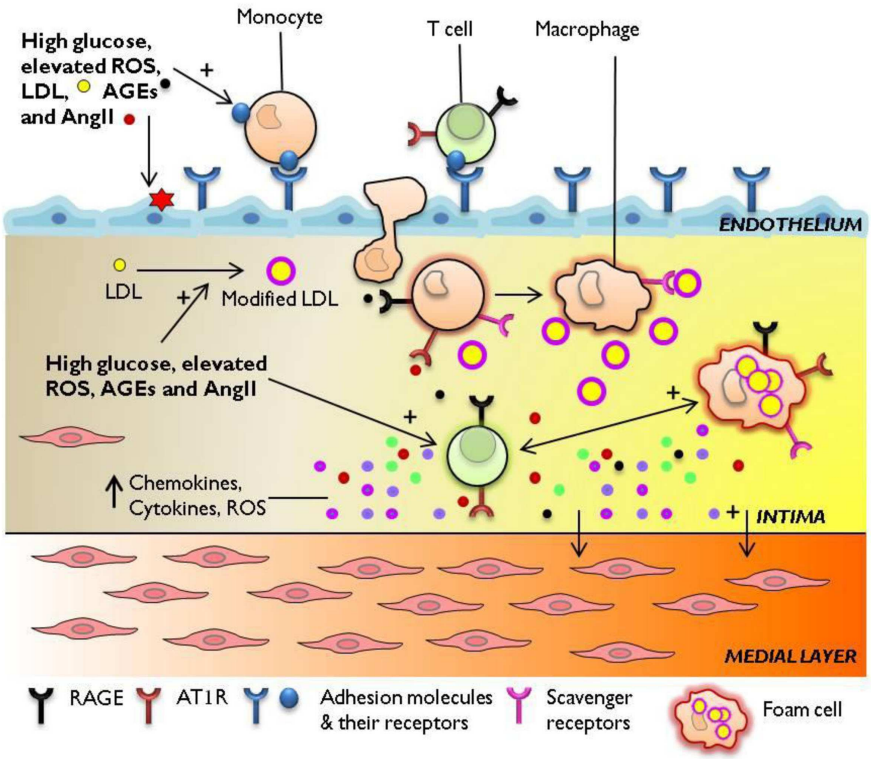

FIGURE 1 | Immune mechanisms engaged in diabetes-accelerated atherosclerosis. Diabetes-associated hyperglycemia, hyperlipidemia, and oxidative stress render the endothelium dysfunctional, leading to the retention and oxidation of LDL molecules in the intimal space. The increased expression of adhesion molecules E-selectin, ICAM-1, VCAM-1 at the endothelial membrane, and upregulation of chemotactic molecules such as MCP-1 facilitate the continuous infiltration of immune cells to the inflamed aorta. Resident and monocyte-derived macrophages engulf LDL to form foam cells which release a host of pro-inflammatory cytokines, protease, and ROS. Activated T cells recruited from the circulation to the lesion also secrete cytokines which amplify pro-inflammatory cellular immune responses in the diabetic plaque. The diabetes-mediated increase in vascular inflammation drives the development and progression of atherosclerosis. AGEs, advanced glycation end-products; AT1R, angiotensin II type 1 receptor; ICAM-1, intercellular adhesion molecule-1; LDL, low-density lipoprotein; MCP-1, monocyte chemotactic protein-1; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule-1.

lesional SMCs promote the accumulation and activation of leukocytes in the atherosclerotic lesion (Braun et al., 1999; Raines and Ferri, 2005). Of note, the expression of VCAM-1, ICAM-1, and fractalkine is confined to SMCs within atherosclerotic vessels (or lesion-prone areas) but not in healthy vessels (O'Brien et al., 1993; Endres et al., 1997; Barlic et al., 2007); providing further evidence of the pro-atherogenic role of these molecules. In response to diabetes-associated atherogenic stimuli, such as Ang II (Ruiz-Ortega et al., 2003) and ROS (Su et al., 2001), SMCs elaborate an array of extracellular matrix proteins which facilitate the retention of inflammatory cells and stabilization of the plaque including the formation of a fibrous cap. Diabetes stimulates and sustains the inflammatory "synthetic" phenotype of vascular SMCs. Hyperglycemia enhances the migration and proliferation of resident SMCs within the intimal lesion which contributes to the accelerated formation of advanced atherosclerosis in diabetes (Suzuki et al., 2001). Furthermore, exposure of cultured SMCs to high glucose or AGEs significantly increases the production of ROS by Nox enzymes (Inoguchi et al., 2000; Wautier et al., 2001; Gray et al., 2013). More specifically, recent evidence has identified a central role for the Nox1 isoform in glucose (Gray et al., 2013) and Ang II-induced vascular superoxide production (Lassègue et al., 2001). Therefore, vascular SMCs represent key players in diabetes-mediated pro-inflammatory and pro-oxidant responses that drive atherosclerosis.

MACROPHAGES ARE A SOURCE OF PRO-OXIDANT, PRO-INFLAMMATORY MOLECULES WHICH FUEL PRO-ATHEROGENIC PROCESSES IN THE VESSEL WALL

The macrophage plays a critical role in the initiation and progression of the atherosclerotic lesion. Circulating monocytes are recruited to inflamed regions of the endothelium where they engulf trapped LDL to form foam cells, histologically visible as the early fatty streak (Ross, 1997). Uptake of glycated LDL by human monocyte-derived macrophages occurs to a greater extent than for native LDL (Klein et al., 1995). Interestingly, glycoxidized LDL increases scavenger receptor expression to a greater extent than glycated LDL or oxLDL alone (Lam et al., 2004). Therefore, hyperglycemia in combination with elevated ROS in diabetes increases macrophage avidity for LDL, and thus foam cell formation, which ultimately activates other macrophages, infiltrating adaptive "effector" cells as well as resident vascular cells leading to accelerated atherosclerosis.

The lipid-laden macrophage acts as a frustrated phagocyte, leaking ROS (refer to Nox section), and metalloproteases (MMPs) into the extracellular space which catalyze the degradation of the extracellular matrix proteins and trigger apoptosis of SMCs that support the plaque's fibrotic cap, ultimately enhancing the risk of plaque rupture (Shah et al., 1995). Macrophages function in response to environmental cues (Waldo et al., 2008). Monocyte-derived macrophages cultured in high glucose (25 mM) conditions display increased expression and activity of MMP-9 without affecting metalloproteinase inhibitor (TIMP-1) expression (Death et al., 2003). In line with these findings, Devaraj et al. (2006) showed that monocyte superoxide anion release, pro-inflammatory cytokines interleukin (IL) 6, and IL1β were significantly elevated in type 1 diabetic subjects compared with in control subjects. These striking effects of hyperglycemia on macrophage activity may provide some insight into the significantly increased incidence of plaque rupture, thrombosis, and ultimately myocardial infarction in diabetic patients compared to their non-diabetic counterparts (Silva et al., 1995).

INTERACTIONS BETWEEN LEUKOCYTES WITHIN THE LESION ENGAGE INFLAMMATORY PATHWAYS THAT DRIVE ATHEROSCLEROSIS

Monocyte-derived macrophages act as sentinels of pro-inflammatory signals, able to be activated by diverse stimuli and release a range of mediators that contribute to pro- as well as anti-atherogenic processes (Moore and Tabas, 2011). A plethora of cytokines are detected in atherosclerotic vessels, and the reader is directed to excellent reviews by Tedgui and Mallat (2006) and Ait-Oufella et al. (2011). Activated macrophages and foam cells release a number of pro-inflammatory cytokines and chemokines which facilitate the continual recruitment of monocytes and T lymphocytes to the atherosclerotic lesion. Of note, the predominance of IFNγ-producing T lymphocytes in the lesion is likely to contribute significantly to local macrophage activation (de Boer et al., 1999). Further gene expression analysis of advanced human plaques has revealed a cytokine profile indicative of a pro-inflammatory, Th1-type cellular immune response (Frostegård et al., 1999). The increased expression of CD40 and MHC-II molecules on plaque macrophages (and other plaque-associated vascular cells) also suggests an enhanced capacity of these cells to function as antigen-presenting cells to T lymphocytes (Hansson et al., 1991; Li et al., 1993; Mach et al., 1997). The potent immunostimulatory and pro-atherogenic effects of IFNγ are well established (Gupta et al., 1997), and so interactions between macrophages and T lymphocytes that continuously upregulate IFNγ production are expected to contribute to the progression of the lesion.

THE CYTOKINE PROFILE OF ACTIVATED VASCULAR CELLS/LEUKOCYTES IS ALTERED IN THE DIABETIC SETTING

Macrophages represent a heterogeneous population of distinct subsets capable of differentially affecting Th1- or Th2-type responses (Mills et al., 2000). Lesion macrophages adopt predominantly a pro-inflammatory and pro-atherogenic role indicative of the M1-subtype (Khallou-Laschet et al., 2010; Wilson, 2010; Shalhoub et al., 2011). Furthermore, hyperglycemia interferes with the ability of IL4 to polarize macrophages to the alternatively activated M2 state, characterized by wound repair and anti-inflammatory functions (Auffray et al., 2009; Parathath et al., 2011). A comprehensive review of macrophage differentiation and activation pathways in atherosclerosis can be found by Shalhoub et al. (2011). In a similar manner, hyperglycemia is associated with increased activation of pro-inflammatory human T lymphocytes and decreased suppressor function of T regulatory cell subsets (Marfella et al., 2003; Lindley et al., 2005; Stentz and Kitabchi, 2005). Therefore, not only does the diabetic micro-environment amplify the magnitude and array of new, potentially immunogenic stimuli but it also skews the leukocyte repertoire in favor of adopting a pro-inflammatory functional phenotype. Moreover, high glucose may interfere with the immunomodulatory capacity of T cell and

macrophage subsets leading to an unabated prolongation of the inflammatory process.

Patients with diabetes have an increased risk of infection attributable to defects in both innate and adaptive immunity (Geerlings and Hoepelman, 1999). Consistent with these findings in humans, streptozotocin (STZ)-induced diabetes in rodents predisposes to delayed innate (Chin et al., 2012) and adaptive (Vallerskog et al., 2010) responses irrespective of the challenge, leading to increased susceptibility to infection. High glucose conditions have been shown to decrease T and B lymphocyte proliferation, diminish cell viability, and increase apoptosis via mechanisms likely to involve increased oxidative stress (Rubinstein et al., 2008). This seems counter-intuitive in a disease characterized by secondary complications driven by immune cell-mediated inflammation, however the high glucose and high ROS diabetic milieu provides the ideal setting for chronic activation of innate and adaptive cells to non-microbial, modified macromolecules (described in section above). Therefore, it is important to discuss diabetes-induced alterations in the immune system in the context of any insults (infection) or complications (e.g., atherosclerosis) that accentuate as well as catalyze further derangement in both arms of the immune response.

SIGNALING PATHWAYS IMPORTANT TO HYPERGLYCEMIA-INDUCED VASCULAR PATHOPHYSIOLOGY

AGE/RAGE

Advanced glycation end products and their cell surface receptor, RAGE, have been implicated in the amplification and progression of immune-inflammatory responses that underscore diabetic complications (Schmidt et al., 2001; Basta et al., 2004; Jandeleit-Dahm et al., 2008; Bierhaus and Nawroth, 2009). Atherosclerotic lesions in diabetic *ApoE* KO mice display increased accumulation of AGEs and enhanced expression of RAGE (Kislinger et al., 2001; Wendt et al., 2002; Soro-Paavonen et al., 2008). Ligation of RAGE by AGE stimulates endothelial pro-inflammatory gene expression (VCAM-1, E-selectin) and ROS production in a NADPH-oxidase dependent manner (Schmidt et al., 1995; Wautier et al., 2001; Higai et al., 2006). In macrophages, AGE–RAGE interaction prompts cell chemotaxis and cytokine release via a mechanism involving the activation of NADPH oxidase (Kirstein et al., 1990; Wautier et al., 2001). RAGE also appears to play a role in T lymphocyte activation and differentiation, mediating Th1-type responses such as the synthesis of IFNγ (Chen et al., 2008). Recent work by Akirav et al. (2012) found that RAGE is expressed intracellularly in human T cells following TCR activation but constitutively on T cells from patients with diabetes. The current evidence supports a critical role of AGE/RAGE signaling in the chronic activation of the immune-inflammatory processes that accelerate atherosclerosis in diabetes.

ANG II/AT1R

The renin-angiotensin system (RAS) plays an important role in the regulation of vascular contractility and also represents a primary target for diabetes-induced vascular dysfunction and inflammation. Most of the known pathophysiological effects of Ang II are mediated via the activation of the Ang II type 1 (AT1) receptor. The production of Ang II and the expression of its AT1R are upregulated in the aorta of diabetic mice (Candido et al., 2002, 2004). Signaling via the AT1R has been shown to upregulate the RAGE pathway in diabetic atherosclerosis (Ihara et al., 2007). Furthermore, Ang II induces activation with increased adhesion molecule expression in endothelial cells, monocytes, and T lymphocytes (Hahn, 1994; Tummala et al., 1999; Hoch et al., 2009). Most of the signaling events secondary to Ang II-AT1R binding are redox sensitive, such as NFκB activation, and rely on the production of ROS by Nox enzymes (Pueyo et al., 2000; Alvarez and Sanz, 2001; Liu et al., 2003). Treatment of diabetic mice with an AT1R blocker such as Candesartan attenuated ROS production and Nox activity leading to improvements in endothelial function (Oak and Cai, 2007). Recent work by Valente et al. (2012) revealed a direct physical association between the AT1R and the Nox1-NADPH-oxidase isoform in vascular SMCs, however the potential functional and regulatory implications of this relationship remain to be explored in the context of hyperglycemia. A common feature of AGE and Ang II signaling is the convergence on Nox-derived ROS as secondary mediator molecules required to elicit the effects of RAGE and AT1 receptor binding, respectively.

NOX/ROS

Hyperglycemia induces activation of the Nox family NADPH-oxidase enzymes and the consequent ROS production contributes to the pathophysiological complications of diabetes (Gao and Mann, 2009). Nox enzymes are the major source of ROS in the vessel wall, differentially expressed in vascular and infiltrating inflammatory cells (Griendling and Ushio-Fukai, 1997; Lassègue and Clempus, 2003). In particular, the isoforms Nox1, Nox2, Nox4, and Nox5 play important roles in a wide range of physiological and pathological processes relevant to cardiovascular disease (Sorescu et al., 2002; Bedard and Krause, 2007; Lassègue and Griendling, 2010). Increased ROS production in the diabetic aorta positively correlates with upregulated expression and activity of Nox1, Nox2, and Nox4 (Guzik et al., 2002; Wendt et al., 2005; Hwang et al., 2007). Nox1 KO mice are protected from endothelial dysfunction in diabetes (Youn et al., 2012) while studies in Nox4 KO mice have reported a vasculo-protective role for endogenous Nox4 (Schröder et al., 2012). Furthermore, recent work by Gray et al. (2013) in Nox1 and Nox4/*ApoE* DKO mice identified a key role for Nox1 but not Nox4-derived ROS in diabetes-accelerated atherosclerosis which is consistent with previous reports suggesting different (patho)physiological functions for Nox isoforms in the vasculature.

Reactive oxygen species are both a product, and modulator, of leukocyte recruitment, activation, and function. Ligation of VCAM by leukocytes activates endothelial NADPH oxidase which results in enhanced ROS production (Deem and Cook-Mills, 2004). In macrophages, Nox2 (gp91phox) is the major source of ROS contributing to the respiratory burst, while Nox4 is inducible by oxLDL (Lee et al., 2010; Tavakoli and Asmis, 2012). T lymphocytes also express a phagocyte-type Nox that is activated after TCR stimulation (Jackson et al., 2004). Further activation of vascular Nox by NFκB-dependent pro-inflammatory cytokines, including TNFα, reaffirms the relationship between oxidative stress and immune-mediated inflammation in diabetes (Park et al., 2006; Gauss et al., 2007; Miller et al., 2007). The potential impact of

diabetes-mediated changes in Nox on ROS-sensitive leukocyte functions represents a relatively unexplored area of investigation.

ATHERO-PROTECTIVE MECHANISMS: FOCUS ON ANTIOXIDANTS

We have discussed some of the key pro-inflammatory, pro-atherosclerotic effects of Ang II/AT1R and AGE/RAGE signaling pathways that are aberrantly activated in response to hyperglycemia. However, it is important to note that these same pathways can elicit anti-inflammatory effects, and the reader is encouraged to consult Goh and Cooper (2008), Daugherty et al. (2010), and Thomas et al. (2010) for detail on alternative AGE receptors, AT2R, and ACE2. While pro-inflammatory signaling cascades tend to predominate in diabetes, the inactivation of some important athero-protective pathways by the diabetic milieu further exposes the diabetic vasculature to pro-oxidant insults that accelerate atherogenesis. Major anti-oxidant defense systems such as superoxide dismutases (SODs), and glutathione peroxide 1 (Gpx1) are critical in the maintenance of redox balance in the intracellular and extracellular spaces. Diabetic *ApoE/GPx1* DKO mice show significantly more aortic atherosclerosis associated with enhanced macrophage recruitment, RAGE, and VCAM-1 expression compared to the diabetic *ApoE* KO mice (Lewis et al., 2007). Therefore, a decrease in anti-oxidant capacity in diabetes in combination with elevated ROS production from various sources, contributes to the pro-oxidant diabetic milieu (Feillet-Coudray et al., 1999; Vessby et al., 2002).

AN EMERGING ROLE FOR EPIGENETIC MODIFICATIONS IN DIABETIC COMPLICATIONS

The effect of hyperglycemia on the transcription and translation of genes coding proteins involved in the inflammatory response persist beyond the time of exposure. A growing number of studies utilizing the bio-informatic power of epigenetic medicine have already identified chromatic modifications characterized by histone methylation and acetylation sites, and their associated enzymes, involved in glucose-mediated changes in gene expression (Brasacchio et al., 2009; Pirola et al., 2011; Keating and El-Osta, 2012; Miao et al., 2012). El-Osta et al., identified a role for Set7, a H3K4-specific methyltransferase, in transient hyperglycemia-mediated chromatin changes at the promoter of the NF-κB subunit p65 resulting in increased gene expression of p65, MCP-1, and VCAM-1 in vascular endothelial cells that persist during subsequent normoglycemia; a phenomenon termed "glycemic memory" (El-Osta et al., 2008; Okabe et al., 2012). Similarly, *ex vivo* culture of vascular SMCs from type 2 diabetic *db/db* mice exhibit a sustained atherogenic and pro-inflammatory phenotype with concomitant depletion of the H3K9-specific methyltransferase, Suv39h1, despite restoration of euglycaemia (Villeneuve et al., 2010). Further investigations suggest that regulation of pro-inflammatory gene expression by Suv39h1 is dependent on glucose-mediated elevations in the microRNA (miR)-125b (Villeneuve et al., 2008).

Recent advances in cancer research have demonstrated cross-talk between miR machinery and DNA methylation (Ting et al., 2008; Yan et al., 2011). While there is growing evidence supporting glucose-induced alterations in chromatin structure and miR expression in the pathogenesis of diabetic complications (Reddy and Natarajan, 2011; Villeneuve et al., 2011), little is known about the interplay of these components in diabetes.

Epigenetic mechanisms play a central role in the pathobiology of endothelial (Piconi et al., 2004; El-Osta et al., 2008) and SMCs (Villeneuve et al., 2008, 2010) in high glucose conditions, yet remain a relatively unexplored area in the context of immune cell-mediated vascular inflammation. Monocytes and lymphocytes, immune cells with well-characterized roles in atherosclerosis (Hansson and Hermansson, 2011), display distinct profiles of histone acetylation and methylation in diabetic patients with corresponding changes in inflammatory gene expression (Miao et al., 2007, 2008). Additionally, Miao et al. (2012) identified marked differences in human leukocyte antigen (HLA)-expression in monocytes from diabetic patients compared to controls which related to differences in the histone acetylation status of the HLA promoter region. In light of these findings, it would be very interesting to explore the epigenetic status of various immune cell populations, especially adaptive immune cells which naturally develop "memory" subsets [present also in complex atherosclerotic lesions (Stemme et al., 1992)] that respond rapidly to repeated challenges of the same antigen. Poorly controlled diabetes whereby blood glucose regularly spikes to pathological levels may be sufficient to reactivate memory subpopulations, leading to rapid and robust inflammatory responses that accelerate atherosclerosis and increase risk of plaque rupture events in diabetes. Future studies examining chromatin modifications in immune cells intimately involved in the pathogenesis of diabetic complications will improve our understanding of the mechanisms underlying glycemic memory and the sustained pro-inflammatory state of the diabetic vasculature.

THERAPEUTIC STRATEGIES WITH CLINICAL PROMISE

Therapeutic strategies against diabetes-associated atherosclerosis can be targeted at various stages of disease pathogenesis. Interventions that block signaling via RAGE (Soro-Paavonen et al., 2008) or the AT1R (Candido et al., 2004) have proved effective in reducing plaque formation in mouse models with diabetes. There is a growing body of evidence to suggest that a large number of the pro-inflammatory signaling cascades triggered by hyperglycemia converge on Nox-derived ROS making it an excellent target for novel therapies (Sedeek et al., 2012). To date, studies examining pharmacological inhibition of Nox-derived ROS in experimental models of chronic inflammatory diseases including liver fibrosis (Jiang et al., 2012), diabetic nephropathy (Sedeek et al., 2010), and atherosclerosis (Gray et al., 2013) have yielded positive results, and much anticipation surrounds the clinical evaluation of specific Nox inhibitors (Kim et al., 2011). The effects of immune modulation on atherosclerosis and the emerging inflammatory cell/cytokine-directed therapies have been addressed in a recent review by Little et al. (2011). Increasing evidence demonstrates a strong association between circulating oxLDL-immune complexes and cardiovascular risk in diabetic patients (Lopes-Virella et al., 1999, 2011a,b; Orchard et al., 1999). Immunization with oxLDL or other candidate plaque antigens have shown promise in animal models (Palinski et al., 1995; Ameli et al., 1996; George et al., 1998) however evidence demonstrating the pathogenic properties of antibodies to modified LDL (summarized by Lopes-Virella

and Virella, 2010) may hamper the clinical utility of this strategy. Cytokine-based therapies that skew macrophage or T cell to anti-inflammatory and regulatory phenotypes have been effective in mouse models of atherosclerosis (Namiki et al., 2004; Sasaki et al., 2009; Cardilo-Reis et al., 2012). The establishment of highly atherosclerosis-specific antigens or cytokines in combination with cell- or tissue-focused delivery systems will be pre-requisites for more fine-tuned immunomodulatory therapies. Overall, significant challenges continue to plague attempts to translate laboratory findings to the clinic and greater emphasis on human studies is required to fully realize the therapeutic potential of targeting immunological mechanisms in disease (Davis, 2008; Libby et al., 2011). A systems approach to immunology, as outlined in a recent review by Brodin et al. (2013), may impart more successful clinical results for novel therapies.

CONCLUSION

An extensive body of experimental and clinical evidence has improved our understanding of the immune-inflammatory processes involved in vascular disease. However, there are still considerable challenges in alleviating the burden of vascular complications in diabetic patients. In this review we have discussed that, in addition to high glucose, pathological increases in AGEs, Ang II, and ROS induce a chronic state of vascular inflammation. Specifically, we have described how diabetes-mediated aberrations in vascular-leukocyte interactions result in increased accumulation of pro-inflammatory leukocytes to the atherosclerotic vessel wall. Future studies that explore the pro-inflammatory effects of RAGE, AT1R, or Nox-signaling pathways in the diabetic vasculature are likely to offer novel ways of targeting the immune responses inappropriately activated or inactivated in diabetes.

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Association of intercellular adhesion molecule 1 (ICAM1) with diabetes and diabetic nephropathy

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Diabetes and diabetic nephropathy are complex diseases affected by genetic and environmental factors. Identification of the susceptibility genes and investigation of their roles may provide useful information for better understanding of the pathogenesis and for developing novel therapeutic approaches. Intercellular adhesion molecule 1 (ICAM1) is a cell surface glycoprotein expressed on endothelial cells and leukocytes in the immune system. The *ICAM1* gene is located on chromosome 19p13 within the linkage region of diabetes. In the recent years, accumulating reports have implicated that genetic polymorphisms in the *ICAM1* gene are associated with diabetes and diabetic nephropathy. Serum ICAM1 levels in diabetes patients and the *icam1* gene expression in kidney tissues of diabetic animals are increased compared to the controls. Therefore, ICAM1 may play a role in the development of diabetes and diabetic nephropathy. In this review, we present genomic structure, variation, and regulation of the *ICAM1* gene, summarized genetic and biological studies of this gene in diabetes and diabetic nephropathy and discussed about the potential application using ICAM1 as a biomarker and target for prediction and treatment of diabetes and diabetic nephropathy.

Keywords: intercellular adhesion molecule 1, diabetic nephropathy, end-stage renal disease, type 1 diabetes mellitus, type 2 diabetes mellitus

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases in which the patients have high blood glucose levels. Its epidemic has become a national and global crisis. Based upon the figures today, at least 366 million people at the worldwide have diabetes. By the year 2030, this number is expected to be double (Bonow and Gheorghiade, 2004; Wild et al., 2004; Cornell and Dorsey, 2012; Lam and LeRoith, 2012). There are two major types of diabetes. Type 1 diabetes (T1D), previously called juvenile diabetes or insulin-dependent diabetes, develops on the basis of autoimmune destruction of pancreatic β-cells, which results in insulin deficiency. It mostly affects young people (<20 years old) but occurs also in adults (Lightfoot et al., 2012). Type 2 diabetes (T2D) is the most common form of diabetes and accounts for approximately 85–90% of all diabetic patients. In T2D, hyperglycemia results from a combination of impaired insulin secretion and insulin resistance. When the pancreatic β-cells loose the ability to compensate for insulin resistance in liver, skeletal muscle, and adipose tissues, hyperglycemia becomes manifest (Alberti and Zimmet, 1998). Diabetes patients often develop macro- and/or micro-vascular complications. Diabetic nephropathy (DN) is one of serious complications and occurs in 30–40% of diabetic patients (Heerspink and de Zeeuw, 2011; Marshall, 2012). This diabetic complication is characterized by pathophysiological changes in glomerular hyperfiltration, renal hypertrophy, tubular function and then progress to proteinuria and reduction of glomerular filtration rate (GFR). The patients with DN exhibit persistent proteinuria, hypertension, declining renal function, and increased premature mortality largely as a result of cardiovascular disease. DN is the most common single cause of end-stage renal disease (ESRD). Once overt DN occurs, it progresses slowly or rapidly to the most advanced stage of chronic kidney disease which needs dialysis or transplantation treatment (Marshall, 2004; Shields and Maxwell, 2010; Weil et al., 2010; Thomas and Groop, 2011). The treatment cost for diabetes patients has been increasing staggering in the recent decades and becomes a further burden of the healthcare system. Diabetes and DN are multi-factorial diseases, which are influenced by both genetic and environmental factors (Satko et al., 2005; Pitkäniemi et al., 2007; Ashcroft and Rorsman, 2012; Gonzalez-Bulnes and Ovilo, 2012; Morahan, 2012). Therefore, identification of the susceptibility genes in development of diabetes and diabetic complications and investigation of their roles are of importance to provide useful information for improvement of the prevention and medication programs.

Intercellular adhesion molecule 1 (ICAM1, OMIM: 147840) is a cell surface glycoprotein and expressed in endothelial cells

Abbreviations: ACR, urinary albumin/creatinine ratio; AER, albumin excretion rate; CD54, cluster of differentiation 54; DN, diabetic nephropathy; ESRD, end-stage renal disease; GFR, glomerular filtration rate; GoKinD, Genetics of Kidneys in Diabetes; HDL, high-density lipoprotein; HWE, Hardy–Weinberg equilibrium; ICAM1, intercellular adhesion molecule 1; LD, linkage disequilibrium; LDL, low-density lipoprotein; LFA, leukocyte adhesion protein; SNP, single-nucleotide polymorphism; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; UTR, un-translation region.

and leukocytes in the immune system. This endothelial- and leukocyte-associated transmembrane protein has been known for its importance in stabilizing cell–cell interactions and facilitating leukocyte endothelial transmigration. Recently, the accumulating reports from genetic studies in diabetic patients with and without DN and from biological studies with diabetic animal models have implicated that ICAM1 may play a role in the pathogenesis of diabetes and DN. In this review, we will summarize the genetic and pathophysiological relevance of ICAM1 and discuss about the possible role of ICAM1 in the development of diabetes and DN as well as the perspectives of the ICAM1 research.

GENOMIC DNA STRUCTURE, mRNA AND PROTEIN OF ICAM1

The *ICAM1* gene (GeneID: 3383) is located in chromosome 19p13.2 and spans 15,775 base pairs (bp) along the short arm of this chromosome (10,381,517–10,397,291 bp from *pter*). Its aliases include cluster of differentiation 54 (CD54) and cell surface glycoprotein P3.58 (BB2). **Figure 1** demonstrates genomic structure, regulation, and variation of the *ICAM1* gene. There are seven exons and six introns in the *ICAM1* gene. A relatively large un-translation region (UTRs) resides respectively at both 5′ and 3′-sequences of the gene, while the translation start point is located in exon 1. From the 2–6 exon, there is one non-synonymous single-nucleotide polymorphism (SNP) each, by which the amino acid changes of ICAM1 protein are caused. ICAM1 is a transmembrance glycoprotein molecule of the immunoglobulin superfamily and characterized by five distinct immunoglobulin-like domains, a transmembrance domain and a cytoplasmic tail (van de Stolpe and van der Saag, 1996). ICAM1 protein is 505 amino acids in the length, the molecule weights between 80 and 114 kDa depending upon the levels of glycosylation, which varies among cell types and environments (Newman et al., 1990). Interestingly, there are several transcription binding proteins including NF-kappaB, NF-kappaB1, STAT3, δCREB, STAT1, STAT1α, and STAT1β, which may up-regulate the *ICAM1* gene activity (Roebuck and Finnegan, 1999). MicroRNA (miR-221) may down-regulate the gene expression in the region of 3′-UTR of *ICAM1* (Gong et al., 2011). There are CpG islands for methylation in the promoter of the *ICAM1* gene. A study in tumor endothelial cells has demonstrated that the *ICAM1* gene activity can be epigenetically silenced by promoter histone modifications (Hellebrekers et al., 2006). All regulatory factors are of importance to control the ICAM1 activities in immune-related processes. Our research has been focused on investigating whether alterations in the *ICAM1* gene structure and function are associated with the development of diabetes and DN.

LINKAGE OF THE *ICAM1* GENE TO DIABETES AND DIABETIC NEPHROPATHY

Diabetes and DN are multi-factorial diseases, which are influenced by both genetic and environmental factors. To search for the susceptibility genes for the diseases, genome-wide scan linkage analysis has been used. This is a family-based approach to investigate if the genetic markers such as microsatellites or SNPs that span the whole genome and co-segregate with disease phenotypes. Microsatellites are simple repeats of 1–6 bp in genome and (CA)n is the most common form. A total of ∼30,000 microsatellites present high levels of inter- and intra-specific polymorphisms and distribute in whole genome (often in intergenic DNA regions and rarely in the sequences of the genes). SNPs are the substitutions of nucleotides in genomic DNA. In general speaking, bi-allelic SNPs are the most common type. Tri-allelic ones and small insertions/deletions are also included. C/T is the most common SNP

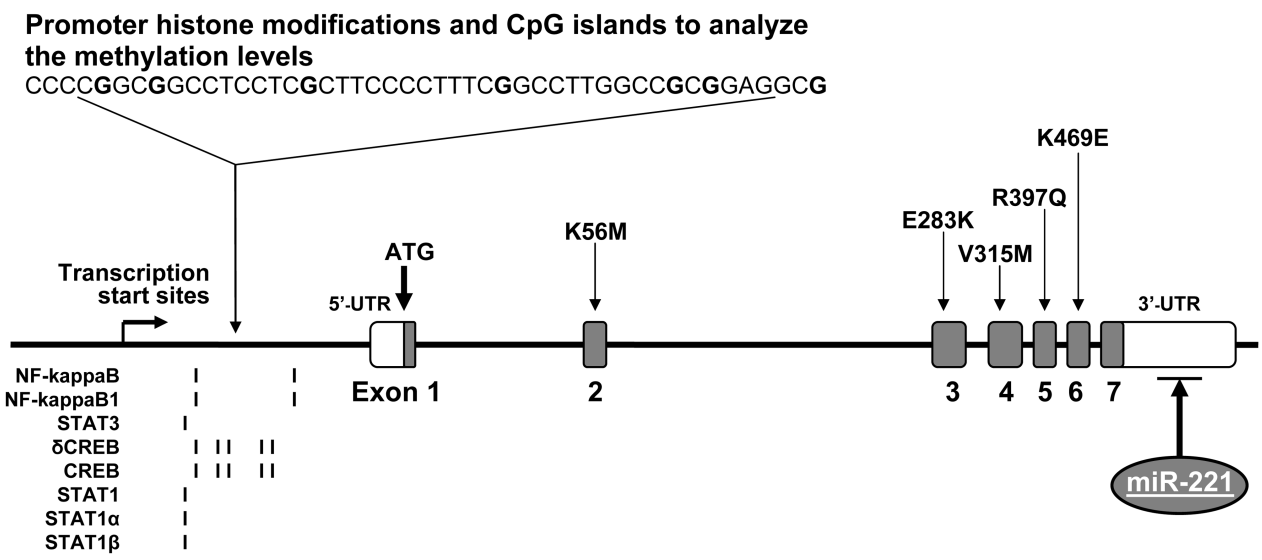

FIGURE 1 | Genomic DNA structure, variation, and regulation of the *ICAM1* gene. The *ICAM1* gene is consisted of seven exons and six introns. There are several non-synonymous SNPs, which reside one each in exons 2–6. In the gene promoter, there are CpG islands for methylation and epigenetically silence by histone modifications as well as transcription binding sites for the proteins, including NF-kappaB, NF-kappaB1, STAT3, δCREB, STAT1, STAT1α, and STAT1β. In addition, microRNA (miR-221) in the region of 3′-UTR may down-regulate the *ICAM1* gene expression.

in the human genome. SNPs reside in the coding region of the genes are called cSNPs, which include non-synonymous SNPs (with amino acid changes) and synonymous SNPs (without amino acid changes). Most of SNPs are located in non-coding regions of the genes, including promoter, intron, and UTRs. SNPs in the promoter region may alter transcription binding site and thereby affect the transcriptional activity of the gene. Moreover, a number of SNPs are found in inter-genic sequences. Up to date, more than 40 million SNPs are recorded in the public SNP databases, which are freely available for research use. Using the genetic markers, the linkage analysis allows us to identify the genome that is transmitted within families along with the disease phenotypes of interest at a genome-wide scale. Based on finding a statistical signal, the probability of co-segregation of a disease with a chromosomal locus is given (Gulcher, 2012). In this approach of genome-wide scan, highly polymorphic genetic markers distributed across the genome are genotyped in large family pedigrees or in affected and discordant sibling pairs.

By using the approach of genome-wide scan and linkage analyses, several chromosomal regions including 19p13 have been predicted to link with diabetes and DN. Mein et al. (1998) have previously conducted genome wide scan analysis in 93 affected sibpair families and 263 multiplex families from the UK and indicated that loci in chromosome 19p13 are linked to T1DM. Later on, the linkage of T1DM to chromosome 19p13 is replicated by the study with 2658 affected sib-pairs in USA (Concannon et al., 2009). Interestingly, lipid-related traits such total cholesterol, triglycerides and low-density lipoprotein (LDL) concentrations in T2DM are linked to chromosome 19p13 and this finding has been replicated in Caucasians, African-American and Hispanic families (Imperatore et al., 2000; Adeyemo et al., 2005; Malhotra and Wolford, 2005). Several interesting candidate genes, including insulin receptor, resistin and *ICAM1*, are involved in the region of chromosome 19p13. The linkage of LDL concentrations to chromosome 19p13 has been replicated in the study with 612 individuals from 28 Amish families in USA (Pollin et al., 2004). Furthermore, Leon et al. (2007) have performed a genome-wide scan study in 1251 African Americans (AA) and 1129 European Americans (EA) hypertensive siblings from the Hypertension Genetic Epidemiology Network study and indicated that loci in chromosome 19p are linked with albumin to creatinine ratio (ACR) when both AA and EA subjects are combined in the analyses. Particularly, Kathiresan et al. (2008) have analyzed the genome-wide scan data from three studies including 8816 T2D subjects and found six new loci associated with LDL, cholesterol, HDL, and triglycerides. One of the loci is located in chromosome 19p13. Previously, Arya et al. (2006) have conducted a genome-wide scan and linkage study and suggested that loci in both chromosomal arms of 19p13.2 and 19q13.4 may be linked to birth weight in T2D families of both Mexican Americans and EA. By the analyses of the combined metabolic syndrome and echocardiographic factors, Kraja et al. (2008) have found the linkage of blood pressure in T2D patients of AA and EA with the region chromosome 19p13. Taking together with the information from above briefly described genome-wide scan studies, it is clear that there are the loci in chromosome 19p may confer the susceptibility risk to diabetes and DN.

ASSOCIATION OF THE *ICAM1* GENETIC POLYMORPHISMS WITH DIABETES AND DIABETIC NEPHROPATHY

Several research groups including ours have reported the genetic association studies of the *ICAM1* gene in T1DM and DN. Guja et al. (1999) reported that the transmission of the G allele of SNP K469E (A/G) is increased in Romanian T1DM families. One year later, Nishimura et al. (2000) replicated that this K469E polymorphism is associated with adult-onset T1DM in a Japanese population. However, the association of K469E polymorphism in the *ICAM1* gene with T1DM was not found in Danish, Finnish, and British Caucasian (Nejentsev et al., 2000). Furthermore, Nejentsev et al. (2003) demonstrated that another synonymous SNP G241R in the *ICAM1* gene was associated with T1DM. All these previous studies were designed for analysis of one or two SNP(s). In order to ascertain whether the *ICAM1* genetic polymorphisms are associated with T1DM and DN, we conducted the comprehensive genetic association studies. The studied SNPs including K469E and G241R were selected based upon the information of their position in the *ICAM1* gene and their linkage disequilibrium (LD) values in the HapMap. Our data from single marker association analyses indicated that K469E and another intronic polymorphism (rs281432) were significantly associated with T1DM in Swedish Caucasians (Ma et al., 2006). Interestingly, these two SNPs are located in intron 2 and exon 6, respectively. According to the data of pair-wise LD values for SNPs in the *ICAM1* gene, a relatively strong LD ($/D'/ \geq 0.7$) existed to extend over the region between these two SNPs. Due to the large 5′- and 3′- UTRs in exons 1 and 7, the LD block covers almost the whole coding region of the gene. Further multiplex marker association analysis was done and the common haplotype C-A constructed by C allele from K469E and A allele from rs281432 was found to be associated with T1D (Ma et al., 2006). Later on, we found that K469E polymorphism in the *ICAM1* is associated with DN in T1D patients of Americans of European descent and selected from the Genetic of Kidney Diseases in Diabetes (GoKinD) study (Mueller et al., 2006). However, no association of G241R in the *ICAM1* gene with T1DM and DN in Swedish and GoKinD populations was found (Ma et al., 2008). In patients with T2D, the K469E polymorphism in the *ICAM1* gene was found to associate with plasma fibrinogen levels and diabetic retinopathy (Kamiuchi et al., 2002; Yokoyama et al., 2005; Liu et al., 2006; Petrovic et al., 2008; Vinita et al., 2012). Up to date, there is, however, no report regarding the association of *ICAM1* genetic polymorphism with DN in T2D.

Interestingly, we have observed that genotype distribution of K469E polymorphism in the *ICAM1* gene presents a high heterozygous index (∼50%; Ma et al., 2006). **Figure 2** represents the genotype distribution of the *ICAM1* E469K polymorphism in Swedish non-diabetic control subjects, T1D patients without and with DN. From non-diabetic control subjects, to T1D patients without DN and the patients with DN, the frequencies of the carriers with heterozygous genotype are increased, while the carriers with 469E homozygous genotype decreased. In order to avoid the possibility that high heterozygous index may be caused by genotyping errors, we confirmed the genotyping experiments with two different techniques such as dynamic allele-specific hybridization (DASH) and pyrosequencing (Ronaghi et al., 1998; Howell et al., 1999). In the human genome, there are segmental duplications

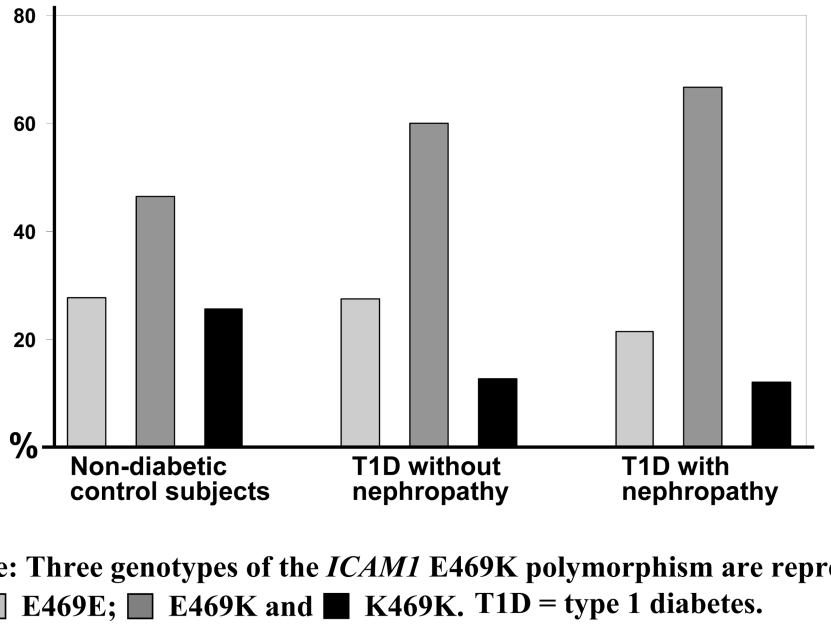

FIGURE 2 | Genotype distribution of the *ICAM1* K469E polymorphism. The genotype distribution of the *ICAM1* K469E polymorphism is represented from a genetic association study in Swedish population (Ma et al., 2006). Three genotypes of the *ICAM1* K469E polymorphism are shown in as light gray color for K469K, gray for K469E, and dark for E469E. Obviously, the heterozygous index is high compared to the percentage of homozygous and increased from the group of non-diabetic control subjects, to type 1 diabetes (T1D) patients without diabetic nephropathy and the patients with diabetic nephropathy.

(duplicons) with >90% sequence similarity between the copies, which may cause specific allelic and genotypic diversities, such as high heterozygous index in complex diseases (Venter et al., 2001; Shaw and Lupski, 2004). To ascertain whether K469E SNP is involved in a duplicon, we further performed a cloning and sequencing analysis and found that no duplication resides in the gene region (Ma et al., 2006). K469E is a non-synonymous SNP in exon 6 of the *ICAM1* gene, which causes the amino acid changes of the ICAM1 protein. We have submitted ICAM1 amino acid sequences with K469 and 469E alleles respectively into SWISS-MODEL (Peitsch, 1995; Arnold et al., 2006) to understand the changes of ICAM1 protein. There are 532 amino acids in the protein sequence of ICAM1, K469 is wild-type and has 100% identified homology. Compared to the DIMER image of wild ICAM1 protein, however, the structure of ICAM with mutant 469E is significantly changed. Although the modeling analysis implicates that the K469E polymorphism in the *ICAM1* gene may have functional effect, further investigation with transfection of 469E allele into cells such human embryonic kidney (HEK) 293A or with *icam1* knock-out mouse model is necessary in order to further understand the pathogenic mechanism.

POSSIBLE ROLE OF ICAM1 IN DEVELOPMENT OF DIABETES AND DIABETIC NEPHROPATHY

In general speaking, ICAM1 proteins act as ligands and the primary receptors for ICAM1 are integrins, which mediate cell–cell interactions and allow signal transduction. Specifically, ICAM1, unlike most integrin-binding proteins, does not contain an RGD (Arg-Gly-Asp) motif to promote integrin binding (van de Stolpe and van der Saag, 1996), but is targeted to two integrins of the β2 subunit family, i.e., leukocyte adhesion protein-1 (LFA-1) and Mac-1 (integrin, alpha M; Janeway, 2001). Thus, based upon the interaction with these two molecules, ICAM1 has a role for two important immune-related functions: T lymphocytes activation and leukocyte–endothelial cell interaction. The role of ICAM1 in the development of diabetes and DN has not been fully explored. Recent studies, however, have provided the information to predict that ICAM1 is involved in the pathogenesis of diabetes and DN (Sahakyan et al., 2010a,b).

Diabetic nephropathy is a progress disease, which is categorized into stages based upon urinary albumin excretion (UAE) values. The early phase, which can be reversed, is microalbuminuria. The reduction of renal function begins with proteinuria. Clinical investigation has demonstrated that soluble ICAM1 levels in stored blood samples from T1D patients are higher compared to non-diabetic control subjects. High ICAM1 levels in T1D patients are associated with a relative risk of 1.67 (95 CI 0.96–2.92, $P = 0.03$) of developing incident sustained microalbuminuria after adjustment for baseline age, sex, duration of diabetes, and randomized treatment assignment (Lin et al., 2008). Furthermore, Astrup et al. (2008) have reported that soluble ICAM1 levels are associated with all-caused mortality and cardiovascular morbidity in T1D patients with DN. The similar findings have been observed in T2D patients. Soluble ICAM1 levels are significantly correlated with albuminuria in T2D patients (Rubio-Guerra et al., 2007). T2D patients with diabetic micro-angiopathic complications have higher soluble ICAM1 levels in comparison with diabetic group without micro-angiopathic complications and healthy control subjects

(Mastej and Adamiec, 2008). The findings from clinical investigations have been supported by studies with diabetic animal models such as the db/db mice and streptozotocin-induced rats. Compared to non-diabetic rats, serum and urinary ICAM1 levels in streptozotocin-induced rats are found to be increased, which are parallel with the elevation of UAE (Qian et al., 2008). Furthermore, evidence has indicated that ICAM1 is overexpressed in glomeruli diabetic rats (Watanabe et al., 2011) and in tubular epithelial cells of kidney in T2D db/db mice (Kosugi et al., 2009). Therefore, ICAM1 may play a role in the development of diabetes DN and possible mechanism is shown in **Figure 3**. In a diabetic condition with hyperglycemia, the *ICAM1* gene transcription in the nuclei is increased and the *ICAM1* gene expression on the surface of endothelium cells is up-regulated. ICAM1 binding activity with LFA-1 is increased and more lymphocytes from blood are transferred into cells in glomeruli and peritubular capillaries of nephron in kidney. Consequently, injure of kidney glomeruli and tubular occurs and the proteins are excreted to urine. In this figure, however, two questions still remain. First, how is ICAM1 gene activity stimulated by high blood glucose levels? Second, how does ICAM1 elevation cause kidney tubular and glomeruli injury?

ICAM1 AS A BIOMARKERS FOR PREDICTION OF DIABETIC NEPHROPATHY

Biomarkers are substances and structures that can be measured in biological samples such as urine, blood, saliva, DNA, and protein. A biomarker can be used as an indicator of a particular disease state such as diabetes and DN (Caveney and Cohen, 2011). Currently, the biomarker used for prediction and diagnosis of DN is UAE. This biomarker, however, is less valuable for early prediction and diagnosis of DN. Therefore, researchers have searched for novel biomarkers of DN in order to improve the diagnosis approach and prevention program (Hellemons et al., 2012). As we described above, genetic and biological studies have implicated that ICAM1 plays a role in the development of diabetes and DN. First, the *ICAM1* gene is located in a linkage region with diabetes and DN. Second, the K469E polymorphism in the *ICAM1* gene is associated with diabetes and DN. Third, serum ICAM1 levels are gradually increased from low levels in normal albuminuria to high levels in micro-albuminuria and to even higher levels in proteinuria. Therefore, we concluded that ICAM1 is associated with diabetes and DN. This molecule is most likely a useful biomarker for prediction of endothelial dysfunction in diabetes and DN. Further evaluation of ICAM1 as a biomarker in a large cohort of T1D and T2D patients with and without DN needs to be done.

ICAM1 AS A TARGET FOR DRUG DEVELOPMENT

ICAM1 is a molecule involved in many pathways including anti-inflammation. Although the picture of ICAM1 involvement and interaction is complex, experiments have indicated that inhibition of the ICAM1 gene expression may improve the progress of diabetes and DN. Glucagon-like peptide-1 (GLP-1) has

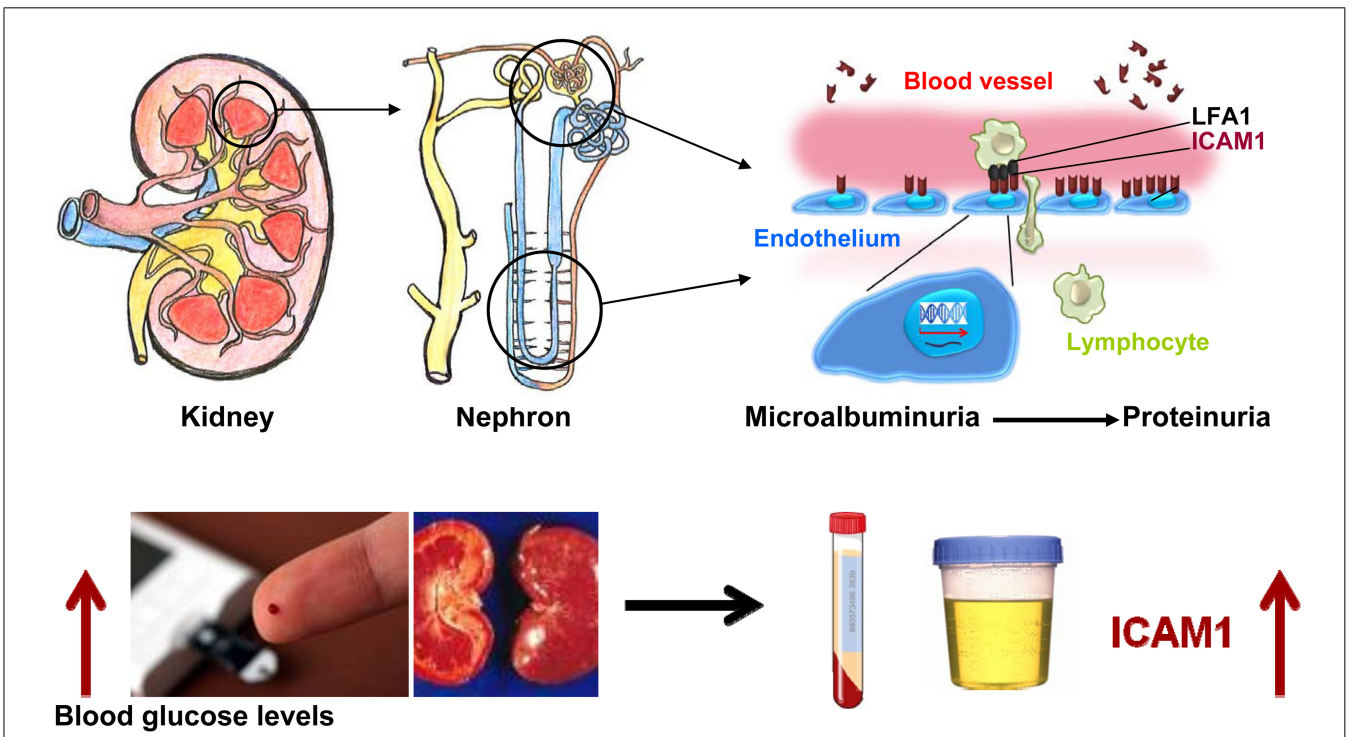

FIGURE 3 | Possible role of ICAM1 in the development of diabetic nephropathy. Under a diabetic condition with hyperglycemia, the *ICAM1* gene transcription in the nuclei is increased and the *ICAM1* gene expression on the surface of endothelium cells is up-regulated. ICAM1 protein binding activity with leukocyte adhesion protein-1 (LFA-1) is increased and more lymphocytes from blood are transferred into cells of glomeruli and peritubular capillaries of nephron in kidney. Consequently, serum ICAM1 levels are increased. Injury in kidney glomeruli and tubular is occurred and the proteins are released to urine.

various extra-pancreatic actions, in addition to its enhancement of insulin secretion from pancreatic islets. Kodera et al. (2011) have demonstrated that GLP-1 receptor agonist, exendin-4, decreases the ICAM1 gene expression and ameliorates albuminuria, glomerular hyperfiltration, glomerular hypertrophy, and mesangial matrix expansion in the diabetic rats without changing blood pressure or body weight. Furthermore, Matsui et al. (2010) have reported that nifedipine, a calcium-channel blocker, blocks the advanced glycation end product (AGE)-induced tubular damage and also inhibits ICAM1 gene activity in tubular cells, which may have benefits in treatment of DN. Liu et al. (2010) have suggested that berberine can ameliorate renal dysfunction in diabetic rats by decreasing ICAM1 gene expression and nuclear factor-kappa B (NF-kappaB) activation. Taking together, the data from these studies suggest that ICAM1 may be a good candidate as target for drug development. Inhibition of ICAM1 gene activity may benefit in treatment of diabetes and DN.

SUMMARY AND PERSPECTIVES

We have described genetic and biological studies of ICAM1 in diabetes and DN. Data implicate that ICAM1 can be used a biomarker for prediction of diabetes and DN and may also be serviced as a target for drug development. To reach these two objectives, however, we need to continuously put efforts into the study of ICAM1 in diabetes research. First, genetic polymorphisms in the *ICAM1* gene are associated with T1D, T2D and DN in T1D. But, no genetic association with DN in T2D has been reported. Although it is known that methylation modification of genomic DNAs and histone proteins is involved in the regulation of ICAM1 gene expression, no epigenetic study of this gene in diabetes and DN has been done. Second, clinical observations in diabetes patients and experimental studies with diabetic animal models have demonstrated that elevation of serum ICAM1 levels and overexpression of the gene in kidney tissues. Inhibition of ICAM1 gene activity with agents may improve the kidney function, suggesting that ICAM1 may be a target for drug development in treatment of diabetes and DN.

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Diabetic nephropathy: the role of inflammation in fibroblast activation and kidney fibrosis

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Kidney disease associated with diabetes mellitus is a major health problem worldwide. Although established therapeutic strategies, such as appropriate blood glucose control, blood pressure control with renin–angiotensin system blockade, and lipid lowering with statins, are used to treat diabetes, the contribution of diabetic end-stage kidney disease to the total number of cases requiring hemodialysis has increased tremendously in the past two decades. Once renal function starts declining, it can result in a higher frequency of renal and extra-renal events, including cardiovascular events. Therefore, slowing renal function decline is one of the main areas of focus in diabetic nephropathy research, and novel strategies are urgently needed to prevent diabetic kidney disease progression. Regardless of the type of injury and etiology, kidney fibrosis is the commonly the final outcome of progressive kidney diseases, and it results in significant destruction of normal kidney structure and accompanying functional deterioration. Kidney fibrosis is caused by prolonged injury and dysregulation of the normal wound-healing process in association with excess extracellular matrix deposition. Kidney fibroblasts play an important role in the fibrotic process, but the origin of the fibroblasts remains elusive. In addition to the activation of residential fibroblasts, other important sources of fibroblasts have been proposed, such as pericytes, fibrocytes, and fibroblasts originating from epithelial-to-mesenchymal and endothelial-to-mesenchymal transition. Inflammatory cells and cytokines play a vital role In the process of fibroblast activation. In this review, we will analyze the contribution of inflammation to the process of tissue fibrosis, the type of fibroblast activation and the therapeutic strategies targeting the inflammatory pathways in an effort to slow the progression of diabetic kidney disease.

Keywords: fibroblasts, kidney fibrosis, EMT, EndMT, cytokines, diabetic nephropathies, inflammation

INTRODUCTION

Diabetic nephropathy is the most common cause of end-stage renal disease (ESRD) worldwide (Ritz et al., 1999; Viswanathan, 1999; Parving, 2001; Remuzzi et al., 2002). Current treatment strategies may slow but in most of cases cannot arrest progression toward ESRD (Lewis et al., 1993; Brenner et al., 2001). Once diabetic nephropathy progresses to ESRD, management with dialysis is expensive and is associated with increased cardiovascular morbidity and mortality compared to non-diabetic ESRD (Parving, 2001; Remuzzi et al., 2002).

Therefore, in an effort to aid in the discovery of novel therapeutic concepts that allow the prevention and retardation of diabetic nephropathy, innovative insight into the pathophysiology of diabetic nephropathy is mandatory (Wolf and Ritz, 2003). Hemodynamic alterations such as hyperfiltration and hyperperfusion caused by hyperglycemia are considered major kidney injury factors, but such alterations are only one aspect of a complex series of pathophysiological alterations related to the presence of glucose metabolism defects.

Various theories have been proposed concerning the pathogenesis of diabetic nephropathy, including proteinuria, genetics, race, hypoxia, ischemia, and inflammation (Fernández Fernández et al., 2012). Individually, the theories proposed thus far may not be able to explain the progression of diabetic nephropathy. Among these theories, inflammation appears to be the critical pathway for the development and progression of diabetic nephropathy.

The role of inflammation in diabetic nephropathy has been reported previously (Mezzano et al., 2003; Rivero et al., 2009; Wada and Makino, 2013). In the context of current theories that are focused on inflammation and fibroblast activation, such as epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndMT), we discuss the role of inflammation in the progression of diabetic kidney disease, with an emphasis on therapeutic strategies.

GENERAL OVERVIEW OF THE BIOLOGY OF DIABETIC NEPHROPATHY

MULTIPLE PROFIBROTIC PATHWAY INVOLVEMENT

Various theories have been proposed regarding the development and progression of diabetic kidney disease. The pathological events that have traditionally been implicated in the development of diabetic nephropathy include poor glycemic control, hypertension, proteinuria, and other such factors. Individual factors such as genetics, race, obesity, and smoking have been shown to contribute

to variations in the onset and progression of kidney disease (Bakris et al., 2003; American Diabetes Association, 2011; Cravedi et al., 2012).

Diabetic organ damage, including diabetic nephropathy, is fundamentally triggered by glucose metabolism defects; normalizing blood glucose levels is essential for diabetic therapy (The Diabetes Control and Complications Trial Research Group, 1993; Ohkubo et al., 1995; UK Prospective Diabetes Study [UKPDS] Group, 1998). Indeed, pancreatic transplantation in overt diabetic nephropathy with sclerotic glomeruli can cure the nephropathy with significant amelioration of renal pathology, but the process takes nearly 10 years (Fioretto et al., 1993, 1998; Fioretto and Mauer, 2011). However, the normalization of blood glucose levels in diabetic patients is challenging, and such intensive therapies in diabetic patients are associated with increased mortality risk and are likely associated with frequent severe hypoglycemia (Ismail-Beigi et al., 2010). Although strict control of blood glucose levels inhibits the progression of urine albuminuria levels, it does not offer any difference in clinical kidney disease outcome (Hemmingsen et al., 2011; Coca et al., 2012; Slinin et al., 2012). Therefore, to prevent diabetic complications, therapeutic strategies are required in addition to those that target blood glucose normalization.

The degree of urine albumin/protein is associated with the progression of kidney disease via the activation of tubular angiotensin-converting enzyme (ACE) inhibitors or inflammatory pathways (Abbate et al., 2006; Cravedi et al., 2012; Erkan, 2012). Strategies to decrease proteinuria with renin–angiotensin system (RAS) blockade have been shown to be partially renoprotective (Lewis et al., 1993; Brenner et al., 2001). However, 30% of diabetic nephropathies occur in the absence of significant proteinuria (Fernández Fernández et al., 2012), suggesting that other pathways have a role in the pathogenesis of the condition, and recent clinical trials raise questions about the significance of strong RAS blockade using a combination of RAS inhibitors in diabetic nephropathy patients (Mann et al., 2008; Parving et al., 2012).

Systemic/intra-glomerular hypertension can result in the onset and progression of diabetic kidney disease. ACE inhibitors and angiotensin receptor blockers (ARBs) have been shown to slow disease progression by controlling hypertension, in addition to decreasing proteinuria (Lewis et al., 1993; Brenner et al., 2001). Non-dihydropyridine calcium channel blockers, aldosterone antagonists, and direct rennin inhibitors (e.g., aliskiren) have been reported to control hypertension and reduce albuminuria/proteinuria, but evidence of long-term, significant clinical renal outcomes remains lacking (Slinin et al., 2012).

Diabetic insults to the kidney are mediated through the various pathogenic pathways described above. Inflammation appears to be the final common pathway and can result in either kidney repair if regulated or fibrosis if the process is uncontrolled.

INFLAMMATION AND KIDNEY FIBROSIS

Fibrosis is a sequence of normal wound healing and repair that is activated in response to injury to maintain the original tissue architecture and ensure normal functional integrity (Pinzani, 2008; Wynn, 2008). Essentially, inflammation is required for tissue repair, except in embryos, where tissue can be repaired without typical inflammation (Bullard et al., 2003; Redd et al., 2004). Inflammation is closely associated with tissue repair, regeneration of parenchymal cells, and filling of tissue defects with fibrous tissue, i.e., scar formation (Wynn, 2007). The inflammatory response to an injury eventually results in either (a) the normal tissue repair process and the regaining of structural and functional integrity or (b) abnormal and uncontrolled tissue repair, which leads to progressive fibrosis with loss of tissue structure and function. Although non-resolving inflammation has been shown to be a major driving force in the development of fibrotic disease, as described above, inflammation is an essential aspect of host defense mechanisms in response to injury (Wynn, 2007). In this regard, progressive fibrosis with sustained inflammation can be considered as a type of chronic wound with defects in the normal healing process (Liu, 2011). Thus, controlling excessive inflammation has great therapeutic potential of inhibiting progressive kidney fibrosis.

Once tissue is injured, inflammatory cells infiltrate the site of injury due to the enrichment of pro-inflammatory niches at the site of injury and directional guidance mediated by chemotactic cytokine concentration gradients (Chung and Lan, 2011). The infiltration of inflammatory cells, such as lymphocytes, monocytes/macrophages, dendritic cells (DCs) and mast cells, precedes the process of tissue fibrosis (Chung and Lan, 2011). At the injury site, activated infiltrated inflammatory cells can synthesize tissue damage factors such as reactive oxygen species (ROS) and produce fibrogenic cytokines and several growth factors (Ricardo et al., 2008; Duffield, 2010; Vernon et al., 2010). Sustained inflammatory cell activation results in profibrotic cytokine pressure within the local microenvironment. The cytokine pressure subsequently primes the fibroblasts at the site of injury and induces tubular epithelial cell phenotypic changes into a mesenchymal-like phenotype that produces a large amount of profibrotic extracellular matrix (ECM) components (Liu, 2011). Therefore, sustained inflammation after the tissue injury could be the initiator, the trigger and the activator of tissue fibrosis progression.

The importance of inflammation in the development and progression of renal fibrosis has been well documented (**Table 1**; Ricardo et al., 2008; Duffield, 2010; Vernon et al., 2010). Inflammation is regulated by the complex interaction of various factors, involving cytokines, chemokines, and adhesion molecules. Renal inflammation is characterized by glomerular and tubulointerstitial infiltration by inflammatory cells, including neutrophils, macrophages, lymphocytes, and other such cells, regardless of the initial injury. Such cellular infiltrates are evident in both experimental models of renal disease and human renal biopsy specimens (Ferenbach et al., 2007). Inflammation is initiated with the entry of neutrophils, which take up cell debris and phagocytose apoptotic bodies (Lee and Kalluri, 2010). Activated neutrophils degranulate to release inflammatory and profibrotic cytokines (Lee and Kalluri, 2010). Subsequently, macrophages infiltrate damaged tissues and play an important role in the production of inflammatory cytokines and profibrotic cytokines (Lee and Kalluri, 2010). The recruitment and activation of T lymphocytes has been shown to be a significant early event in the initiation of renal fibrosis, and it typically precedes the influx of macrophages into the injured kidneys (Lin et al., 2008). The importance of T or B lymphocytes has

Table 1 | Inflammatory cell types and their roles in kidney fibrosis.

Inflammatory cell type	Major roles in the kidney fibrosis process	Reference
Neutrophil	Initiation of inflammation	Lee and Kalluri (2010)
	Uptake of cell debris	
	Phagocytosis of apoptotic bodies	
Lymphocyte	T cell recruitment and activation as an early event in the fibrosis process	Lin et al. (2008), Tapmeier et al. (2010)
	T cell deficiency associated with reduced fibrosis	
	Cytokine production	
Macrophage	M1 macrophages exhibit a pro-inflammatory phenotype, and M2 macrophages do not have a typical inflammatory phenotype	Ricardo et al. (2008), Lin et al. (2009), Duffield (2010), Wang and Harris (2011)
	Generation of various cytokines, chemokines and reactive oxygen species	
Dendritic cell	Capture and deliver antigens to T cells	Heymann et al. (2009), Macconi et al. (2009), Hochheiser et al. (2011a)
	Stage-specific role in kidney fibrosis	
Mast cell	Controversial role in fibrosis	Miyazawa et al. (2004), Kanamaru et al. (2006), Timoshanko et al. (2006), Holdsworth and Summers (2008)
	Mast cell-deficient mice display increased morality and kidney fibrosis in experimental animal models	

been analyzed in genetic mouse models that lack mature B and T lymphocytes and have a V(D)J recombination-activating protein 1 (RAG1) deficiency. RAG1-deficient mice are protected against fibrosis after obstructive injury (Tapmeier et al., 2010).

Similar anti-fibrogenic effects were observed when $CD4^+$ T cells were depleted in wild-type mice after obstructive injury (Tapmeier et al., 2010), whereas reconstitution with purified $CD4^+$ T cells in RAG1-knockout (B, T cell-deficient) mice led to restored fibrogenic responses following obstructive injury (Tapmeier et al., 2010), suggesting that lymphocytes, especially $CD4^+$ T cells, have a critical role in the pathogenesis of renal fibrosis induced by obstructive injury. An analysis of type IV collagen α3 chain-deficient mice, the model of human Alport syndrome, revealed that RAG1 deficiency in mice significantly ameliorated tubulointerstitial injury without amelioration in glomerular basement membrane (GBM) structures (LeBleu et al., 2008), but streptozotocin (STZ)-induced diabetic animal models using the same RAG1-deficient mice displayed no alteration in tubular injury when compared to control diabetic mice, even though RAG1-deficient diabetic mice exhibited low levels of albuminuria (Lim et al., 2010). Macrophage infiltration into the kidney cortex was the same in the STZ-induced diabetic RAG1-deficient and control diabetic mice, suggesting that the major mechanism for the inflammatory sequence was not affected by the absence of lymphocytes in their model (Lim et al., 2010); however, these results must be confirmed in additional studies, such as those using much stronger diabetic kidney fibrosis models (Sugimoto et al., 2007), to determine whether this observation is generalizable in diabetic nephropathy. It is not clear how or whether the various inflammatory response processes can affect disease-specific responses and subsequent tubule-interstitial injury from fibrosis.

Evidence from renal biopsies has shown that macrophage accumulation in diabetic kidneys predicts declining renal function (Duffield, 2010; Wang and Harris, 2011). STZ-induced diabetic animal models indicate that macrophage accumulation is associated with kidney fibrosis (Chow et al., 2004; Sugimoto et al., 2012). It is believed that macrophages play crucial roles in renal fibrogenesis (Duffield, 2010; Wang and Harris, 2011). Monocytes are recruited from circulating blood into the injured sites in response to tissue damage through cytokine-directed navigation, and subsequently recruited monocytes are differentiated into two broad but distinct subsets of macrophages: activated (M1) macrophages and alternatively activated (M2) macrophages (Ricardo et al., 2008; Lin et al., 2009). It is believed that M1 macrophages exhibit a typical pro-inflammatory phenotype via the generation of various chemokines, as well as ROS. M1 macrophages display pathogenic functions that lead to further tissue injury and fibrosis. In diabetic animal models, the depletion of the chemokines intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) diminishes macrophage accumulation and subsequent inflammation and tissue damage (Chow et al., 2005, 2007b). Immunohistological analysis has revealed that macrophages accumulated in diabetic kidney injury sites exhibit inducible nitric oxide release, CD169, and phosphorylated p38 mitogen-activated protein kinases (Adhikary et al., 2004; Chow et al., 2007a,b). Macrophage scavenger receptor-A-deficient mice are protected from interstitial fibrosis in diabetic nephropathy associated with the amelioration of microinflammation (Usui et al., 2007). The depletion of macrophages has been shown to protect and restore renal fibrosis after various injuries in non-diabetic animal models. The reconstitution of macrophages can worsen existing fibrotic lesions, thereby demonstrating that they have a profibrotic role in renal fibrogenesis (Henderson et al., 2008; Ko et al., 2008a; Lin et al., 2009). Macrophage activation status is also a major determinant of their pro-fibrogenic ability. The infusion of toll-like receptor 9 (TLR9) agonist-activated macrophages exaggerated disease progression in doxorubicin-induced nephropathy in mice, whereas

resting macrophages did not induce disease progression (Wang et al., 2008).

Dendritic cells originate from the same bone marrow myeloid progenitor cells as macrophages, and they abundantly accumulate in normal kidney interstitium (John and Nelson, 2007; Kaissling and Le Hir, 2008; Teteris et al., 2011). DCs have important roles in the regulation of immune tolerance and the mounting of robust immune responses to pathogens. Macconi et al. (2009) reported that DCs can produce antigenic peptides from albumin through a proteasome-dependent pathway in a remnant kidney model. Subsequently, such peptides activate $CD8^+$ T cells, suggesting that in proteinuric diseases, renal DCs capture and carry filtered antigens to T cells, leading to the production of pro-inflammatory cytokines. The crucial roles of DCs in renal disease progression and fibrogenesis have been demonstrated in DC-depleted mice, which exhibit renal protection via the overexpression of the model antigens, ovalbumin and hen egg lysozyme, in glomerular oocytes and nephrotoxic serum nephritis models (Heymann et al., 2009; Hochheiser et al., 2011a). Interestingly, later nephrotoxic nephritis (NTN) models revealed that DC depletion in late-stage nephritis halted disease progression but that early-stage depletion augmented disease progression, suggesting that DCs have a disease phase-specific role in certain disease models (Hochheiser et al., 2011a,b). The significance of DCs in diabetic kidney fibrosis remains unclear (Tu et al., 2010).

The role of mast cells in renal fibrogenesis remains controversial and unclear (Kanamaru et al., 2006; Timoshanko et al., 2006; Holdsworth and Summers, 2008). In diabetic kidney disease, mast cell numbers have been correlated with interstitial fibrosis (Hiromura et al., 1998; Sakamoto-Ihara et al., 2007). However, analyses of mast cell-deficient experimental animal models have suggested that mast cells have renal-protective roles. Indeed, in experiments, mast cell-deficient mice exhibited increased mortality and histopathological deteriorations in anti-GBM syndrome disease models (Kanamaru et al., 2006). Interstitial fibrosis was augmented in mast cell-deficient mice compared to control animals, which was demonstrated using a puromycin amino nucleoside-nephritis model (Miyazawa et al., 2004).

The evidence described above clearly demonstrates that the activation of inflammatory lymphocytes, macrophages, and DCs is essential for fibrotic kidney disease initiation and progression, including diabetic kidney disease.

INFLAMMATION: FIBROBLAST ACTIVATION

The local accumulation of profibrotic cytokines in the microenvironment following kidney injury leads to the activation of ECM-producing cells, which are essential for renal fibrogenesis. As discussed above, almost all cell types in the tubulointerstitium of the kidneys, such as residential fibroblasts, tubular epithelial cells, vascular smooth muscle cells, and a subset of macrophages, are responsible for producing ECM. The fundamental matrix-producing cells that generate a large amount of interstitial matrix components, including fibronectin and type I and type III collagens, are fibroblasts (Strutz and Zeisberg, 2006). Profibrotic cytokine transforming growth factor-beta (TGF-βs) has been shown to play an essential role in this process, and the inhibition of TGF-βs or the TGF-β-stimulated smad transcriptional factor signaling pathway blockade has been shown to exhibit anti-fibrotic effects (Border and Noble, 1994; Miyazono, 2000; Kanasaki et al., 2003, 2011; RamachandraRao et al., 2009; Takakuta et al., 2010; Hills and Squires, 2011; Lan, 2011; Sharma et al., 2011; Choi et al., 2012).

TGF-βs modulate the overall response by affecting different receptors and downstream signaling. Active TGF-βs have profibrotic effects, and latent TGF-βs have an anti-fibrotic effect (Lan, 2011; Meng et al., 2013). The smad pathway is a downstream signaling pathway that can be influenced by non-TGF-βs molecules, such as angiotensinogen and advanced glycation end products. Among the smad molecules, smad 2 and smad 7 are likely renoprotective, and smad 3 is pathogenic (Meng et al., 2010, 2013; Lan, 2011; Lan and Chung, 2012).

In diseased kidneys, activated fibroblasts express α smooth muscle actin (αSMA) and are often referred to as myofibroblasts. These types of cells possess unique contractile properties (Strutz and Zeisberg, 2006). In the renal fibrosis process, renal myofibroblasts are believed to be an activated fibroblast phenotype that essentially contributes to ECM production and deposition in tubulointerstitial fibrosis (Strutz and Zeisberg, 2006). Several studies have examined the origin, activation, and regulation of these matrix-producing myofibroblasts (Grande and Lopez-Novoa, 2009; Meran and Steadman, 2011).

Figure 1 briefly summarizes the five well-reported sources of matrix-producing myofibroblasts, including the activation of residential fibroblasts, differentiation of pericytes, recruitment of circulating fibrocytes, and conversion of tubular epithelial cells and endothelial cells into mesenchymal cells (Barnes and Gorin, 2011). The relative contribution to and even the existence of each particular myofibroblast-generating pathway in renal fibrosis is still a topic of intense debate (Zeisberg and Duffield, 2010). Difficulty with identifying and tracking the lineage of fibroblasts, matrix-producing myofibroblasts, and other cell types due to the lack of specific markers has made the theory very controversial. Despite this controversy, there is no doubt that such matrix-producing fibroblasts exhibit significant heterogeneity.

INFLAMMATION: ACTIVATION OF RESIDENTIAL FIBROBLAST AND PERICYTES

Historically, all matrix-producing myofibroblasts were thought to originate from residential fibroblasts by phenotypic activation following renal injury and inflammation (Hewitson, 2009; **Figure 2**). This concept has recently been debated and challenged (Strutz and Zeisberg, 2006; Grande and Lopez-Novoa, 2009; Zeisberg and Duffield, 2010).

Fibroblasts are localized in the interstitial space between the capillaries and the epithelia in normal kidneys. They are organized as a network spread throughout the renal parenchyma, where they stabilize and organize the tissue architecture (Kaissling and Le Hir, 2008). Morphological analysis has revealed that these fibroblasts are stellate shaped and display a rough endoplasmic reticulum, collagen-containing granules and actin filaments (Strutz and Zeisberg, 2006). They exhibit multiple cell processes, which enable them to interact with the tubular and capillary basement membranes (Kaissling and Le Hir, 2008). In quiescent states, interstitial fibroblasts express CD73 (also known as ecto-5′-nucleotidase)

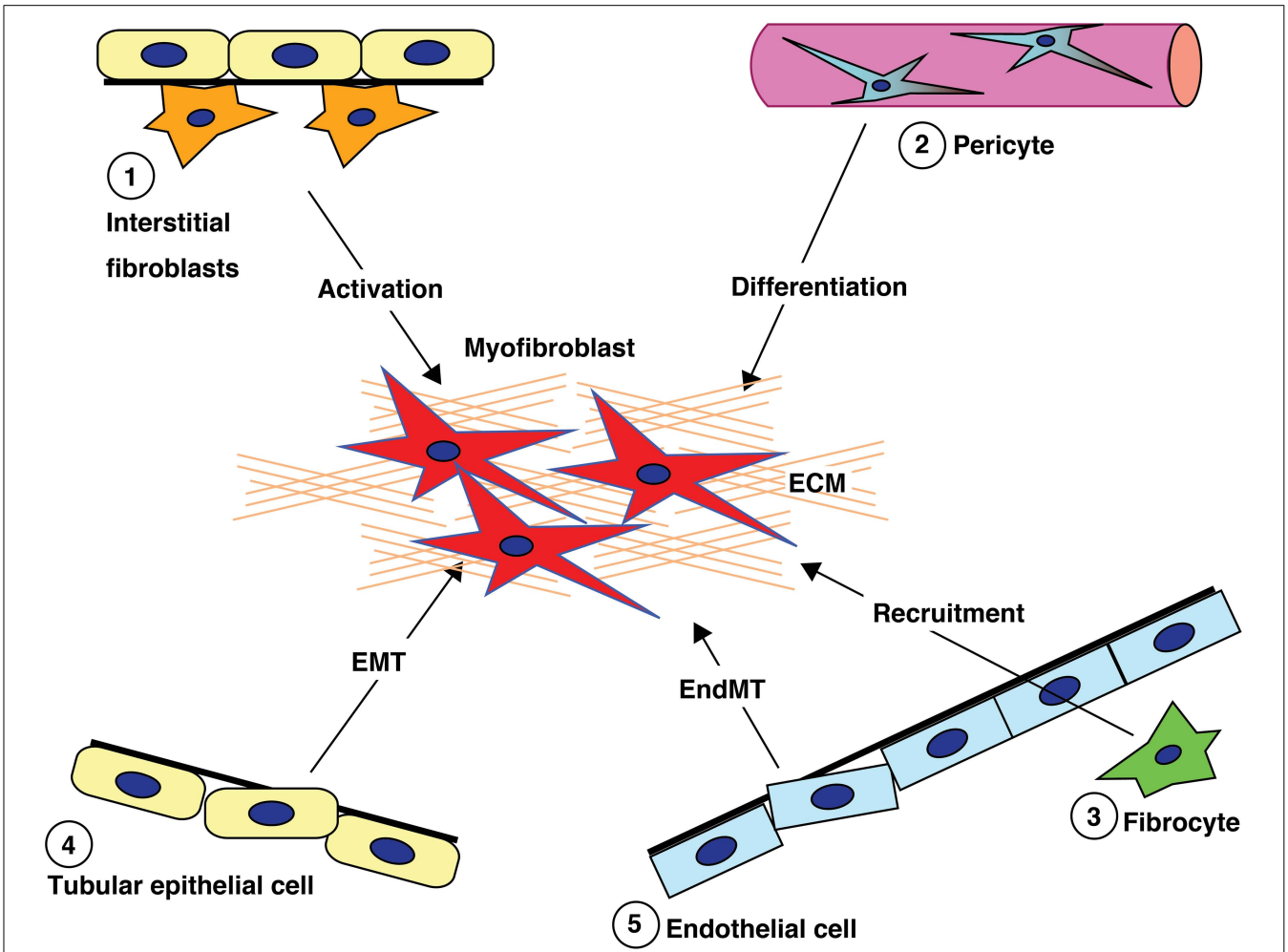

FIGURE 1 | Diverse origins of fibroblasts. Kidney fibrosis is a well-coordinated event originating from various sources (1, interstitial cells; 2, pericytes; 3, fibrocytes; 4, tubular epithelial cells; 5, endothelial cells) and processes (recruitment, activation, differentiation, epithelial-to-mesenchymal transition, and endothelial-to-mesenchymal transition) that contribute to myofibroblast activation/formation.

in their plasma membrane and are responsible for producing erythropoietin (Kaissling and Le Hir, 2008; Paliege et al., 2010). These cells also express platelet-derived growth factor receptor β (PDGFRβ; Floege et al., 2008) and fibroblast-specific protein 1 (FSP1; also known as S100A4), a small protein that exhibits a calcium-binding motif and is associated with the cytoskeleton (Wynn, 2007). Under normal conditions, fibroblasts are responsible for maintaining the homeostasis of the interstitial matrix against physiologic conditions by producing an essential, basal level of ECM components. Following profibrotic cytokine inflammation and mechanical stress, these interstitial fibroblasts undergo phenotypic change into myofibroblasts by expressing αSMA, and they begin to produce a large amount of ECM components. Myofibroblasts can express both FSP1 and PDGFRβ. In addition, myofibroblasts can also express *de novo* vimentin (an intermediate filament protein). The myofibroblast phenotype is indeed a mixture of both matrix component-producing activated fibroblasts and αSMA-expressing highly contractile smooth muscle cells.

Fibroblasts become activated by stimulation with cytokines, including TGF-βs, connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), and fibroblast growth factor 2 (FGF2; Liu, 2011). In addition to cytokine-mediating reactions, direct cell–cell interactions (with leukocytes and macrophages), ECM–integrin interactions, hypoxia, and hyperglycemia could contribute to the activation process (Chow et al., 2004, 2007a; Liu, 2011). In tubulointerstitial fibrosis, renal fibroblasts maintain an activated status, even after the initial injury and insult has ceased (Liu, 2011). Activated fibroblasts are characterized by two key features: proliferation and myofibroblastic activation (Strutz and Zeisberg, 2006). However, the proliferation of residential fibroblasts is also controversial, and some reports have indicated that no proliferation occurs in unilateral ureteral obstruction renal fibrosis models (Yamashita et al., 2005). Myofibroblastic activation is illustrated by αSMA expression and matrix production (Strutz and Zeisberg, 2006). Both fibroblasts and myofibroblasts have the ability to proliferate when stimulated with cytokines. This fibroblast proliferation results in the expansion of

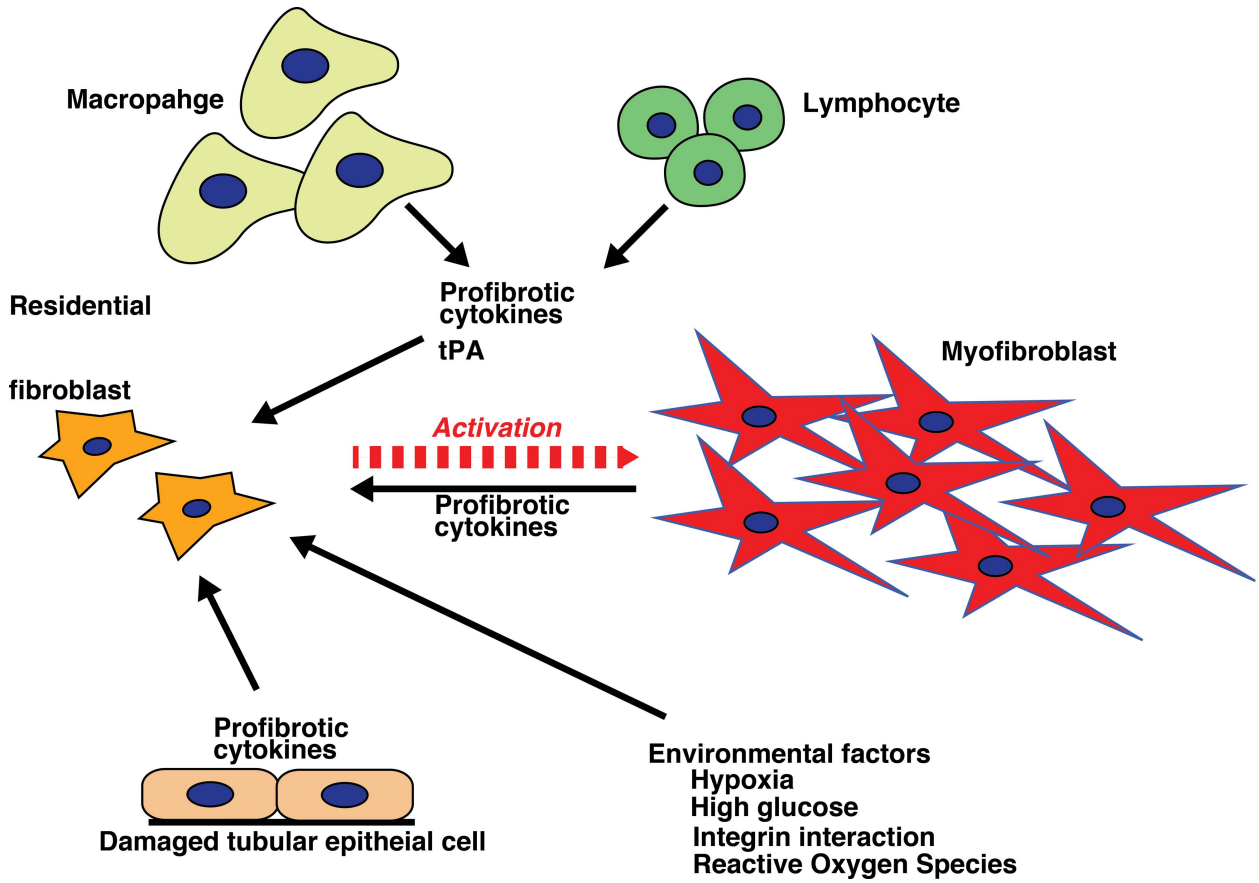

FIGURE 2 | Activation of fibroblasts. Internal milieu changes (hypoxia, high glucose, integrin interactions, and production of reactive oxygen species) and profibrotic inflammatory cytokines from varied cell sources (macrophages, lymphocytes, and damaged tubular epithelial cells) result in fibroblast activation, which initiates and sustains the process of fibrosis.

the fibroblast population and ECM deposition in the interstitial space in damaged kidneys (Strutz and Zeisberg, 2006). Fibroblasts are activated and proliferate in response to several cytokines and mitogens, such as PDGF, TGF-β, basic FGF2, and CTGF. These cytokines are also potentially derived from activated macrophages (Strutz et al., 2000; Bottinger, 2007; Hewitson, 2009; Phanish et al., 2010; Boor and Floege, 2011; Ostendorf et al., 2012).

Tissue-type plasminogen activator (tPA), which can also be derived from activated macrophages, is another critical factor in kidney fibrosis (Hu et al., 2007, 2008; Hao et al., 2010; Lin et al., 2010). tPA plays crucial roles in the pathogenesis of renal interstitial fibrosis through a variety of mechanisms, such as protection from apoptosis and induced mitogenesis (Hu et al., 2007, 2008; Hao et al., 2010; Lin et al., 2010). In renal interstitial fibroblasts, tPA also induces the expression of matrix metalloproteinase-9 (MMP-9), which causes destruction of the tubular basement membrane (TBM), subsequently leading to tubular EMT (Hu et al., 2006). tPA also acts independently of its protease activity and induces myofibroblast activation of quiescent interstitial fibroblasts through low density lipoprotein (LDL) receptor-related protein 1 (LRP-1)-mediated recruitment of β1 integrin signaling in rat kidney fibroblasts (Hu et al., 2006, 2007). Two downstream effectors of integrin signaling, focal adhesion kinase and integrin-linked kinase (ILK), control fibroblast proliferation and matrix production, respectively (Hao et al., 2010). tPA also acts as a survival factor that protects renal interstitial fibroblasts/myofibroblasts from apoptosis, resulting in the expansion of the myofibroblast population in diseased kidneys.

Some studies have suggested that vascular pericytes are a major source of myofibroblasts in fibrotic kidneys (Lin et al., 2008; Humphreys et al., 2010; Schrimpf and Duffield, 2011). However, this concept remains controversial (Zeisberg and Duffield, 2010), largely due to difficulties in defining what constitutes a pericyte. Pericytes are one subset of the stromal cells, which offer endothelial partial support by covering capillary walls to aid in stabilization. The markers for pericytes used in several reports that indicate pericyte–myofibroblast conversions, such as PDGFRβ, are not absolutely specific and are indeed expressed in many cell types, including fibroblasts. Fibroblasts are intricately connected to the capillaries via cell processes in the renal interstitium (Liu, 2011). Some reports have suggested that once kidneys are damaged, pericytes no longer adhere to the endothelium and that thereafter, these cells migrate, proliferate, and finally differentiate into myofibroblasts (**Figure 2**; Lin et al., 2008; Humphreys et al., 2010). In

addition, the blockade of PDGFR signaling by imatinib reduces the number of myofibroblasts and restores renal damage in UUO models (Chen et al., 2011). These results also do not entirely support pericyte conversion into myofibroblasts but do indicate that PDGF signaling is a crucial player in kidney fibrogenesis (LeBleu and Kalluri, 2011). Although the pericyte conversion concept is interesting, it requires further study to verify whether pericytes and interstitial fibroblasts are, in fact, the same entity (Kaissling and Le Hir, 2008).

INFLAMMATION: BONE MARROW-DERIVED CELL RECRUITMENT

Fibrocytes, which are a subset of bone marrow-derived circulating monocytes, also display fibroblast-like features in the peripheral blood and are another possible source of fibroblasts (Herzog and Bucala, 2010). Human fibrocyte precursors can be co-purified together with $CD14^+$ monocytes (Peng et al., 2011). In mice, the fibrocyte transition from monocytes is enhanced with the expression of CD11b, CD115, and Gr1 (Niedermeier et al., 2009). The transition is stimulated by direct contact with activated CD4+ lymphocytes and is mediated via a mammalian target of rapamycin (mTOR)–PI3-dependent pathway (Niedermeier et al., 2009). Differentiated fibrocytes are negatively regulated by the Fcγ receptors CD64 and CD32, as indicated by the inhibition of such receptors with serum amyloid P, which inhibits fibrocyte accumulation in human and experimental animal models (Pilling et al., 2003, 2006, 2007) through immunoreceptor tyrosine-based inhibitory motif-dependent mechanisms (Pilling et al., 2006). Fibrocyte differentiation is stimulated by the Th2 cytokines interleukin (IL)-4 and IL-13 and TGF-β1, which is aided by integrin β1 and inhibited by Th1 cytokines such as interferon-gamma (IFN-γ), tumor necrosis factors (TNF), and IL-12. TLR2 agonists indirectly inhibit fibrocyte differentiation, and it is likely that this inhibition involves the mechanisms by which other cell types in the peripheral blood mononuclear cell population secrete unknown fibrocyte differentiation inhibitory factors (Maharjan et al., 2010). As TLR2 is a receptor for immunopathogens, it is possible that some bacterial signals can inhibit fibrocyte differentiation and may thus slow wound closure (Maharjan et al., 2010).

Fibrocytes display the characteristics of both fibroblasts and hematopoietic cells, as they are spindle-shaped and express the hematopoietic cell marker CD45. Fibrocytes have the ability to produce type I collagen (Pilling et al., 2009; Wada et al., 2011). Interestingly, fibrocytes also display certain chemokine receptors, such as CCR1, CCR2, CCR7, CXCR4 in mice (Sakai et al., 2006; Mehrad et al., 2009; Ekert et al., 2011; Scholten et al., 2011) and CCr2, CCR3, CCr5, and CXCR4, as well as the β1 integrin subunit, and semaphorin 7a in humans (Abe et al., 2001; Mehrad et al., 2009; Ekert et al., 2011; Gan et al., 2011). Following kidney injury, fibrocytes have been hypothesized to mobilize, infiltrate the renal parenchyma and participate in fibrogenesis. The differentiation of fibrocytes is also regulated through the other inflammatory cells, such as $CD4^+$ T cells, via the production of cytokines (Niedermeier et al., 2009). The profibrotic inflammatory cytokines IL-4 and IL-13 induce the differentiation of fibrocytes, whereas anti-fibrotic cytokines such as IFN-γ and IL-12 inhibit fibrocyte differentiation, as expected (Shao et al., 2008). Interestingly, the calcineurin inhibitor cyclosporine promotes fibrocyte differentiation, suggesting a possible explanation for cyclosporine-induced nephrotoxicity in clinical settings (Niedermeier et al., 2009). The inhibition of angiotensin II type-1 receptor signaling prevents the accumulation of fibrocytes in the kidneys as well as the bone marrow in mouse models of renal fibrosis (Sakai et al., 2008).

Nevertheless, the significance of fibrocytes in renal fibrogenesis remains controversial. Similar to the issues with other theories, there are no specific markers for these cells to allow a clear distinction of fibrocytes from other types of cells, such as monocytes, macrophages, fibroblasts, and myofibroblasts. Adding to the controversy, it has been shown that there are subpopulations of fibrocytes (Pilling et al., 2009). The role of fibrocytes, or bone marrow-derived cells, in renal fibrosis is inconsistent, yet (Iwano et al., 2002; Roufosse et al., 2006; Lin et al., 2008; Niedermeier et al., 2009) studies have shown that a considerable ratio of all collagen-producing fibroblasts in a mouse model of UUO that originate from fibrocytes or bone marrow-derived cells (Iwano et al., 2002; Niedermeier et al., 2009); however, other studies using the same model have reported contradictory results (Roufosse et al., 2006; Lin et al., 2008, 2009). If there is a specific fibrocyte lineage, it must contribute to the tissue repair process. The role of bone marrow cells in tissue repair has been reported in studies of genetic defects in type IV collagen α3 chain knockout mice, the model of human Alport syndrome (Sugimoto et al., 2006). Therefore, some bone marrow cell lineages must be involved in tissue repair. As fibrosis is the end result of uncontrolled tissue repair and wound healing processes, it is reasonable that fibrocytes have a role in fibrogenesis when they accumulate excessively at the injured site.

INFLAMMATION: EMT AND EndMT

During embryogenesis, the epithelia exhibit high plasticity, and they can alternate between epithelia and mesenchyme through the processes of EMT and mesenchymal-to-epithelial transition (MET; Thiery, 2002; Kalluri and Neilson, 2003). When organ development is completed, the epithelia typically display specialized functions, and this specialization is believed to be a terminal differentiation (Gumbiner, 1992; Yeaman et al., 1999). However, recent biological evidence has shed new light on the plasticity of epithelia, which were formerly considered terminally differentiated cells. Using human renal biopsy samples, the number of tubular epithelial cells with EMT features has been shown to be associated with serum creatinine and the degree of damage (Rastaldi et al., 2002). In addition, EMT has been reported in canine glomerulonephritis (Aresu et al., 2007). More than 100 studies have demonstrated the potential significance of EMT in kidney fibrosis by examining the phenotypic conversion of tubular cells in animal models as well as in renal biopsy samples from various kidney disease patients (Yang and Liu, 2002; Zeisberg et al., 2003; Hertig et al., 2008; Boonla et al., 2011; Togawa et al., 2011). Morphological evidence indicates that epithelial cells do indeed traverse the disrupted TBM into the interstitium after kidney injury in UUO in mice (Yang et al., 2002). EMT in human diseases, such as inflammatory bowel diseases and several cancers, has been reported (Kalluri and Weinberg, 2009). This evidence strongly suggests that epithelial plasticity and the occurrence of EMT play a role in certain human disease conditions.

Using a genetic-lineage tracking, Iwano et al. (2002) reported that over one-third of FSP1$^+$ interstitial fibroblasts originated from the tubular epithelia in a mouse model of obstructive nephropathy. Although such evidence strongly suggests the presence of EMT in tissue fibrosis, some recent experimental studies using lineage-tracing have reported that the epithelial or endothelial origin of fibroblasts is unclear (Humphreys et al., 2010; Li et al., 2010). These reports were questioned in a review (Zeisberg and Duffield, 2010). EMT-associated matrix-producing mesenchymal cells definitely exist, but EMT in fibrogenesis does not necessarily indicate de novo production of fibroblast from tubular epithelial cells (**Figure 3**), which is the extreme outcome of EMT (Kalluri and Weinberg, 2009; Zeisberg and Duffield, 2010; **Figure 3**). The number of epithelial cells undergoing EMT does not necessarily need to be identical or even similar to the number of cells that become fibroblasts (Zeisberg and Duffield, 2010). Fibroblasts proliferate at the injured site regardless of their origin. Therefore, even if the original EMT-derived fibroblasts were a minor population to begin with, they would proliferate and constitute a large portion of the fibroblasts, producing huge amounts of ECM components (Kalluri and Weinberg, 2009; Zeisberg and Duffield, 2010). Furthermore, as described above, EMT is a dynamic and even reversible process, and thus, intermediate EMT in tubular epithelial cells is more abundant compared to the extreme fibroblast end points of EMT. Therefore, the ratio of epithelial cells proceeding to complete EMT and complete conversion into fibroblasts is reasonably very low and depends heavily on disease model factors. It is likely that factors such as longer exposure to the micro-inflammatory milieu and the persistence of raised cytokine pressure are more likely to induce EMT (Liu, 2004; Kalluri and Weinberg, 2009; Zeisberg and Duffield, 2010).

Epithelial-to-mesenchymal transition is an important and useful therapy target in treating kidney disease. The blockade of EMT with various agents, such as bone morphogenetic protein (Zeisberg et al., 2003; Sugimoto et al., 2007), hepatocyte growth factor (Yang and Liu, 2002), ILK inhibitor (Li et al., 2009b), Wnt antagonists (Surendran et al., 2005; He et al., 2009), and paricalcitol (Tan et al., 2006), as well as the induction of endogenous heat shock protein (Wynn, 2007; Zhou et al., 2010), has been shown to ameliorate renal fibrosis and preserve kidney function in various animal models. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a master regulator of oxidative stress and a potential drug target for diabetic nephropathy (Pergola et al., 2011; Li et al., 2012), has an inhibitory effect on the EMT process via interaction with heme oxygenase-1 (Shin et al., 2010). In addition, it was recently shown that BMP7 receptor Alk3 in renal tubules is essential for anti-fibrogenesis and tissue repair in the kidneys; Alk3-agonistic compounds were also shown to exhibit renal protection in experimental kidney fibrosis models, including models of diabetic nephropathy; this renal protection was associated with the inhibition of EMT, inflammation, and apoptosis (Sugimoto et al., 2012).

Fibroblasts and/or myofibroblasts can be derived from capillary endothelium through the EndMT (Zeisberg et al., 2008; Li et al., 2009a). EndMT is considered to be a unique form of EMT, as endothelial cells are a specialized type of epithelia. Zeisberg et al. (2008) investigated the contribution of EndMT in renal fibrosis in three mouse models of chronic kidney disease, including STZ-induced diabetic nephropathy. When analyzed according to the presence of endothelial marker CD31 and the (myo)fibroblast markers αSMA and FSP1 in conjunction with lineage tracing, approximately 40% of all FSP-1-positive and 50% of αSMA-positive cells in STZ kidneys were also CD31 positive (Zeisberg et al., 2008), suggesting a significant contribution of EndMT in kidney fibrosis. Li et al. (2009a) also reported a contribution of EndMT in diabetic glomerular sclerosis as well as interstitial fibrosis in early diabetic mice.

Biological analysis has revealed that EndMT is also regulated by a combination of various inflammatory cytokine pressures. Although the role of inflammatory cytokines in EndMT and kidney fibrosis has not been clearly established, it is likely that inflammation is a critical mediator in EndMT. In an inflammatory

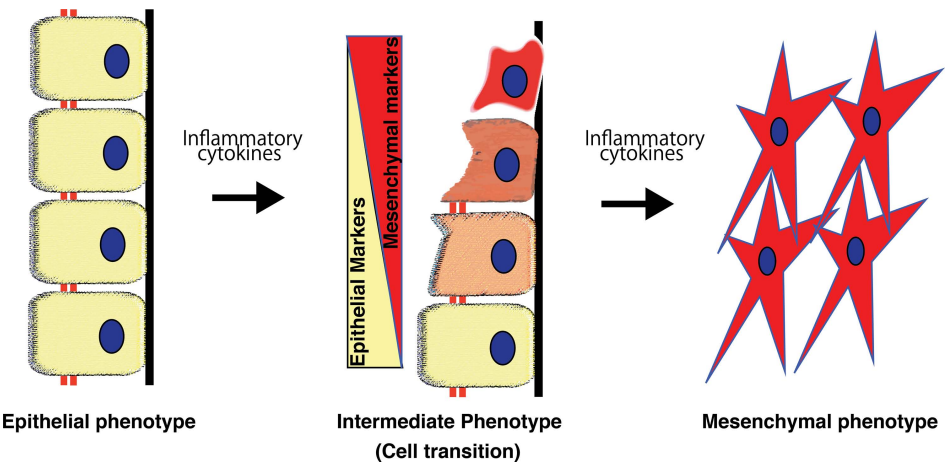

FIGURE 3 | Process of epithelial-to-mesenchymal transition. Epithelial-to-mesenchymal transition is a phenomenon that is well-orchestrated by inflammatory cytokines and results in epithelial cells gradually losing their cell markers and acquiring the mesenchymal phenotype.

bowel disease model, Rieder et al. (2011) clearly demonstrated that either a combination of TGF-β1, IL-1β, and TNF-α or activated lamina propria mononuclear cell supernatants induced morphological and phenotypic changes consistent with EndMT; each inflammatory cytokine alone had limited effects on endothelial phenotypic changes (Rieder et al., 2011). Another recent report indicated that the inflammatory transcriptional factor nuclear factor-kappaB (NFκB) determined inflammation-induced EndMT (Maleszewska et al., 2012). These reports suggest that inflammation has a crucial role in the pathogenesis of EndMT. TGF-βs, IL-1β, and TNF-α expression are indeed increased at the site of kidney injury in diabetes (Lim and Tesch, 2012). It has been shown that IL-1, IL-6, IL-18, and TNF-α are likely relevant in diabetic nephropathy.

Fibroblasts heterogeneity has been established, and such heterogeneity could be explained in part by various fibroblast origins, such as residential fibroblast activation, pericyte conversion, fibrocyte origination, and EMT/EndMT. In addition, kidney fibroblasts in diabetic kidneys could be somewhat different from those of other kidney diseases. Fibroblasts isolated from diabetic skin ulcers exhibited diverse morphological and functional characteristics when compared to normal skin fibroblasts (Loots et al., 1999; Xu et al., 2012b). It also likely that fibroblast activation from fibroblasts, pericytes, or fibrocytes is an early event (Kaissling and Le Hir, 2008), whereas EMT and/or EndMT contribute at a late stage after longer, sustained injury (**Figure 2**; Liu, 2004). In each type of fibroblast activation process and the sequential process of kidney fibrogenesis, inflammatory cells and their produced cytokines play a crucial role.

VARIATION IN SUSCEPTIBILITY FOR DIABETIC NEPHROPATHY: THE ROLE OF VARIATION IN INFLAMMATORY RESPONSE

Variations in conventional risk factors, which include variations in blood sugar control, blood pressure control, and proteinuria control, can contribute to variations in diabetic nephropathy susceptibility (Fernández Fernández et al., 2012).

Genetic polymorphisms in a variety of molecules involved in pathogenic pathways of diabetic nephropathy could contribute to the variation in individual susceptibility (Brorsson and Pociot, 2011). Genetic polymorphisms can affect important pathogenic pathways such as the renin–angiotensin pathway, the inflammatory cytokine pathway, the nitric oxide pathway, the bradykinin pathway, the Wnt pathway, Notch signaling, and the matrix metalloprotease-related pathway, and have an impact on the development and progression of diabetic nephropathy (Maeda, 2008; Brorsson and Pociot, 2011; Kure et al., 2011).

The cytokine milieu is more important than individual cytokine levels because cytokines exhibit pleiotropism, redundancy, synergism, antagonism, *trans*-modulation, and *trans*-signaling. The effect of the overall cytokine milieu, rather than individual cytokine levels, influences this effect (Navarro-Gonzalez et al., 2011; Elmarakby and Sullivan, 2012). The variations in the cytokine response that is mediated by genetic variation can contribute to the variations observed in diabetic nephropathy.

Variations in TLRs also modulate the response based on the level of antigenic exposure and their location in particular organs (Huebener and Schwabe, 2012). Too much immunity results in autoimmune disorders, and too little immunity results in infections. Thus, optimal immunity requires a balance between autoimmunity and opportunistic infections (Thomas, 2010; Fujio et al., 2012). There are no exact cutoff values that define high, optimal, or low immunity levels for inflammatory mediators. Acute and transient elevations in cytokines lead to different activity than does chronic low-grade elevation. Infection and injury that produce transient stimulus of the immune or inflammatory systems resolve without resulting in fibrosis (Serhan et al., 2007). If an infection or injury is chronic (e.g., tuberculosis or non-healing wounds), fibrosis can result (dos Santos et al., 2012; Franchi et al., 2012).

Persistent injury or inflammation after an unknown period of time can result in permanent changes in the inflammatory response, resulting in the unregulated production of profibrotic factors. Even controlling the inciting stimulus at that time may not be able to prevent the progression of the disease due to epigenetic alterations (Gaede et al., 2008; Bechtel et al., 2010; Xu et al., 2012a).

A multifaceted and sequential approach may be needed for the management of diabetic kidney fibrosis (Gaede et al., 2008). Diabetic kidney disease pathways are redundant with respect to the multiple pathogenic factors that can activate the final fibrosis pathway. At different stages of disease, different pathogenic pathways predominate, necessitating the initiation of therapies in a sequential manner.

Glycemic control is beneficial for preventing micro-vascular complications in newly diagnosed diabetic patients, as reported by the DCCT and UKPDS trials, but it is not beneficial in established diabetic patients for the prevention of significant outcomes of diabetic nephropathy, as reported in recent trials (Hemmingsen et al., 2011; Coca et al., 2012; Slinin et al., 2012). Late-stage diabetes, hypertension, and proteinuria may be the predominant causes of the progression of diabetic nephropathy, necessitating antihypertensive/antiproteinuric measures to prevent further disease progression.

Multiple and sequential management includes the initial blood sugar control and blood pressure control, followed by anti-inflammatory, anti-EMT/EndMT, and anti-fibroblast activation medications at different stages of diabetic kidney disease (Goel and Perkins, 2012).

PERSPECTIVE: SLOWING THE PROGRESSION OF DIABETIC KIDNEY DISEASE – THE ROLE OF INFLAMMATORY PATHWAYS

Existing therapies targeting glucose control, blood pressure control, and proteinuria reduction, alone or in combination, have failed to slow or reverse the progression of diabetic nephropathy. As is evident in the above discussion, the role of inflammation in the initiation and progression of diabetic nephropathy is undisputed.

Basic studies using various animal models have demonstrated pro-inflammatory cytokine gene activation in diabetic nephropathy (Navarro et al., 2005, 2006; Navarro-Gonzalez et al., 2009). Various existing drugs, which have anti-inflammatory activity, have been shown to slow or reverse diabetic kidney disease.

ANTI-INFLAMMATORY THERAPIES

An aldosterone antagonist (spironolactone) inhibits NFκB, thereby inhibiting MCP-1 (Han et al., 2006). Pioglitazone, a peroxisome proliferator-activated receptor (PPAR) agonist, has been shown to slow diabetic nephropathy by downregulating various pro-inflammatory and profibrotic genes, such as NFkB, CCL2, TGF-β1, plasminogen activator inhibitor-1 (PAI-1), vascular endothelial growth factor (VEGF), etc. (Ko et al., 2008b). ACE inhibitors and ARBs suppress NFκB signaling and thereby suppress the inflammatory response (Kim et al., 2011). Pentoxifylline, TNF-α receptor fusion proteins and chimeric monoclonal antibodies have been shown to decrease TNF-α, a pro-inflammatory cytokine, thereby decreasing fibrosis (Navarro-Gonzalez et al., 2009). Bardoxolone mesylate is a novel triterpenoid agent with Nrf-2 agonistic activity that has been shown to have anti-inflammatory and anti-oxidant activity. It inhibits NFκB and Janus kinase/signal transducers and activators of transcription (JAK-STAT) signaling and acts as an anti-oxidant and inflammatory modulator (Zhang, 2013). This compound was expected to be a useful drug for diabetic nephropathy in an early clinical trial (Pergola et al., 2011); however, further clinical trials evaluating the renoprotective effects of this drug against diabetic nephropathy were terminated due to increased cardiac events in the drug-treated group (Zhang, 2013). Finally, cyclooxygenase (COX)-2, a pro-inflammatory enzyme responsible for the formation of inflammatory prostanoids, is induced in diabetic kidneys (Komers et al., 2001), and preclinical data suggest that COX-2 inhibitors decrease proteinuria and preserve glomerular structure in animal models of diabetic nephropathy (Cheng et al., 2002; Komers et al., 2007; Matsunaga et al., 2007; Nasrallah et al., 2009; Quilley et al., 2011). However, clinical trials have not yet revealed any significant clinical outcomes of COX-2 inhibition on diabetic nephropathy (Sinsakul et al., 2007; Cherney et al., 2008a,b). In addition, the enhanced expression of COX-2 is involved in maintaining adequate renal hemodynamics and function in some patients with diabetic nephropathy (Khan et al., 2001). Therefore, the inhibition of COX-2 would be harmful in a certain set of diabetic nephropathy patients.

In view of the current understanding of the central role of inflammation in diabetic nephropathy progression, future studies may focus on maintaining epithelial integrity, halting the process of EMT/EndMT transition and controlling the cytokine milieu.

Future diabetic nephropathy research could be directed toward inhibiting the inflammatory pathway in conjunction with conventional therapeutic strategies (Navarro and Mora, 2006; Rivero et al., 2009), although a few fundamental questions remain. Indeed, in contrast to the experimental animal model studies, anti-inflammatory therapy has been notably ineffective in halting kidney fibrogenesis in clinical settings. Furthermore, anti-inflammatory therapy has worsened clinical outcomes in patients with lung fibrosis and systemic sclerosis (Wynn, 2011; Murray et al., 2012). These reports suggest that anti-inflammatory therapy would not be effective or could even worsen clinical outcomes in patients with established diabetic nephropathy and fibrosis. Additionally, there is a possibility that inflammation is an important initiator of fibrosis, as described above, although in the advanced chronic fibroproliferative stage, inflammation would act in an alternative manner to facilitate organ repair and protection. Therefore, examining stage-specific inflammation in diabetic nephropathy and its associated fibrosis, as well as identifying reliable biomarkers, is required before inflammation can be used as a therapeutic target in the treatment of this condition.

CONCLUSION

Diabetic nephropathy is a devastating kidney disease that contributes to the majority of end-stage kidney disease cases worldwide, and this condition is associated with a higher frequency of cardiovascular events. Diabetic nephropathy is associated with the activation of a variety of pathways that lead to the progression of kidney disease. Among these pathways, inflammatory pathway activation plays a central role. The inflammatory pathway leads to the activation and recruitment of the fibroblasts, which in turn initiate and sustain the fibrotic process. Apart from conventional glucose control and blood pressure control, targeting the specific pathways that lead to the activation of inflammation and fibroblasts could be a new and effective intervention in the management of diabetic nephropathy.

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Anti-neurotrophic effects from autoantibodies in adult diabetes having primary open angle glaucoma or dementia

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Aim: To test for anti-endothelial and anti-neurotrophic effects from autoantibodies in subsets of diabetes having open-angle glaucoma, dementia, or control subjects.

Methods: Protein-A eluates from plasma of 20 diabetic subjects having glaucoma or suspects and 34 age-matched controls were tested for effects on neurite outgrowth in rat pheochromocytoma PC12 cells or endothelial cell survival. The mechanism of the diabetic glaucoma autoantibodies' neurite-inhibitory effect was investigated in co-incubations with the selective Rho kinase inhibitor Y27632 or the sulfated proteoglycan synthesis inhibitor sodium chlorate. Stored protein-A eluates from certain diabetic glaucoma or dementia subjects which contained long-lasting, highly stable cell inhibitory substances were characterized using mass spectrometry and amino acid sequencing.

Results: Diabetic primary open angle glaucoma (POAG) or suspects ($n = 20$) or diabetic dementia ($n = 3$) autoantibodies caused significantly greater mean inhibition of neurite outgrowth in PC12 cells ($p < 0.0001$) compared to autoantibodies in control diabetic ($n = 24$) or non-diabetic ($n = 10$) subjects without glaucoma ($p < 0.01$). Neurite inhibition by the diabetic glaucoma autoantibodies was completely abolished by 10 μM concentrations of Y27632 ($n = 4$). It was substantially reduced by 30 mM concentrations of sodium chlorate ($n = 4$). Peak, long-lasting activity survived storage ×5 years at 0–4°C and was associated with a restricted subtype of Ig kappa light chain. Diabetic glaucoma or dementia autoantibodies ($n = 5$) caused contraction and process retraction in quiescent cerebral cortical astrocytes effects which were blocked by 5 μM concentrations of Y27632.

Conclusion: These data suggest that autoantibodies in subsets of adult diabetes having POAG (glaucoma suspects) and/or dementia inhibit neurite outgrowth and promote a reactive astrocyte morphology by a mechanism which may involve activation of the RhoA/p160 ROCK signaling pathway.

Keywords: autoantibodies, diabetes mellitus, open angle glaucoma, dementia, neurite outgrowth

INTRODUCTION

Glaucoma is a chronic neurodegenerative disorder affecting retinal ganglion cells (RGC) and is a leading cause of blindness worldwide (Quigley, 2011). Glaucoma has been reported to increase in older adults with type 2 diabetes mellitus (Klein et al., 1994; Goldacre et al., 2012), and in certain populations having Alzheimer's dementia (Tamura et al., 2006), although the mechanisms for these associations are unclear. The aim of the present study was to test the hypothesis that autoantibodies having anti-endothelial and anti-neuronal effects increase in older adults with type 2 diabetes and primary open angle glaucoma (POAG). We examined 20 older adults with type 2 diabetes and glaucoma, 34 age-matched adults without glaucoma (24 diabetic and 10 non-diabetic subjects), and 5 adults having diabetes and dementia for plasma autoantibodies which could inhibit neurite outgrowth in PC12 cells or decrease endothelial cell (EC) survival.

Humoral autoimmunity has been implicated in the etiology of certain forms of glaucoma (Tezel and Wax, 2004) including evidence for increased circulating autoantibodies to optic nerve head heparan sulfate proteoglycans (HSPG) in subsets of normal tension or POAG (Tezel et al., 1999). Anti-neuronal HSPG autoantibodies (having anti-neuronal and anti-endothelial effects) were reported in adult type 2 diabetes in association with certain microvascular complications including painful neuropathy (Zimering et al., 2011). The diabetic neuropathy plasma IgG autoantibodies induced EC contraction, EC apoptosis, and inhibited neurite outgrowth through a mechanism involving activation of the Rho A/Rho kinase signaling pathway (Zimering and Pan, 2009; Zimering et al., 2011). The Rho A/Rho kinase signaling pathway is present in diverse cell types (neurons, astrocytes, and endothelial-like trabecular meshwork cells) implicated in the pathophysiology of glaucoma (Tura et al., 2009; Kumar

and Epstein, 2011; Kameda et al., 2012). Our hypothesis is that autoantibodies capable of binding to HSPG expressed on neurons, astrocytes, and ECs and activating Rho A/Rho kinase signaling may be capable of mediating axonal injury, ischemia, and glial reactivity underlying early glaucomatous changes (Crish and Calkins, 2011) leading to vision loss.

We now report that plasma IgG autoantibodies from diabetic POAG or suspects ($n = 20$) significantly inhibited neurite outgrowth in PC12 cells ($p < 0.0001$) compared to autoantibodies from diabetic ($n = 24$) or non-diabetic ($n = 10$) subjects without glaucoma. The neurite outgrowth inhibitory activity in four of four diabetic (POA) glaucomatous plasma autoantibodies tested was completely abolished by co-incubating PC12 cells with 10 μM concentrations of Y27632, a selective Rho kinase inhibitor. The neurite-inhibitory activity was also significantly reduced in the presence of 30 mM sodium chlorate which substantially reduces HSPG expression in PC12 cells. Taken together, these data suggest possible involvement of cell surface HSPG and activation of the Rho A/Rho kinase signaling pathway in the mechanism for PC12 neurite inhibition by diabetic glaucoma plasma autoantibodies.

MATERIALS AND METHODS

SUBJECTS

Informed consent for the Investigational Review Board (IRB) approved substudy to the Veterans Affairs Diabetes Trial (VADT) was obtained from all subjects. Fifteen of 89 subjects enrolled at the VA New Jersey Healthcare System (VANJHCS) study site to the VADT had a diagnosis of POAG or glaucoma suspect. Eleven of the 15 VADT subjects were included in the study based on the availability of an aliquot of plasma stored at −70°C necessary for protein-A affinity chromatography to obtain IgG autoantibodies. The four excluded subjects included two non-Hispanic white glaucoma subjects, one African-American glaucoma suspect and one non-Hispanic white glaucoma suspect. A control group of 21 VADT subjects without glaucoma or suspicion of glaucoma was randomly selected from among the remaining patients in whom baseline plasma was available for analysis. Twenty-two additional diabetic or non-diabetic glaucoma subjects or controls all evaluated in an IRB-approved VANJHCS study were selected for further analysis of the association between diabetic POAG and plasma autoantibodies.

DIAGNOSTIC METHODS AND SUBGROUPS

Glaucoma or suspects

All subjects were evaluated by the optometry and/or ophthalmology staff at the Veterans Affairs New Jersey Healthcare System. The diagnosis of POAG was based on findings from a combination of test modalities including: dilated fundoscopic examination, applanation tonometry, gonioscopy, pachymetry, periodic Humphrey visual field testing, or the results of Humphrey visual field tests, dilated fundoscopic examination, and tonometry performed by outside ophthalmologists and reported to optometry/ophthalmology specialists at the VANJHCS. Glaucoma suspect is defined as a high risk individual with asymmetric cup to disk (C/D) enlargement, equivocal neural rim narrowing, or retinal nerve fiber layer thinning, but without definite evidence for visual field loss. Subjects with increased intraocular pressure (IOP) alone, but without C/D enlargement were not included in the primary analysis, e.g., ocular hypertension ($n = 1$). Because many patients were already being treated with IOP-lowering medications at the time of study enrollment, it was not possible to distinguish subgroups of normal tension glaucoma vs. high-pressure POAG. Patients with secondary forms of glaucoma such as neovascular glaucoma ($n = 1$), or corticosteroid-associated glaucoma ($n = 1$) were excluded from the analysis. Subjects with other ocular or neuropathologies previously associated with potent EC autoantibodies, but lacking diabetic painful neuropathy, e.g., central retinal artery occlusion ($n = 1$), dementia ($n = 1$), or stroke ($n = 1$) were excluded from the analysis. Painful diabetic neuropathy was defined as the presence of characteristic findings on clinical examinations performed by expert neurologists as previously reported (Zimering et al., 2011). Diabetic nephropathy was defined as urinary albumin excretion ≥300 mg/g creatinine or urinary protein excretion ≥500 mg/g creatinine.

Diabetes and dementia (n = 3 + 2)

In three VADT patients who developed dementia at the end of the clinical trial, neurite-inhibitory activity in PC12 cells was analyzed (**Figure 1**) as a control for the results in glaucoma, another type of chronic neurodegenerative disorder. Patient #1 was a 68-year-old African-American male with POAG, long-standing type 2 diabetes without significant diabetic retinopathy who was diagnosed with glaucoma at the onset of his VADT participation. His mild visual field loss was stable over a 7-year observation period; he was diagnosed with Alzheimer's type dementia. Patient #2 is a 71 year old non-Hispanic white male with type 2 diabetes, ocular hypertension without baseline visual field loss whose visual fields were stable and cup/disk ratio was normal over a 5-year observation period; he was diagnosed with dementia secondary to traumatic brain injury. Patient #3 was a 75-year-old non-Hispanic white male with long-standing type 2 diabetes, no evidence of glaucoma, and mixed fronto-temporal and multi-infarct dementia.

Two additional subjects having diabetes and dementia were included in the analysis of EC inhibitory activity in stored protein-A eluates (**Table 3**). Patient #4 is a 70-year-old VADT subject who had type 2 DM, mild diabetic retinopathy, and later developed Alzheimer's type dementia. Patient #5 is a 53-year-old type 1 DM (non-VADT) patient who had a wide spectrum of microvascular complications including nephropathy leading to end stage renal disease, multiple recurrent small vessel strokes (Zimering, 2010) progressing to dementia, proliferative diabetic retinopathy, autonomic and painful neuropathy, and glaucoma possibly due to steroid use.

Blood drawing

Baseline plasma samples were obtained from study subjects prior to initiation of treatment in the VADT substudy. Plasma bFGF was determined with a sensitive specific two-site IRMA as previously described (Zimering et al., 2009a). Bioactivity in protein-A eluate fractions was previously shown to be stable for 5 years or longer at −20°C (Zimering and Thakker-Varia, 2002).

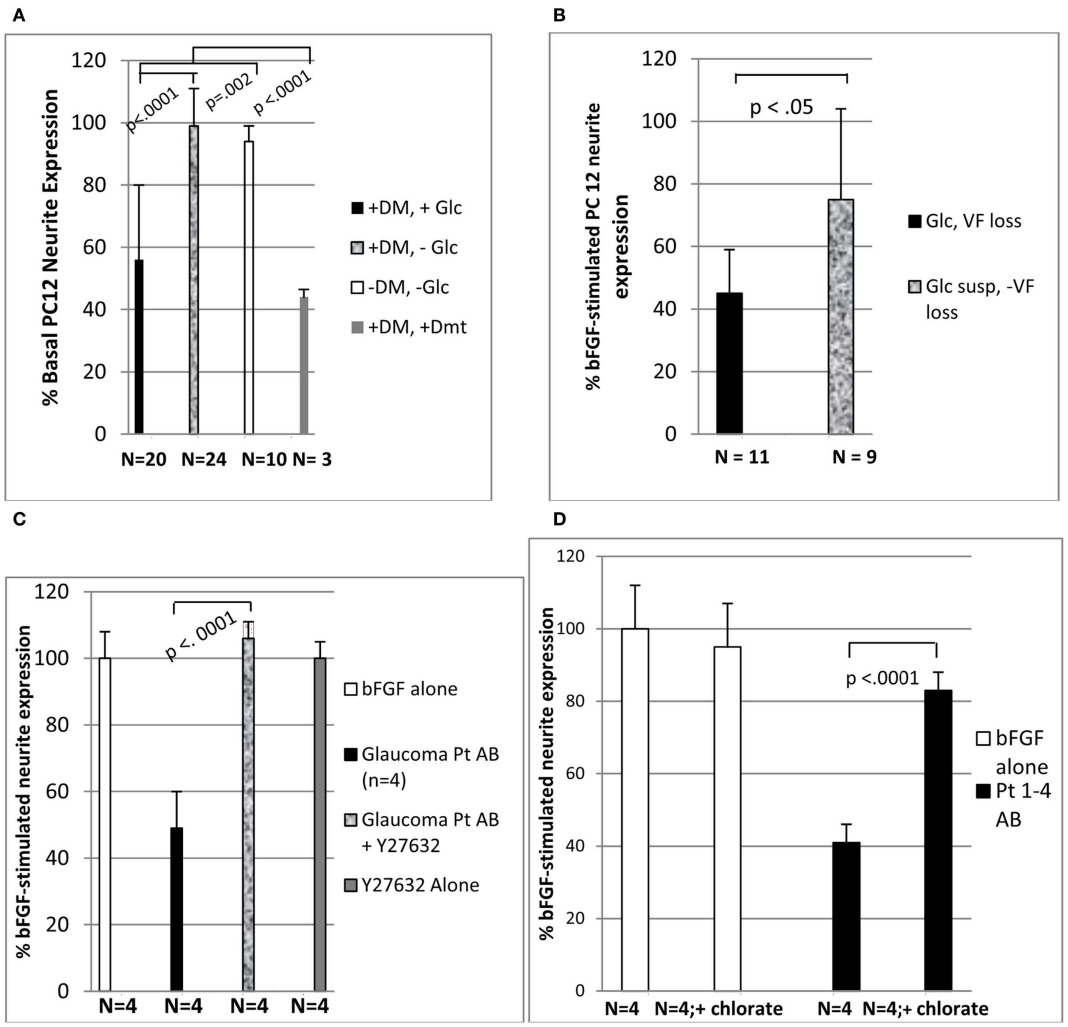

FIGURE 1 | Diabetic glaucomatous autoantibodies inhibit neurite expression in PC 12 cells (A) highest neurite-inhibitory activity was associated with glaucomatous vision loss (B) and was eliminated by treatment with Y27632 (C) or 30 mM sodium chlorate (D). (A-D) Thirty microgram per milliliter concentrations of the protein-A eluate fraction of plasma was incubated with PC12 cells in the presence or absence of 10 μM concentrations of Y27632 **(C)** or 30 mM concentrations of sodium chlorate **(D)** as described in Section "Materials and Methods." Results (mean ± 1 SD of four different protein-A eluates) are expressed as % neurite expression compared to cells incubated with 10 ng/mL concentrations of basic fibroblast growth factor alone [i.e., 100%, open bars **(C,D)**]. DM, diabetes mellitus; Glc, glaucoma or suspect (susp); Dmt, dementia; VF, visual field.

Neurite outgrowth inhibition assay

Undifferentiated rat pheochromocytoma PC12 cells obtained from ATCC (Manassa, VA, USA) were grown in DMEM containing 10% horse serum, 5% fetal calf serum, and 10 ng/mL bFGF was added in order to induce neuronal differentiation. Test protein-A eluate fractions (1:50 dilution = 30 μg/mL protein) were added to cells in duplicate or triplicate and incubated at 37˚C for 48 h. Next the proportion of cells expressing a neurite of ≥2 cell diameters in length was counted and compared to the proportion of bFGF-stimulated neurite cell expression in dishes containing bFGF without added test fractions as previously described (Zimering et al., 2011). Rho kinase-dependence of the neurite inhibition was assessed by co-incubating cells in the presence or absence of 10 μM concentrations of Y27632, a selective inhibitor for the Rho-associated protein kinase, p160ROCK (Uehata et al., 1997). Dependence of neurite-inhibitory effects in the diabetic protein-A eluates on cell surface sulfated proteoglycans was tested by comparing results in to pheochromocytoma PC12 cells grown in the presence or absence of 30 mM sodium chlorate, a treatment which substantially reduces HSPG expression in the cells (Hoogewerf et al., 1991).

Endothelial cell survival assay

Bovine pulmonary artery ECs (Clonetics, Inc., San Diego, CA, USA) were grown in Medium 199 plus 10% fetal calf serum and EC growth medium (EGM, Clonetics, Inc., San Diego, CA, USA). EC survival in the presence or absence of diabetic plasma protein-A eluates (30 μg/mL) was assessed after 48 h incubation as previously described (Zimering et al., 2011). Results represent the mean (±1 SD) of quadruplicate determinations.

Human lymphoblast cell culture

Human lymphoblasts (GM 01500) from a patient having light chain amyloidosis (AL disease) were obtained from the Human Mutant Repository, Coriell Institute (Camden, NJ, USA), and grown in RPMI 1640 containing 2 mM L-glutamine, 10% fetal calf serum. The cell supernatant was subjected to sequential protein-A affinity chromatography followed by antihuman IgG affinity chromatography in order to obtain partially purified monoclonal human IgG for testing of EC bioactivity.

Protein-A affinity chromatography

The IgG fraction of plasma was isolated using protein-A affinity chromatography as previously reported (Zimering et al., 2011).

Antihuman IgG affinity chromatography

The protein-A eluate fraction obtained after applying 1.0 mL of the human lymphoblast (GM 01500) cell supernatant to a protein-A affinity column (1.0 mL) was adjusted to pH 7.0 by adding 1 M Tris pH 9.0. The eluate was then diluted 1:1 with an equal volume of 100 mmol/L Tris pH 8.0 and applied to a goat antihuman IgG agarose column (1.0 mL), washed with 5 mL of 100 mmol/L Tris pH 8.0, and eluted stepwise with 5× 1.0 mL aliquots of 100 mmol/L sodium citrate, pH 3.0. The second eluate fraction contained nearly all of the recovered protein and it was adjusted to pH 7.0 with 1 M Tris pH 9.0 prior to sterile filtration and testing of biological activity.

Cerebral cortical astrocytes

Rat cerebral cortices were dissociated and placed in polylysine-coated T-75 flasks containing minimal essential medium without phenol red and 15% fetal bovine serum (having low estradiol concentration)-NM-15. They were fed on day 3 with fresh NM-15 medium. On day 10, the flasks were shaken overnight at 250 rpm. The next day, the medium was removed, the cells were rinsed with warm PBS, and then fresh NM-15 containing 10 mM AraC was added to the cells. Two days later, the cells were trypsinized and plated at 250,000 cells per dish in polylysine-coated 35 mm dishes containing NM-15. After 2–3 weeks in serum-containing medium, a small percentage of the astrocytes (1–5%) spontaneously differentiated from a flat polygonal shape into cells having a "stellate" appearance, bearing one or more thick processes located proximal to the cell body or several thin long branching processes located distal to the cell body. The acute effects of diabetic plasma autoantibodies on astrocyte morphology were assessed in cells that had been maintained for 2–3 weeks in culture. Cell viability was good under these long-term culture conditions.

Time-lapse photomicroscopy

Stellate-appearing astrocytes (and surrounding less differentiated cells) were imaged under high power magnification using a Nikon TMS microscope at 200× magnification. Following bath application of a 1:100 dilution (3–20 μg/mL) of protein-A eluates from diabetic glaucoma or control subjects, cells were observed continuously for acute change in morphology. Baseline and follow-up images were captured using a Nikon camera connected to the microscope with a phototube every 5 min for a period up to 30–45 min after addition of protein-A eluates.

Heparin Sepharose affinity chromatography

Heparin Sepharose (HS) affinity chromatography was carried out as previously reported (Zimering et al., 2009a).

SELDI mass spectrometry

Mass spectrometry was carried out as previously reported (Zimering et al., 2011).

Protein sequencing

Amino acid sequencing was carried out on the Applied Biosystems (Foster City, CA, USA) Procise®494 protein sequencer using standard Edman sequencing. The resulting chromatographs were analyzed using Model 610A software.

Furin digests of diabetic protein-A eluates

Two microliters of an aqueous solution containing human recombinant furin (≥2000 units/mL) was added to 40 μL of protein-A eluates (8 μg protein) from each of three diabetic subjects in buffer containing 100 mmol/L Tris, pH 7.0, and 200 μM calcium. Following 180 min incubation at 25°C, 4 μL aliquots of furin-treated or -untreated protein-A eluates were added in quadruplicate to ECs for testing of biological activity. The remaining aliquot of furin-treated and -untreated paired samples was subjected to mass spectrometry which revealed a decrease in the peak 23 kDa apparent light chain MW species (present in untreated samples) in paired samples subjected to furin digestion.

Chemicals

Protein-A agarose was obtained from Pierce Chemical Co. (Rockford, IL, USA). Goat antihuman IgG (whole molecule) agarose was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Human recombinant furin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were analytical grade.

Protein determinations

Protein concentrations were determined by a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

Statistics

All data are the mean ± 1 SD as indicated. Comparisons were made by paired and unpaired Student's *t*-tests, or Fischer's exact test as indicated.

RESULTS

ASSOCIATION BETWEEN DIABETIC GLAUCOMA OR GLAUCOMA SUSPECT AND RACE

Diabetic subjects with POAG or POAG suspects included a significantly higher proportion of African-American or subjects of Afro-Caribbean descent compared to diabetes without glaucoma (**Table 1**). Mean baseline diastolic blood pressure was significantly higher in diabetes having POAG or suspects than in diabetes without glaucoma (**Table 1**). It was also higher in a control group of non-diabetic subjects without glaucoma (**Table 1**). There was a trend of a significant association ($p = 0.06$) between low plasma basic fibroblast growth factor (0–3.4 pg/mL) and an increased proportion of diabetic subjects having POAG or suspects vs. no POAG or suspect (100 vs. 69%; **Table 1**).

Table 1 | Baseline clinical characteristics in the study subjects.

Risk factor	Diabetes			No diabetes
	No Glc ($N=24$)	Glc or susp ($N=20$)	*P*-value*	No Glc ($N=10$)
Age (years)	65.2 ± 8.3	69.6 ± 12.3	0.16	68.0 ± 16.7
Race (NHW/AA/H)	(22/1/1)	(9/10/1)	0.0006^	(5/4/1)
BMI (kg/m^2)	32.0 ± 5.1	32.0 ± 6.9	0.99	31.2 ± 7.7
DM duration (years)	11.0 ± 6.9	13.9 ± 10.0	0.28	NT
Hba_1c (%)	8.3 ± 1.4	8.3 ± 1.5	0.95	NT
Syst bp (mm Hg)	129.9 ± 10.9	133 ± 13.6	0.48	124 ± 7.4
Diast bp (mm Hg)	67.6 ± 8.9	74.6 ± 9.1	0.01	76 ± 7.0[a]
Total Chol (mg/dL)	172 ± 14	176 ± 41	0.78	174 ± 51
Low bFGF (%)	69 (16)	100 (11)	0.06^	NT
Insulin use (%)	39	60	0.18^	NT

*Results are mean ± 1 SD or proportion; (), number tested; Glc, glaucoma; susp, suspect; bp, blood pressure; Chol, cholesterol; Low bFGF, basic fibroblast growth factor = <4 pg/mL; ^ Fischer's exact test; *P-value for comparison of diabetics with or without glaucoma. NHW, non-Hispanic white, AA, African-American, H, Hispanic, [a] P = 0.016 compared to diabetes without glaucoma.*

Table 2 | Association between diabetic glaucoma or suspect and co-morbid microvascular complications or family history of dementia.

Risk factor	Diabetes		
	No Glc ($N=24$)	Glc or susp ($N=20$)	*P*-value*
ME, AMD (%)	12.5	30	0.26
Nephropathy (%)	25	15	0.48
Painful neuropathy (%)	25	65	0.014
Inhibitory EC Act[c] (%)	25	65	0.014
FH of dementia (%)	0 (20)	27 (11)	0.04

*Results are proportion; (), number tested; Glc, glaucoma, susp, suspect, *Fischer's exact test, comparison of diabetics with or without glaucoma; ME, macular edema, AMD, age-related macular degeneration, FH, family history in a 1° relative of Alzheimer's dementia; [c] significant inhibitory endothelial cell (EC) activity, defined as ≤90% of basal cell number as described in Section "Materials and Methods."*

ASSOCIATION BETWEEN DIABETIC GLAUCOMA (SUSPECT) AND PAINFUL NEUROPATHY OR FAMILY HISTORY OF DEMENTIA

Low plasma basic fibroblast growth factor was associated with a significantly increased occurrence of EC inhibitory autoantibodies in older adult type 2 diabetes from the VADT (Zimering et al., 2009a). Since baseline EC inhibitory autoantibodies predicted an increased risk for certain microvascular complications in the VADT substudy (Zimering et al., 2009b), we tested for associations between diabetic POAG and EC inhibitory autoantibodies or co-morbid diabetic microvascular complications. A significantly higher proportion of diabetic subjects with POAG or suspects (65 vs. 25%) had inhibitory EC autoantibodies compared to the proportion of diabetic subjects without glaucoma ($p = 0.014$; **Table 2**). Painful neuropathy was significantly more prevalent among diabetic POAG or suspects compared to diabetes without POAG ($p = 0.014$; **Table 2**). First-degree relatives (mother, father) of diabetic POAG or suspects were significantly more likely to have had Alzheimer's dementia as a contributory cause of death compared to the parents of diabetic subjects without glaucoma (3/11 vs. 0/20; $p = 0.04$, **Table 2**).

NEURITE OUTGROWTH INHIBITORY ACTIVITY IN DIABETIC PROTEIN-A ELUATES: ASSOCIATION WITH GLAUCOMATOUS VISION LOSS OR DEMENTIA

Mean PC12 neurite outgrowth inhibitory activity in the protein-A eluates (1:50 dilution; ∼30 μg/mL) of plasma from diabetic POAG or suspects significantly exceeded mean inhibitory activity from diabetes without glaucoma (55.7 ± 24 vs. 99.0 ± 12%; $p < 0.0001$; **Figure 1A**). It was also significantly more inhibitory ($p = 0.002$) compared to mean activity in protein-A eluates from subjects without diabetes and without glaucoma (94.2 ± 5%; **Figure 1A**). The protein-A eluates of three diabetic subjects who later developed dementia had the most potent mean neurite-inhibitory activity (44 ± 3%). It significantly exceeded mean inhibitory activity in IgG from diabetes without glaucoma ($p < 0.0001$, **Figure 1A**). Diabetic glaucomatous vision loss ($n = 11$ subjects) was associated with significantly more inhibitory mean PC 12 neurite activity compared to mean activity in the protein-A eluates from nine diabetic POAG suspects (45.5 ± 14 vs. 74.6 ± 31; $p < 0.05$; **Figure 1B**).

EFFECT OF RHO KINASE INHIBITOR ON NEURITE-INHIBITORY ACTIVITY IN DIABETIC PLASMA PROTEIN-A ELUATES

The Rho A/Rho kinase signaling pathway plays an important role in axonal pathfinding during development (Dickson, 2001). Y27632 is a selective inhibitor of Rho kinase (Ishizaki et al., 2000). Diabetic glaucomatous plasma autoantibodies (30 μg/mL) from four African-American patients having moderate or severe progressive glaucomatous visual loss caused significant inhibition of neurite outgrowth in PC 12 cells (**Figure 1C**). The mean inhibitory activity from the four protein-A eluates was significantly reduced ($p < 0.001$) by co-incubating PC12 cells with 10 μM concentrations of Y27632 (**Figure 1C**). Similar concentrations of Y27632 alone had no significant effect on PC12 cell neurite expression compared to cells exposed to bFGF (10 ng/mL) without Y27632 (**Figure 1C**).

EFFECT OF REDUCED SULFATED PROTEOGLYCAN EXPRESSION IN PC 12 CELLS

Sodium chlorate substantially reduces the sulfation of proteoglycans in various cells (Hoogewerf et al., 1991). To test for possible involvement of sulfated proteoglycan (e.g., HSPG) in the mechanism for neurite inhibition in diabetic glaucomatous plasma protein-A eluates, PC12 cell cultured in the presence or absence of 30 mM sodium chlorate were incubated with the protein-A eluate fractions (30 μg/mL) of plasma from four diabetic subjects with POAG. The mean neurite-inhibitory activity in all four protein-A eluates (Pt 1–4 AB) (assayed individually) was significantly reduced ($P < 0.0001$) in the PC12 cells cultured in the presence of 30 mM sodium chlorate compared to PC12 cells grown in standard medium (DMEM with 1% FCS) in the absence of chlorate

ion (solid bars, **Figure 1D**). PC12 cell viability was unaffected by the presence of 30 mM chlorate. Neurite expression did not differ significantly in cells grown in the presence of bFGF with or without 30 mM chlorate (open bars, **Figure 1D**).

EFFECT OF DIABETIC PLASMA (POA) GLAUCOMATOUS AUTOANTIBODIES ON ASTROCYTE MORPHOLOGY

Astrocytes play critical role(s) in maintenance of the blood-brain barrier (Allen et al., 2010), protect neurons against glutamate-induced excitotoxicity, and modulate blood flow to actively firing neurons. To facilitate these important neuronal support roles, astrocyte processes lie in close apposition to basement membranes, capillaries, and neuronal synapses. Since the expression of astrocytic processes, or stellation, is inhibited by activity in the RhoA/Rho kinase signaling pathway (Abe and Misawa, 2003), we next tested whether autoantibodies from diabetic POAG or control subjects could inhibit astrocyte stellation *in vitro*. Plasma autoantibodies (1:100 dilution = 3–20 μg/mL) from five of five diabetic glaucoma or suspects tested ($n = 8$ experiments) caused retraction of thick processes located proximal to the astrocyte cell body (e.g., **Figures 2A,B**). Withdrawal of proximally located processes was most rapid (occurring within 2–5 min) and more extensive leading to disruption connectivity among processes in neighboring cells after bath application of low concentration (3 μg/mL) of diabetic POAG plus dementia autoantibodies (**Figures 2C,D**). The retraction of thick astrocytic processes (induced by diabetic glaucoma + dementia autoantibodies) was prevented by pretreating cells (for 10 min) with 5 μM concentrations of Y27632

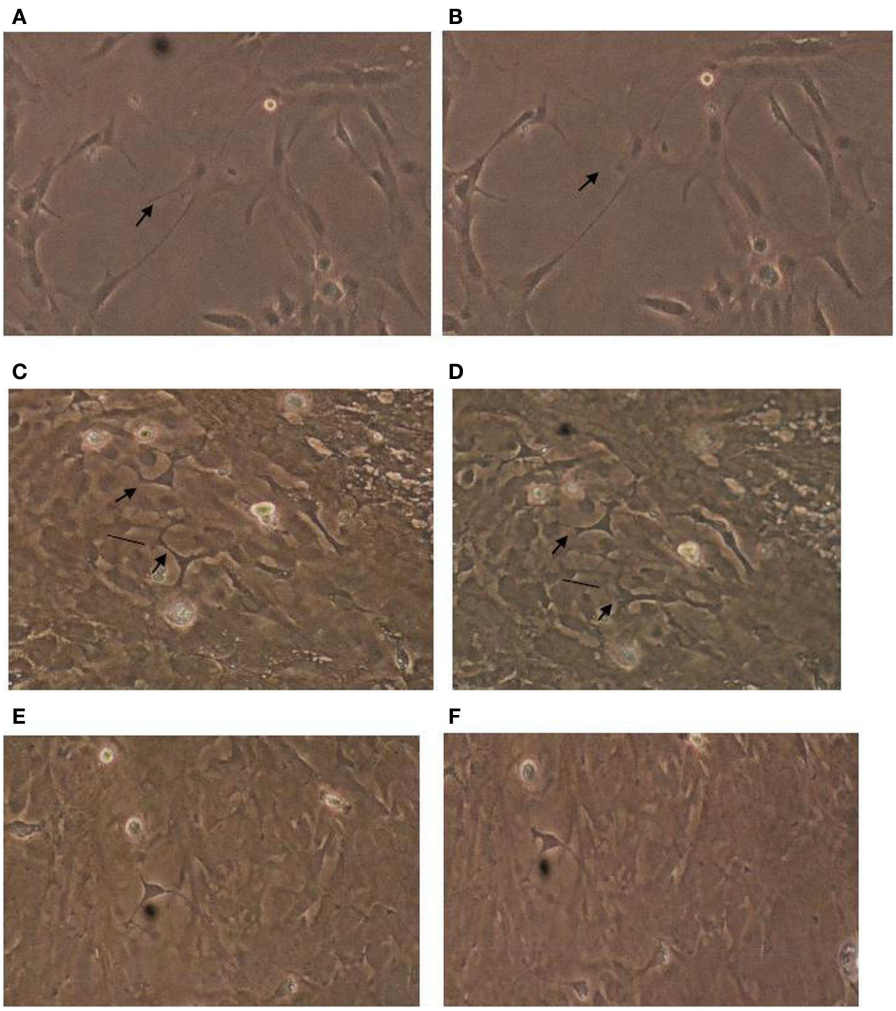

FIGURE 2 | Effect of diabetic glaucomatous autoantibodies on astrocyte morphology. Morphology in cerebral cortical astrocytes **(A)** before or **(B)** 30 min after addition of 10 μg/mL concentration of protein-A eluate from type 2 DM and glaucoma; **(C)** before or **(D)** 10 min after addition of 3 μg/mL concentration of the protein-A eluate fraction from type 2 DM having glaucoma and Alzheimer's type dementia (Pt 1). Antibodies caused varying degrees of withdrawal of thick astrocyte processes associated with increased stress fiber expression (arrows), and cell contraction (vertical lines). Astrocytes cultured in the presence of 10 μM concentrations of Y27632 for 10 min before **(E)** and after **(F)** addition of a 3 μg/mL concentration of the Pt 1 protein-A eluate fraction did not undergo similar changes in their appearance. Results similar to those shown in **(A–D)** were obtained in eight experiments using 3–10 μg/mL concentrations of autoantibodies from five different diabetic glaucoma patients. Much less if any acute change in morphology was observed ($n = 4$ experiments) using 10–20 μg/mL concentrations of protein-A eluate from four type 2 DM subjects without glaucoma.

(**Figures 2E,F**; $n = 2$ experiments). Y27632 alone had no effect on astrocyte morphology (not shown in **Figure 2**). Control plasma autoantibodies (10–20 μg/mL) from four of four age-matched, non-diabetic subjects without glaucoma (or suspicion of glaucoma) tested had little or no significant effect on the morphology of astrocyte thick processes ($n = 4$ experiments, not shown in **Figure 2**). The active autoantibodies (10–20 μg/mL) from two of the diabetic glaucoma subjects that caused retraction of thick astrocyte processes had no apparent significant effect on morphology of thinner more distally located processes found in several highly differentiated, extensively branching astrocytes ($n = 2$ experiments, each observed for 30 min).

FURIN TREATMENT UNMASKED POTENT ENDOTHELIAL CELL INHIBITORY ACTIVITY IN DIABETIC DEMENTIA PROTEIN-A ELUATE

Mean EC inhibitory activity in the protein-A eluates from subsets of diabetic macular edema or nephropathy ($n = 6$) or in diabetic dementia plasma ($n = 5$) significantly exceeded mean activity in protein-A eluates from diabetic non-glaucoma or glaucoma suspects (**Table 3**). After 9 months storage of Patient 4 and 5 diabetic dementia protein-A eluates, a significantly more inhibitory form of EC activity appeared spontaneously (**Table 3**) having similar magnitude to the inhibitory activity recovered from the Patient 1 diabetic dementia and glaucoma protein-A eluate following brief exposure to the proprotein convertase (PC) furin (**Table 3**). Mass spectrometry or amino acid sequencing of long-term stored protein-A eluates from four of four diabetic dementia plasmas tested revealed either peak 23 kDa MW species or 86–89% amino acid sequence homology to human kappa (κ) light chain variable regions. In an adult type one DM patient (Patient 5) who suffered a severe unremitting course of microvascular and neuropathic complications (Zimering, 2010) including multi-infarct dementia and steroid-induced glaucoma, the protein-A eluate retained its potent neurite-inhibitory activity after 5 years storage at 0–4°C. Half-maximal inhibition in EC survival occurred at concentrations of Patient 5 IgG (30 nM) which were comparable to the half-maximal inhibitory concentrations (10 nM) observed in the purified monoclonal IgG_2 kappa light chains obtained from a clone of human lymphoblasts (GM01500) from a patient having multiple myeloma and systemic light chain AL (data not shown). Using HS affinity chromatography, we were able to obtain a highly purified peak EC inhibitory fraction in the Patient 5 protein-A eluate which eluted from HS column with 0.1 M NaCL. Comparison of the amino acid sequences in the Patient 5 peak, HS-purified fraction to its starting material protein-A eluate or to AL disease monoclonal IgG_2 kappa light chains revealed the presence of proline at amino acid position 12 in the peak HS-purified Patient 5 diabetic eluate only. Proline at amino acid position 12 is characteristic of a restricted subgroup of kappa II gene-encoded sequences (Wu and Kabat, 1970).

Table 3 | Effect of long-term storage of protein-A eluates or furin digestion on EC activity.

Diabetic subset	Endothelial cell bioactivity (%)		
	Before	After	*P*-value*
LONG-TERM STORAGE (9–60 MONTHS)			
No glaucoma or susp ($n = 4$)	107.3 ± 6.1	108 ± 6.3	0.87
Glaucoma suspect ($n = 4$)	98.7 ± 5.3	86.8 ± 20.2	0.20
Macular edema/nephr. ($n = 6$)	81.2 ± 5.8†	86.5 ± 14.8	0.47
Dementia ($n = 5$)	81.2 ± 3.3†	66.5 ± 4.5 (2)	0.01
FURIN			
Pt 1: glaucoma + Alz dementia ($n = 1$)	80 ± 8	65 ± 3	0.02
Pt 2: OHT + dementia ($n = 1$)	82 ± 11	87 ± 4	0.63

*Results are mean, ± 1 SD, endothelial cell bioactivity was determined as described in Section "Materials and Methods." *P-value from Student's t-test comparing results before and after storage or furin treatment † $P < 0.0001$ compared to mean activity (before storage) in group of glaucoma suspects. OHT, ocular hypertension.*

DISCUSSION

The present data are the first to suggest that plasma autoantibodies from a subset of older adult type 2 diabetes having POAG or suspects cause significant inhibition of neurite outgrowth in PC 12 cells via a mechanism which may involve binding to cell surface HSPG and activation of intracellular Rho kinase. Our results are consistent in part with earlier reports of increased titers of autoantibodies specific for (optic nerve head) HSPG in subsets of normal tension or primary angle glaucoma having a systemic autoimmune disease (Tezel et al., 1999).

Unlike systemic lupus erythematosus (SLE), type 2 diabetes is not a systemic autoimmune condition. Yet autoantibodies capable of inducing EC apoptosis were described in subsets of SLE nephropathy (van Paassen et al., 2007) or in older adult type 2 diabetes in association with macular edema, nephropathy, and/or painful neuropathy (Zimering and Pan, 2009). The present finding of a significant association between the occurrence of diabetic painful neuropathy and POAG is novel. Taken together with the chlorate sensitivity of the PC12 neurite-inhibitory activity in diabetic glaucoma autoantibodies it suggests HSPG (or a closely related sulfated proteoglycan) as a possible common target for autoantibodies in diabetic POAG and diabetic painful neuropathy.

Heparan sulfate glycosaminoglycans (GAGs) play important roles in early brain development (Inatani et al., 2003) including ensuring retinal axon guidance to the optic nerve head (Ogata-Iwao et al., 2011) and the correct topographic arrangement of axons in the optic nerve (Lee et al., 2004). HSPG are ubiquitously expressed on the cell surface of ECs, mesenchyme-derived cells and neurons. Following CNS injury, astrocyte HSPG increases its sulfation (Properzi et al., 2008). Basic fibroblast growth factor is a known neurotrophic and survival factor in RGC (Soto et al., 2006) which requires HSPG for its biological activity. Anti-HSPG autoantibodies may interfere with the survival-promoting effects of locally available bFGF in RGCs or contribute to generally low plasma bFGF in diabetic glaucoma. Autoantibody-induced neurite retraction may deprive optic axons of trophic support from postsynaptic target neurons. Reversal of astrocyte stellation in association with stress fiber expression and astrocyte contraction suggests that the diabetic glaucoma autoantibodies which induced morphological astrocyte reactivity (Tura et al., 2009) may lead to loss of glial-neuron interaction(s) which is an important source of trophic support in RGCs.

Ten of eleven diabetic subjects who suffered POAG-related visual field loss were African-American or of Afro-Caribbean origin, groups especially prone to develop POAG or glaucomatous blindness (Marshall, 1989). Our findings of generally low plasma bFGF among diabetic glaucoma or suspects is consistent with an earlier report of a significant association between low plasma bFGF and African-American race in a multi-ethnic VADT substudy of older adults having type 2 diabetes (Zimering et al., 2008). Higher mean diastolic blood pressure in the current diabetic glaucoma group may reflect (in part) inclusion of a higher proportion of African subjects. It is also consistent with a reported association between increased diastolic blood pressure and raised IOP in older adults having diabetes (Klein et al., 1994).

The prevalence of glaucoma among blacks living in certain geographically isolated areas is striking. It suggests possible involvement of population- or race-specific hereditary predisposition factor(s). For example, in the Barbados Eye study, the prevalence of POAG among older adult Afro-Caribbean men and women (age 70 years or above) was 17%; it was eight times higher than in Barbadian whites, and could not be accounted for by diabetes or hypertension (Leske et al., 1994). One possible contributory factor is known hereditable differences in autoantibody subclass concentrations. African-American children had higher concentrations of plasma total IgG and the IgG_2 subclass compared to Caucasian children (Shackelford et al., 1985). Km1 is a marker in the constant region of kappa light chains which occurs with 3.7-fold higher frequency in African-American compared to Caucasian populations (Granoff et al., 1984). Km1 was associated with increased IgG_2 antibody response to certain polysaccharide antigens in African-American children (Granoff et al., 1984); and the IgG_2 antibody response (associated with Km1) was predominated by expression of a particular subgroup of less prevalent kappa light chain (κ IIa) genes (Lucas et al., 1991). A kappa II gene is predicted to have encoded the peak inhibitory kappa light chain in the eluate from (type 1 DM) Patient 5 having severe disease manifestations (Zimering, 2010). In contrast, in Caucasian adults, an increased IgG_2 antibody response to several different common polysaccharide bacterial antigens was associated with Gm23, a marker in the heavy chain constant region (Ambrosino et al., 1985). Gm23 was reported to be associated with a significantly increased risk of diabetic retinopathy (Stewart et al., 1993). Our finding of a significantly increased risk for Alzheimer's dementia among the parents of diabetic glaucoma or suspects is consistent with the possibility that a subset of autoantibodies having highly potent (neurite-, EC and glial-) inhibitory properties may reflect inheritance of a particular subgroup of kappa light chain genes. Increased prevalence of such genes as the kappa II (Ig) gene subgroup in persons of African descent may reflect an earlier evolutionary selection pressure that acted to increase the prevalence of a survival-promoting, beneficial allele. The later occurrence of slow neurodegenerative diseases such as open angle glaucoma or Alzheimer's dementia in persons harboring potent kappa light chain autoantibodies is consistent with the concept of "antagonistic pleiotropy," advanced by Williams (1957). Williams postulated that senescence-associated mortality occurs through the selection of genes which confer an early reproductive (survival) benefit yet harbor additional latent harmful effects to the organism which are expressed during aging.

Glaucoma is a slowly progressive neurodegenerative disease whose severity is significantly affected by elevated IOP (Sommer, 1996; Quigley, 2011). IOP is thought to mediate remodeling of the optic nerve head extracellular matrix (ECM) in part through the activation of membrane-type matrix metalloproteinases (MT-MMP) (Agapova et al., 2003). Furin is a proprotein convertase (PC) which is expressed in vascular endothelial and trabecular meshwork cells under conditions of increased hemodynamic or mechanical stress (Negishi et al., 2001; Remacle et al., 2008). In our prior work, long-term storage of certain cancer sera protein-A eluates was associated with the spontaneous appearance of highly potent EC inhibitory substances which were excitotoxic in rat embryonic hippocampal neurons and had MW and amino acid sequence characteristics of Ig kappa half-light chains (Zimering et al., 2011). The current preliminary data are consistent with the possibility that furin or a closely related PC which can recognize and cleave substrates at multi-basic amino acid sequences (Remacle et al., 2008) causes gain-in-inhibitory function through cleavage of certain IgGs at specific recognition sequences(s). One possible recognition site (perhaps giving rise to kappa half-light chains) is the K-$(X)_3$-K-R sequence which is located in the variable-constant switch region in certain κ light chains. The beta site amyloid precursor protein cleaving enzyme 1 (Scholefield et al., 2003), BACE1 (β-secretase), has been implicated in Alzheimer's disease pathogenesis because it generates amyloid β-peptide from its precursor protein. Of interest, β-secretase (BACE1) is under negative regulatory control through its binding to heparan sulfate (Scholefield et al., 2003). Certain members of the PC family are expressed at the cell surface in association with HSPG (Mayer et al., 2008). Thus localization of anti-HSPG autoantibodies at such cell regions might provide a mechanism for dual activation of β-secretase and for proteolytic processing of autoantibodies leading to more highly potent, long-lasting inhibitory substances.

A limitation of our study is that it is small and the results principally reflect the experience of older African-American men having POAG. More study in women and other racial groups is needed to confirm the present findings and to determine whether PC12 neurite-inhibitory autoantibodies may increase in non-diabetic glaucoma subjects. The mechanism(s) underlying glaucomatous neurodegeneration are complex and multifactorial (Kuehn et al., 2005). It is possible anti-HSPG autoantibodies may occur as a consequence of tissue injury yet have only a limited, bystander role in the neurodegenerative disease process. Although *in vivo* experimental support of a pathogenic role for anti-HSPG autoantibodies in glaucomatous neurodegeneration is lacking, *in vitro* effects of the autoantibodies in ECs, neurons and cerebral cortical astrocytes support a potential role in promoting neurodegeneration underlying glaucoma and dementia. Of interest, in a mouse model of hereditary glaucoma, high-dose irradiation unexpectedly completely prevented the subsequent development of glaucomatous RGC degeneration (Anderson et al., 2005). Basic FGF is released following irradiation and it protects microvascular endothelium against radiation-induced apoptosis (Fuks et al., 1994). More study in a mouse model of irradiation-induced neuroprotection could provide a test for involvement of bFGF or factors (anti-HSPG autoantibodies) which negatively affect bFGF local bioavailability in determining long-term RGC survival. Our data do not

exclude the possible involvement of other kinds of autoreactive antibodies, such as heat shock proteins (HSP) autoantibodies, or cell-mediated immune mechanisms in retinal ganglion degeneration (Wax et al., 2008). For example, immunization with HSP 27 or HSP60 in the Lewis rat induced a glaucoma-like pattern of RGC degeneration via activation of T cells which secreted Fas ligand (Wax et al., 2008). Still evidence suggests that early activation of RGC survival pathways (perhaps mediated in part by bFGF) may prevent or delay Fas ligand-induced RGC apoptosis (Kim and Park, 2005).

The retinal inner limiting membrane (ILM) contains HSPG; and the ILM undergoes thickening during aging (Candiello et al., 2010) and in diabetes. In a subset of diabetic glaucoma or suspects (3 of 20 subjects), protein-A eluates caused mild EC stimulation suggesting that diabetic glaucomatous autoantibodies are heterogeneous and may include immune complexes. Trapping of immune complexes in the retinal ILM might disrupt its barrier function or lead to complement activation. A possible underlying role for humoral autoimmunity in subsets of dementia is suggested by reports that autoantibodies cross-reactive with vascular HSPG increased in serum from older adults having senile, Alzheimer's type dementia (Fillit et al., 1987). Brain tissue from Alzheimer's-type dementia patients showed diffuse deposition of IgG, fibrinogen, and glial hyperreactivity indicative of loss of EC barrier integrity (Ryu and McLarnon, 2009). More study is needed to determine whether plasma diabetic autoantibody PC12 neurite-inhibitory activity may be a useful (early) marker for sustained activation of Rho kinase in diverse cell types including neurons, astrocytes, or ECs involved in mediating slowly, progressive glaucomatous visual field loss, or in a subset of diabetes having dementia.

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Basic fibroblast growth factor predicts cardiovascular disease occurrence in participants from the veterans affairs diabetes trial

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Aim: Cardiovascular disease (CVD) is a leading cause of morbidity and mortality in adults with type 2 diabetes mellitus. The aim of the present study was to test whether plasma basic fibroblast growth factor (bFGF) levels predict future CVD occurrence in adults from the Veterans Affairs Diabetes Trial (VADT).

Methods: Nearly 400 veterans, 40 years of age or older having a mean baseline diabetes duration of 11.4 years were recruited from outpatient clinics at six geographically distributed sites in the VADT. Within the VADT, they were randomly assigned to intensive or standard glycemic treatment, with follow-up as much as seven and one-half years. CVD occurrence was examined at baseline in the patient population and during randomized treatment. Plasma bFGF was determined with a sensitive, specific two-site enzyme-linked immunoassay at the baseline study visit in all 399 subjects and repeated at the year 1 study visit in a randomly selected subset of 215 subjects.

Results: One hundred and five first cardiovascular events occurred in these 399 subjects. The best fit model of risk factors associated with the time to first CVD occurrence (in the study) over a seven and one-half year period had as significant predictors: prior cardiovascular event [hazard ratio (HR) 3.378; 95% confidence intervals (CI) 3.079–3.807; $P < 0.0001$), baseline plasma bFGF (HR 1.008; 95% CI 1.002–1.014; $P = 0.01$), age (HR 1.027; 95% CI 1.004–1.051; $P = 0.019$), baseline plasma triglycerides (HR 1.001; 95% CI 1.000–1.002; $P = 0.02$), and diabetes duration-treatment interaction ($P = 0.03$). Intensive glucose-lowering was associated with significantly decreased hazard ratios for CVD occurrence (0.38–0.63) in patients with known diabetes duration of 0–10 years, and non-significantly increased hazard ratios for CVD occurrence (0.82–1.78) in patients with longer diabetes duration.

Conclusion: High level of plasma bFGF is a predictive biomarker of future CVD occurrence in this population of adult type 2 diabetes.

Keywords: basic fibroblast growth factor, type 2 diabetes mellitus, cardiovascular disease

INTRODUCTION

Intensive diabetes treatment slows the development of retinopathy, nephropathy, and neuropathy (1); however, its role in reducing cardiovascular disease (CVD) events in adult type 2 diabetes is more complex. Evidence from the United Kingdom Prospective Diabetes Study (UKPDS) follow-up study indicated a "delayed" beneficial cardiovascular effect from intensive glucose-lowering in newly diagnosed type 2 diabetes (2). Among more advanced type 2 diabetic subjects from the ACCORD, ADVANCE, and veterans affairs diabetes trial (VADT), however, intensive glucose-lowering either did not show a beneficial effect on lowering the rate of cardiovascular events (3) or it increased cardiovascular mortality (i.e., in ACCORD) (4).

Biomarkers that can predict an increased risk for CVD occurrence in a category of adult diabetic patients undergoing substantial treatment intensification would be valuable for guiding patient regarding treatment selection. Basic fibroblast growth factor (bFGF) is a potent angiogenic and smooth muscle cell mitogen which increases in subsets of advanced adult type 2 diabetes having micro-(albuminuria) (5) and/or abdominal obesity (i.e., increased waist/hip ratio) (6). In a planned secondary analysis to the VADT ($n = 399$), we reported that baseline plasma bFGF was a novel

significant predictor of the time to first post-baseline occurrence of coronary heart disease (CHD) (7). The aims of the present analysis were (1) to test whether baseline plasma bFGF predicts the time to first post-baseline CVD occurrence in risk models that adjust for treatment-duration interaction, (2) to determine whether elevated bFGF at year 1 of treatment predicts the subsequent occurrence of CVD events in the VADT subjects, and (3) to investigate novel mechanisms for vascular cell growth promotion involving bFGF or long-lasting (FGF-like) autoantibodies in diabetic subsets having increased plasma bFGF.

SUBJECTS AND METHODS

STUDY SUBJECTS

The study design of the VADT has been previously reported (8). Patients with renal insufficiency or congestive heart failure were excluded from participation. Baseline insulin use (yes/no), and occurrence of a macrovascular event prior to baseline (yes/no) were stratification variables jointly used when subjects were randomized at each participating VA site. CVD occurrences encompassing ischemic coronary artery disease, congestive heart failure, cerebrovascular disease, and peripheral arterial disease events comprised the pre-specified endpoint of the main VADT study. Informed consent for the Investigational Review Board-approved substudy was obtained from 399 diabetic subjects who had consented to participate in the main VADT at six outpatient sites. As previously described (7), aliquots of EDTA plasma obtained in the morning from fasting subjects were shipped frozen (dry ice) to a central laboratory [Maverick, Boston Veterans Affairs Medical Center (VAMC), Boston, MA, USA] where they were inventoried and stored at −80°C. Archived, coded frozen EDTA plasma from consecutively enrolled patients was shipped to the laboratory of Dr. Zimering [VA New Jersey Health Care System, Lyons, NJ, USA (VANJ)] where basic fibroblast growth factor immunoreactivity (bFGF-IR) assays were performed. All other assays were performed in the Central Laboratory of the VADT (Tufts University, Boston, MA, USA).

The baseline and follow-up (year 3) clinical characteristics in the study subjects are shown in **Table 1**.

PATIENT SUBGROUPS

I. A convenience sample consisting of a first group of 26 plasma specimens was used to test for a correlation between plasma bFGF-IR and endothelial cell growth activity (**Figure 2**). The 26 subjects in this subgroup were consecutively enrolled subjects from three study sites.

Table 1 | Comparison of baseline and follow-up characteristics for Standard and Intensive treatment groups comprising 399 substudy patients.

Variable	Baseline			Follow-up[a]		
	STD (*N* = 200)	INT (*N* = 199)	*P*-value	STD (*N* = 200)	INT (*N* = 199)	*P*-value
Age (years)	58.6	60.1	0.08			
Diabetes duration (years)	10.3	12.4	0.007			
Baseline insulin	47%	48%	0.80			
Male	97%	96%	0.99			
Prior CV event	38%	37%	0.79			
Hypertension	73%	68%	0.26			
Race						
Non-hispanic white	58%	56%	0.73			
African-American	21%	22%	0.79			
Hispanic	17%	19%	0.68			
Current smoking	16%	20%	0.35	11%	13%	0.43
bFGF (pg/mL)	16.0	14.2	0.50	8.1[b]	8.0[b]	0.95[b]
HbA_1C (%)	9.6	9.4	0.37	8.8	7.2	<0.0001
Systolic bp (mm Hg)	130.3	131.5	0.51	128.2	124.5	0.03
BMI (kg/m^2)	31.0	31.0	0.97	31.6	32.5	0.11
Weight (lbs)	212.6	213.2	0.88	216.4	224.3	0.06
Waist/hip ratio	0.99	0.99	0.87	1.00	0.99	0.24
Total chol (mg/dL)	183.2	182.6	0.90	163.4	155.7	0.03
LDL chol (mg/dL)	107.1	107.2	0.96	90.4	86.5	0.21
HDL chol (mg/dL)	36.4	37.6	0.25	38.9	40.1	0.32
Triglycerides (mg/dL)	208.7	184.1	0.16	177.8	148.5	0.006
Serum creat (mg/dL)	1.00	0.99	0.67	1.17	1.15	0.73

Results are means or percentages, and P-values are obtained from the comparison of the two treatment groups.

[a]*Year 3 or previous non-missing value prior to year 3.*

[b]*Year 1 annual visit.*

Chol, cholesterol; creat, creatinine.

II. A convenience sample consisting of a second group of 27 plasma specimens was used to test for an association between plasma bFGF-IR and growth stimulatory activity in protein A eluates. The 27 subjects were consecutively enrolled from three study sites.

MEDICATIONS

Baseline anti-diabetic medications included oral agents and/or insulin, as previously reported (9). Similar classes of anti-diabetic medications were used in patients randomized to the standard or intensive glycemic treatment groups, but at different doses. Substantial proportions of patients were treated with one or more anti-hypertensive medications including angiotensin converting enzyme inhibitors (67%) or angiotensin receptor blockers (7%). Sixty-two percent of patients reported use of a statin at baseline, and 13% of patients reported baseline use of a fibrate medication.

STUDY OUTCOMES

As previously reported (7), CVD was adjudicated by an independent Study Endpoints Committee. The subset of CVD outcomes comprising CHD is defined as myocardial infarction, coronary revascularization, inoperable coronary artery disease, or cardiovascular death. Baseline plasma bFGF-IR was determined in ($n = 399$) subjects and at 1 year post-baseline, a bFGF-IR determination was done due to budgetary constraints in a randomly selected subset of 215 subjects. Data on plasma bFGF-IR was obtained without information about study endpoint occurrence. We separately modeled the association of risk factors with time to first post-baseline cardiovascular (CVD) or to first post-baseline CHD in the 399 subjects for whom such post-randomization data was available.

LABORATORY AND CLINICAL MEASURES

Routine laboratory measures were determined by standardized direct enzymatic assay methods (9). The analyzed blood pressure (BP) was the median value of three consecutive determinations recorded from subjects that had been seated and resting for 5-min.

PLASMA SAMPLES

Basic fibroblast growth factor assays

The determination and stability of basic FGF immunoreactivity (bFGF-IR) in plasma has been previously described (7, 10). Plasma bFGF-IR ranged between 0 and 4 pg/mL in healthy male volunteer blood donors, and there was no effect of age on plasma bFGF level (11).

Cell culture and growth assays

Bovine pulmonary artery (BPA) endothelial cells (Clonetics Inc., San Diego, CA, USA) were cultured under previously described conditions (12). Growth-promoting activity is expressed as a percentage of the control cell number (after 4 days incubation in the presence of test fractions) for cells grown in EGM/M199 with 10% FCS without added test fractions. Each point represents the mean of 4–6 determinations. The intra- and inter-assay coefficients of variation were 4 and 7% at 1:50 dilution of protein-A-eluted fractions from plasma of three diabetic subjects ($n = 3$ assays in each patient).

Protein A affinity chromatography

Protein A chromatography was carried out as previously described (12). Eluate fractions were stored at 0–4°C. All fractions were sterile filtered (Millipore Corp., Bedford, MA, USA; 0.22 μm) before assay for growth-promoting activity.

Chemicals

Recombinant human bFGF was from Austral Biologicals Inc. (San Ramona, CA, USA). All other chemicals and reagents were analytical grade.

Protein determinations

Protein concentrations were determined by a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL, USA).

Statistics

The VADT was conducted using the intention-to-treat principle (9). As previously reported (7), baseline insulin use and baseline cardiovascular event-history (the randomization stratification variables) and glycemic treatment group were included as covariates in models when testing for a bFGF effect on CVD occurrence. Cox proportional hazards regression analysis was used to model the association between baseline risk factors and time to first post-baseline CHD, or CVD occurrence. Backward elimination was used to obtain the best fit model using $P \leq 0.05$ as the cutoff for variable retention in the final model. From our prior work (5), we hypothesized that increased, post-treatment bFGF (determined at year 1) may reflect suboptimal blockade of the renin-angiotensin system which is a known risk factor for occurrence of CVD events (13). Consistent with our prior methodology (14), year 1 bFGF, when used in models of the risk factors associated with time to first post-year 1 CVD occurrence, was dichotomized at the upper limit of normal reported in adult men (4.4 pg/mL) (11).

RESULTS

BASELINE AND FOLLOW-UP CHARACTERISTICS IN THE STUDY PATIENTS

Our subject group was comprised of men having the following means: age: 59 years; diabetes duration: 11.4 years; hemoglobin A_1c: 9.5%; BMI: 31 kg/m^2; and 37% reported a prior macrovascular event at study entry (**Table 1**). Baseline clinical characteristics were similar in subjects randomized to standard and intensive treatment, with the exception of mean diabetes duration, which was significantly longer (12.4 vs. 10.3 years) for intensively treated patients (**Table 1**). At the year 3 study visit, intensive treatment was associated with significantly lower mean glycosylated hemoglobin, systolic BP, plasma total cholesterol, and triglyceride concentrations (**Table 1**). Mean body weight, BMI, plasma HDL cholesterol, and creatinine concentration increased significantly in all subjects after 3 years' treatment (data not shown), but the mean values did not differ significantly when comparing standard to intensive treatment patients (**Table 1**).

FREQUENCY OF OCCURRENCE OF POOLED END POINTS

One hundred and five first CVD events occurred in 105 patients during an average of 6 years of VADT study treatment including

57 primary CVD events in the Standard treatment group and 48 primary CVD events in the Intensive treatment group (data not shown).

ASSOCIATION BETWEEN PLASMA bFGF AND FIRST POST-BASELINE OCCURRENCE OF CVD

The best-fitting model of risk factors associated with the time to first post-randomization occurrence of the main study CVD endpoint ($n = 105$ events in 399 subjects) had as significant predictors: prior CV event [hazard ratio (HR) 3.378; 95% confidence intervals (CI) 3.079–3.807; $P < 0.0001$], age (HR 1.027; 95% CI 1.004–1.051; $P = 0.019$), baseline bFGF (HR 1.008; 95% CI 1.002–1.014; $P = 0.013$), baseline triglycerides (HR 1.001; 95% CI 1.000–1.002; $P = 0.015$), and duration × treatment interaction ($P = 0.033$) (**Table 2**). There was no significant association of baseline use of a statin, either ACEi or ARB, or fibrate medication with time to first post-baseline CVD occurrence. There was no significant association of time to first post-baseline CVD occurrence and baseline triglyceride × treatment × duration ($P = 0.759$), baseline triglyceride × treatment ($P = 0.845$) or baseline HDL cholesterol concentration ($P = 0.441$). The (treatment × duration) adjusted model predicted a significantly decreased CVD HR- (CI = 0.38–0.63) associated with intensive glucose-lowering among patients with 10 or fewer years of baseline known diabetes duration, and a non-significant HR (CI = 0.82–1.78) for subjects with a baseline diabetes duration of 15 years or longer (**Figure 1**).

RISK FACTORS ASSOCIATED WITH ONGOING CVD OCCURRENCE IN ADVANCED DIABETES

As previously reported (7), mean plasma bFGF at year 1 decreased significantly compared to baseline levels, but did not differ between STD and INT patients (8.1 vs. 8.0 pg/mL) (**Table 1**). Of the original 399 study subjects, we were able to obtain repeat plasma bFGF at year 1 in 215 randomly selected patients who did not differ in their mean baseline characteristics from all 399 subjects. Secondary analyses suggests that for patients having the longest baseline duration of diabetes (≥15 years), patient age (HR 1.052; 95% CI 1.006–1.098; $P = 0.0316$) is an important predictor of time to first post-year 1 CVD occurrence (22 events in 71 patients). Excluding age from our model, increased year 1 plasma bFGF (>4 pg/mL) appeared to be a nearly significant predictor (HR 2.439; 95% CI 1.522–3.356; $P = 0.0518$) of time to first post-year 1 CVD occurrence (22 events in 71 patients) among patients with baseline diabetes duration ≥15 years.

INCREASED BIOACTIVITY IN VADT PLASMA HAVING ELEVATED bFGF

Since basic FGF is a potent endothelial and smooth muscle cell mitogen which may contribute to cell proliferation associated with atherosclerosis, we compared endothelial cell bioactivity in plasma from VADT subjects having low vs. elevated (>4 pg/mL) plasma bFGF. Mean activity in the 25–75% ammonium sulfate pellet fraction of plasma (127 ± 10%) from 14, consecutively enrolled VADT subjects with elevated bFGF-IR (>4 pg/mL) significantly exceeded mean activity (113 ± 10%) in 12 consecutively enrolled VADT subjects with normal bFGF-IR (0–4 pg/mL, $P = 0.003$) (**Figure 2**).

Table 2 | Cox proportional hazard ratio: time to first post-baseline CVD event.

Variable	HR	95% CI	*P*-value
Baseline bFGF	1.008	1.002–1.014	0.0126
Prior CV event	3.378	3.079–3.807	<0.0001
Age	1.027	1.004–1.051	0.0188
Triglyceride	1.001	1.000–1.002	0.0153
Intensive treatment			
5 years diabetes duration	0.489	0.275–0.869	
25 years diabetes duration	1.370	0.712–2.637	

n = 399 subjects; HR, hazard ratio; CI, confidence interval.

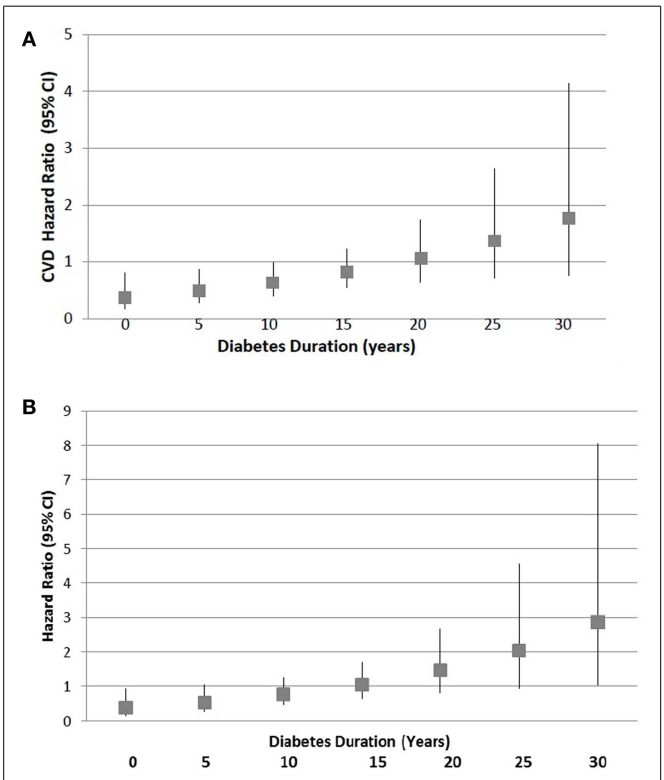

FIGURE 1 | Hazard ratio for post-baseline (A) CVD or (B) CHD occurrence in intensive treatment group by duration of diabetes known at baseline. Squares indicate point estimates and bars denote 95% confidence intervals. Point estimates are obtained from the multivariable adjusted model that includes age, prior CV event, baseline bFGF, treatment, duration, and treatment × duration and are illustrated for 5-year intervals between 0–30 years of baseline diabetes duration.

Basic FGF lacks an amino terminal signal sequence (15), and is sequestered in cells (16). Yet in subsets of endocrine tumor patients having elevated plasma bFGF, we have reported the occurrence of long-lasting, highly potent FGF-like autoantibodies (12, 17). In the present study, we examined the protein A eluates from 27 consecutively enrolled VADT subjects having plasma bFGF-IR ranging from 0 to 29 pg/mL for properties resembling autoantibodies. In 16 of 27 subjects, endothelial

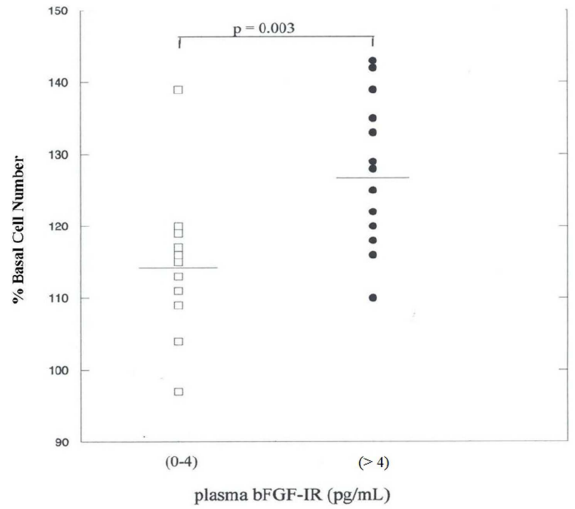

FIGURE 2 | Endothelial cell bioactivity in the 25–75% ammonium sulfate pellet of plasma. Ammonium sulfate pellet fractions from plasma in 26 consecutively enrolled VADT subjects were tested for endothelial cell growth promotion after 4 days of incubation as described in Materials and Methods. Each point represents the mean of four–six determinations.

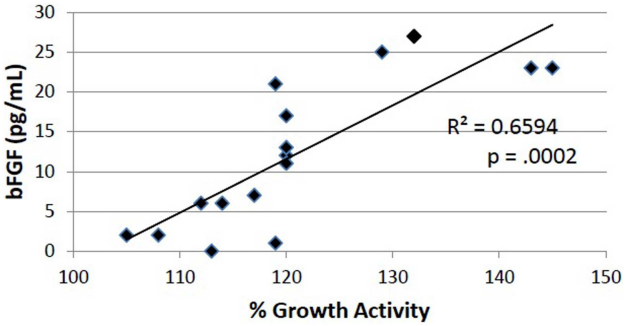

FIGURE 3 | Endothelial cell stimulatory activity in protein A eluate fractions in 16 VADT plasmas was significantly correlated with increasing plasma bFGF-IR concentration. Growth activity was determined after 4 days' incubation as described in Materials and Methods. Each point represents the mean of quadruplicate determinations.

cell bioactivity in the protein A eluate fraction was stimulatory, and there was a significant correlation ($R = 0.81$; $P = 0.0002$) between plasma bFGF level and the level of growth stimulatory activity in the protein A eluate fraction of plasma (**Figure 3**).

DISCUSSION

The current findings suggest that baseline plasma bFGF may be a novel significant predictor of CVD occurrence in a subset of obese, adult male veterans with type 2 diabetes. Following adjustment for a significant treatment − duration interaction, and baseline plasma triglycerides, baseline plasma bFGF was still a significant predictor of the time to first post-baseline CVD occurrence in 399 VADT subjects. This is the first evidence that plasma bFGF is a novel candidate marker of CVD risk in a subset of obese advanced type 2 diabetes.

Plasma basic FGF is low or undetectable in healthy subjects (11), but increases in micro-albuminuric adult type 2 DM (5) or in the presence of coronary artery disease (18). Growth stimulatory activity in the 25–75% ammonium sulfate pellet fraction in VADT plasma having elevated bFGF may be due (in part) to bFGF, or autoantibodies which can bind bFGF or mimic bFGF's growth activity. In support of the latter two possibilities, our preliminary experiments indicated that even low concentrations (1–2 μg/mL) of protein A eluates from subsets of advanced adult type 1 autoimmune diabetes ($n = 2$) or advanced adult type 2 DM having chronic kidney disease ($n = 3$) could be completely neutralized by co-incubating endothelial cells with specific anti-bFGF antibodies. This result suggests that some of the stimulatory activity in protein A eluates from plasma of advanced diabetes subjects may be FGF-like or due to an IgG which can mimic FGF.

Although the tissue sources of increased plasma bFGF in obese subjects with type 2 diabetes are not known, increased waist-hip ratio was significantly associated with substantially increased plasma bFGF in the VADT substudy (6). Angiotensin II stimulates the release of visceral adipocytokines (TNF-alpha, IL-1, IF-gamma) which then act to induce endothelial cell release of bFGF (19). IF-gamma activates macrophage phagocytic function, and promotes B cell isotype switching to the synthesis of IgG (20). Thus the known pro-immune effects from renin-angiotensin system (RAS) activation may act to alter long-lasting IgG autoantibodies to circulating forms of bFGF in diabetic subsets (e.g., VADT) having substantially increased plasma bFGF. Weight gain and/or salt and water retention frequently accompany glycemic treatment intensification, and the latter has been reported to enhance the expression of angiotensin II receptors in rats (21). The current finding of a significant association between baseline bFGF level and increased CVD risk may reflect underlying role(s) for angiotensin II in promoting vascular hypertrophy, atherosclerosis, and immune interactions involving high plasma bFGF. Our preliminary findings of significant or nearly significant associations between advanced age or increased (>4 pg/mL) year 1 plasma bFGF, respectively and the ongoing risk for CVD occurrence in patients having longer-duration of diabetes (≥15 years) are hypothesis-generating and may warrant further investigation. These results are consistent with known RAS activation in older subjects or in those having year 1 plasma bFGF >4 pg/mL (5). Older patients with type 1 diabetes were more likely to experience adverse hemodynamic responses to RAS activation than their younger counterparts (22).

Increased affinity of the FGF-like plasma autoantibodies in diabetic subjects for hydroxyapatite (our preliminary results) suggests that the autoantibodies may localize to calcified areas in advanced coronary atherosclerotic plaque and also may promote the local cellular proliferation typically found with atherosclerosis. Intensive glycemic control may have been associated with a lower rate of CVD occurrences in our subgroup of recent-onset type 2 diabetes by decreasing the occurrence of microvascular damage leading to the increase of bFGF (23). Findings in the VADT substudy of

Reaven et al. (24, 25), namely, that intensive control lowered the subsequent risk of CVD occurrences in subjects having lower baseline coronary calcium, and that albuminuria was a risk factor for progression of coronary calcium, are consistent with the possibility of synergistic interactions between coronary calcium and hydroxyapatite-avid, growth-stimulating autoantibodies.

Atherogenic dyslipidemia has been associated with increased residual cardiovascular risk in obese subjects with type 2 diabetes (26). Consistent with results in the main VADT (9), our substudy group had substantially increased mean baseline plasma triglycerides which remained elevated despite substantial glycemic improvement (**Table 1**). Our multivariable risk model predicts that each 50 mg/dL increase in baseline plasma triglycerides is associated with a 5% increase in the hazard rate for CVD occurrence. The HR associated with increased bFGF (HR 1.008; 95% CI 1.002–1.014) was only modestly increased, but is likely to be significant for the 20% of VADT subjects who had baseline plasma bFGF >20 pg/mL. Patients having an elevation in plasma bFGF of 20 pg/mL compared to control patients would experience 1.17 times the hazard rate for CVD occurrence. The smaller subgroup of patients (6%) having an elevation in bFGF of 50 pg/mL would experience 1.5 times the hazard rate for CVD occurrence compared to control patients with non-elevated bFGF. Taken together with our prior finding of a significant association between markedly increased plasma bFGF (>20 pg/mL) and increased plasma plasminogen activator inhibitor-1 level (6), the current results suggests effects of bFGF on endothelial cells (i.e., proliferation, elaboration of PAI-1) or smooth muscle cells which may contribute to clinically significant increases in CHD or CVD risk in a subset of obese, advanced type 2 DM.

"Vascular metabolic memory" refers to the association between improved glycemic control in adult type 1 diabetes and a substantially reduced risk for later CVD occurrence (27). Since longstanding poor glycemic control has been significantly associated with increased FGF-like plasma bioactivity in adult type 2 DM (5), vascular injury leading to increased plasma bFGF (23) may be one of the unknown mechanisms underlying an association between prior glycemia and future CVD risk.

A limitation of our study is that the results are based on moderate to small sizes and may only reflect the experience of middle-aged and older obese men with treatment-resistant diabetes. The present findings need to be confirmed in other populations with type 2 diabetes. Our finding of a significant treatment x duration interaction effect on the risk for CVD occurrence in adult type 2 diabetes is consistent with results from the main VADT (28), but needs to be interpreted cautiously since it was based on *post hoc* analysis in an embedded subgroup of the main VADT.

In conclusion, the present findings suggest that baseline plasma bFGF may be a marker of CVD risk in adult male veterans with type 2 diabetes. These results suggest that increased plasma bFGF may drive cell proliferation and be involved in the mechanism for increased CVD occurrence in older adults with advanced type 2 diabetes mellitus.

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Immune regulation in T1D and T2D: prospective role of Foxp3+ Treg cells in disease pathogenesis and treatment

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There is increasing evidence that dysregulated immune responses play key roles in the pathogenesis and complications of type 1 but also type 2 diabetes. Indeed, chronic inflammation and autoimmunity, which are salient features of type 1 diabetes, are now believed to actively contribute to the pathogenesis of type 2 diabetes. The accumulation of activated innate and adaptive immune cells in various metabolic tissues results in the release of inflammatory mediators, which promote insulin resistance and β-cell damage. Moreover, these dysregulated immune responses can also mutually influence the prevalence of both type 1 and 2 diabetes. In this review article, we discuss the central role of immune responses in the patho-physiology and complications of type 1 and 2 diabetes, and provide evidence that regulation of these responses, particularly through the action of regulatory T cells, may be a possible therapeutic avenue for the treatment of these disease and their respective complications.

Keywords: inflammation, obesity, Foxp3, Treg, diabetes, metabolic, immune regulation

THE PATHOGENESIS OF TYPE 1 DIABETES

Type 1 diabetes (T1D) is a chronic autoimmune disease resulting from a T cell-dependent (both $CD4^+$ and $CD8^+$) destruction of the insulin-producing β-islets of Langerhans in the pancreas, leading to insulin deficiency and persistent hyperglycemia (**Figure 1**). Upon β-cells destruction, T1D patients lose blood glucose control, which provoke severe hyperglycemia. Even with current insulin replacement therapies secondary complications such as heart disease, blindness, and kidney failure may arise. Diagnosis is typically made early in life, with onset as young as 1 year of age and in most cases before the age of 18. The appearance of diabetes associated autoantibodies in the serum is the first detectable sign of emerging β-cell autoimmunity with over 90% of T1D patients testing positive for at least one at the time of diagnosis. Notable T1D auto-antigens identified include insulin, GAD65 (glutamic acid decarboxylase, 65 kDa isoform), IA2 (insulin auto-antigen 2), and zinc transporter 8 (ZNT8) (Sabbah et al., 1999; Orban et al., 2009). Several reports suggest that insulin is a primary auto-antigen for disease initiation. For example, elimination of pro-insulin or insulin completely abolished insulitis and T1D in NOD mice, while removal of another islet antigen, IGRP, did not show protective effect (Krishnamurthy et al., 2006). Both, genetic and poorly defined environmental factors act together to precipitate disease progression. Most studies confirm a global increase in incidence of T1D, particularly among young children. This likely reflects various environmental changes, although the impact of any individual exogenous factor has not yet been definitively proven. Pathogens such as viruses and bacteria, early exposure to cow's milk, gluten, and meat preservatives and deficiency in dietary Vitamin D or omega 3 fatty acids have been proposed to contribute to the pathogenesis of T1D (Knip et al., 2005). Many epidemiological efforts have been made to understand the potential role of viruses in T1D pathogenesis. It is possible that viral antigenic mimicry could result in cross-reactive responses toward islet antigens. The striking sequence similarities between the 2C protein from coxsackievirus and GAD, a major auto-antigen in T1D, support this notion (Kaufman et al., 1992). Alternatively induction of a pro-inflammatory anti-viral response to infection, could activate innate immune cells, break tolerance, and initiate autoimmunity. It has also been established that in response to the viral infection, endocrine islet cells are able to produce pro-inflammatory cytokines, such as IL-8, IL-6, TNFα, and CXCL10 that could further trigger abnormal immune responses and T1D (Christen et al., 2003; Berg et al., 2006). The gut microbiota, via interaction with the host innate immune system, has been shown to modulate T1D onset (Chervonsky, 2010). For example, in NOD mice, T1D incidence dramatically decreases when mice are exposed to various microbial products. Similarly, the so called "hygiene hypothesis" suggests that the marked increase in T1D incidence in industrialized countries is related to reduced helminth burden therein (Mathis and Benoist, 2012).

Early studies indicate that multiple genes within human leukocyte antigen (HLA) on chromosome 6 are critical susceptibility loci for human autoimmune disease, including T1D. Two T1D associated haplotypes, namely DR4-DQ8 and DR3-DQ2, are present in 90% of children with the disease. Candidate gene studies identify insulin as a second important gene associated with T1D susceptibility, contributing 10% of genetic susceptibility to T1D. Over the last decade, whole genome screens have identified at least 40 other loci associated with T1D. Furthermore,

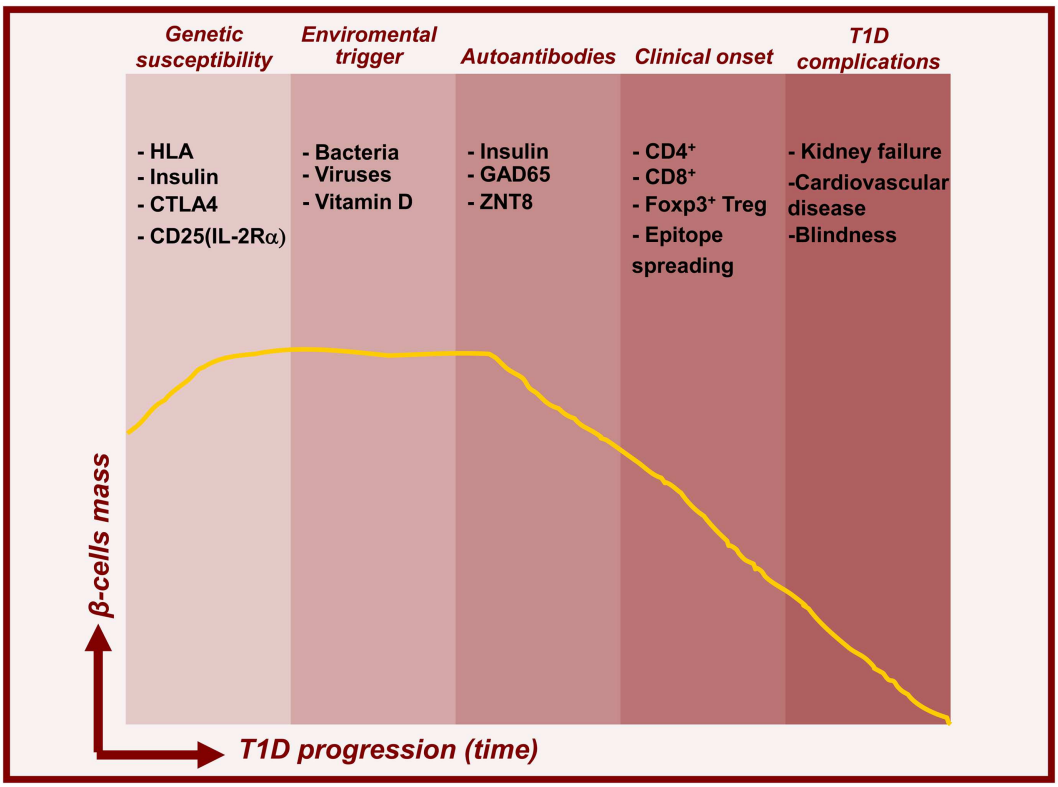

FIGURE 1 | Timelines for type 1 diabetes. Model for temporal relationship between beta-cell mass decline and features of T1D pathogenesis. In addition to genetic predisposition, environmental triggers induce islet autoimmunity and beta-cell death leading to prediabetes and subsequent clinical onset and complications.

mutations in genes found in several of the susceptibility loci, such as *il-2*, *il-2ra*, *Ctla-4*, *PTPN22*, *il-10* have various autoimmune manifestations, including T1D. These genes are associated with the regulation of immune responses either by intrinsically controlling T and B cell reactivity or by enhancing the development and homeostasis of immunosuppression mediated by regulatory T (Treg) cells expressing Foxp3, a DNA-binding forkhead winged helix transcriptional regulator known to drive their lineage development.

Genetic susceptibility in humans and mice are both linked to variations in the IL-2 signaling pathway. In humans, T1D risk is related to the gene region encoding IL-2Rα, whereas in NOD mice the IL-2 gene (*Idd3* locus) confers susceptibility (Lyons et al., 2000). Additionally, it has been established that phosphorylation of STAT5, a crucial IL-2 signaling molecule, is reduced in T1D patients, and could account for diminished Treg cell numbers (Long et al., 2010). Furthermore, proper IL-2 signaling is essential for protection from diabetes in NOD mice. Work from our group and others has demonstrated that protective IL-2 allelic variants favor the expansion and suppressive function of Treg cells directly in the islets (Sgouroudis et al., 2008). Moreover, treatment of diabetic mice with IL-2 increases Treg cell numbers and induces expression of Treg cell-associated proteins such as Foxp3, CD25, ICOS, and CTLA-4. Collectively IL-2 preferentially enhances Treg immunosuppression and down-regulates IFNγ production by pathogenic, islet-infiltrating effector T cells (Teff) (Grinberg-Bleyer et al., 2010).

FOXP3+ TREG CELLS – MASTER REGULATORS OF THE IMMUNE SYSTEM

Natural $CD4^+$ Treg cells which express Foxp3 and develop in the thymus, represent a unique lineage of T cells with the ability to suppress autoimmune and pathological responses (**Figure 2**, top panel) (Piccirillo et al., 2005). They represent 1–10% of thymic and peripheral circulating $CD4^+$ T cells in mouse and human, and are able to down-regulate the activation and function of various immune effector cell subsets. Alternatively, Treg cells can differentiate in the periphery from conventional T cells upon reception of antigen-specific stimulation along with tolerogenic cytokine signals. Natural and induced Treg cells are characterized by the constitutive expression of the IL-2Rα chain (CD25) and preferentially express Foxp3 (Fontenot et al., 2005). The importance of Foxp3 has been demonstrated by mutations in the *foxp3* gene that result in the loss of Treg cell function and the development of multi-organ autoimmunity, including autoimmune diabetes, in IPEX patients and Scurfy mice (Hori et al., 2003; d'Hennezel et al., 2012). Several factors like IL-2 and TGFβ have been identified that can enhance stabilize Foxp3 expression via demethylation of CpG motifs within conserved regions of Foxp3 promoter (Shen et al., 2009; Haiqi et al., 2011). Treg cell mediated suppression

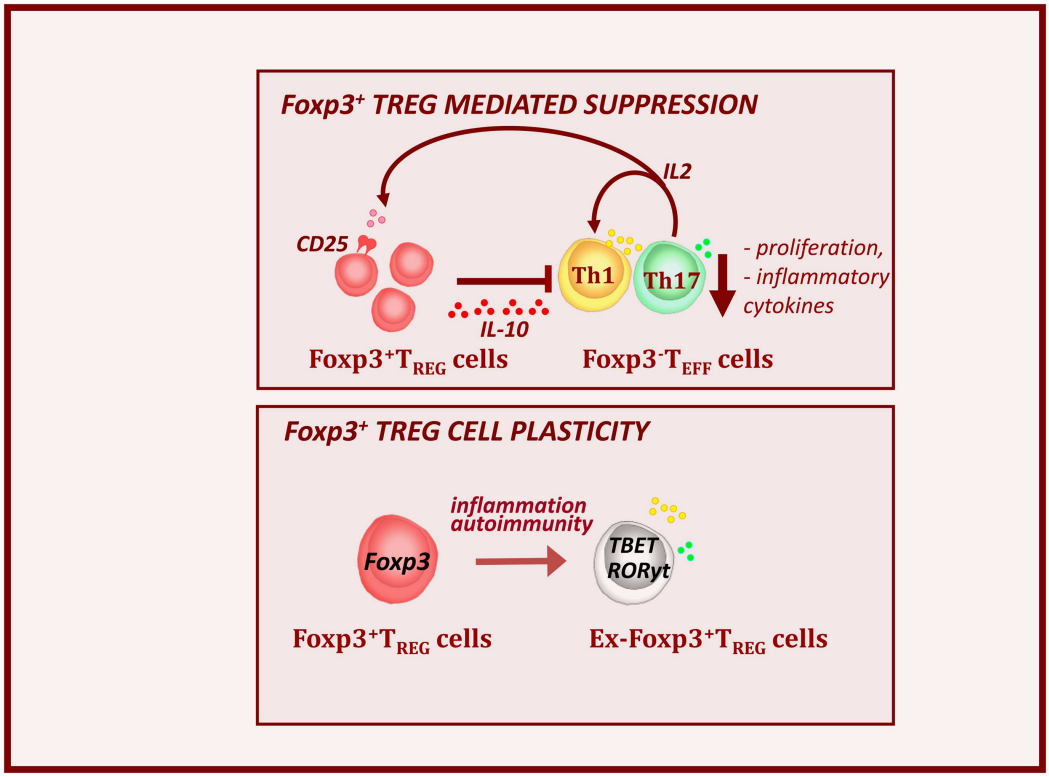

FIGURE 2 | Foxp3+ Treg cells control autoimmunity and T1D pathogenesis. Foxp3+ Treg cells express high levels of IL-2Ra or CD25 and are dependent on Teff-derived IL-2. Alterations in local IL-2 production may precipitate T1D by perturbing Treg cell function. Treg cells produce the immunosuppressive cytokine IL-10. This results to the down-regulation of inflammatory cytokines (IFNγ and IL-17) and decreased expansion of effector cell pools. However during the T1D progression, inflammatory signals, can provoke loss of Foxp3 expression. These, so called ex-Foxp3 Treg cells acquire an effector cell phenotype in terms of transcription factor expression and inflammatory cytokine production and contribute to T1D pathology. As widely observed features of Treg cell biology in autoimmune.

mechanisms are numerous and complex, including several cell surface and soluble factors that directly control activation of effector cells. Suppression is likely mediated via cell–cell contact dependent mechanisms and production of immunomodulatory cytokines such as IL-10 and TGF-β, IL-35, that inhibit DC and T cell activity (Sakaguchi et al., 2009; Shevach, 2009).

THE IMMUNE-PROTECTIVE ROLE OF TREG CELLS IN T1D

The critical importance of Treg cells in autoimmune settings, such as T1D, is well established (Atkinson and Leiter, 1999; Bach and Chatenoud, 2001). In NOD mice, depletion of CD4+CD25+ Treg cells accelerates development of T1D (Salomon et al., 2000; Salomon and Bluestone, 2001). Furthermore, abolishment of co-stimulatory pathways that are vital for Treg homeostasis, such as CD28 and ICOS, in NOD mice exacerbates T1D (Salomon and Bluestone, 2001; Anderson and Bluestone, 2005; Kornete et al., 2012).

We and others have shown that T1D progression is NOD mice is associated with a decrease in numbers and function of Treg cells in the inflamed islets and defects in IL-2 production by effector T cells seem largely responsible (Sgouroudis et al., 2008; Tang et al., 2008; Tritt et al., 2008; Kornete et al., 2012). Overall, this demonstrates that Treg cells function as major controllers of immune homeostasis and tolerance in the periphery. However, more recent finding indicated that Treg cells can become unstable and lose Foxp3 expression in inflamed pancreatic sites during T1D progression (**Figure 2**, bottom panel) (Zhou et al., 2009; McClymont et al., 2011). In the NOD mice, the lack of intra-islet IL-2 results in CD25 down-regulation and consequent reduction in Foxp3 expression. These ex-Foxp3+ T cells produce inflammatory cytokines, such as IFNγ and IL-17 and have increased immunopathogenic potential upon adoptive transfer (Zhou et al., 2009). A similar observation has been made in T1D patients, in which a significant increase in the number of IFNγ and IL-17 secreting Foxp3+ Treg cells was observed (McClymont et al., 2011).

BETA (β) CELLS: REGENERATION AND TRANS-DIFFERENTIATION

The presence of β-cells in patients with long lasting T1D, despite ongoing autoimmunity, suggests that regeneration of β-cells may occur. About 60% of T1D patients enter a clinical "honeymoon phase," lasting between 3 months and 2 years, and characterized by improved insulin secretion to the extent that some patients can discontinue exogenous insulin (Muhammad et al., 1999; Abdul-Rasoul et al., 2006). β-cells have a robust capacity to regenerate by proliferation, likely in response to inflammation-driven signals

(Akirav et al., 2008). Furthermore, recent studies revealed a previously unrecognized plasticity of endocrine cells in the pancreas. However, depending on the nature of experimental β-cell destruction, these studies reached divergent conclusions regarding the origin of new β-cells. Supporting the hypothesis of self-renewal, partial abolition results in β-cell regeneration from surviving β-cells (Dor et al., 2004; Nir et al., 2007). However, when more >99% of β-cells are chemically destroyed, new β-cells are generated either via differentiation of endocrine and islet precursors (Guz et al., 2001; Thyssen et al., 2006) or by spontaneous reprograming of differentiated endocrine cell types such as δ-cells (Fernandes et al., 1997) or α-cells (Chung et al., 2010; Thorel et al., 2010; Zaret and White, 2010).

Several factors have been proposed to influence β-cells regeneration. Inflammation, though driving autoimmune responses, is implicated in β-cells proliferation. For example, while Th1 cell-derived IFN-γ is the main mediator of diabetogenesis in the NOD mice, the transgenic expression of IFN-γ enhances β-cells proliferation and survival (Tuch et al., 1991; Ablamunits et al., 2007). Similarly, inflammatory cytokines such as IL-1β, nitric oxide (NO), and TNF-α were shown to cause β-cell replication (Donath et al., 2003; Luo et al., 2005). More recently, Herold's group demonstrated that increased β-cell proliferation depends on the inflammatory infiltrate itself and immunotherapeutic regimens, namely administration of anti-CD3 mAb and Foxp3$^+$ Treg cells that suppress inflammatory T cells, also decrease β-cell replication (Sherry et al., 2006).

THE CONTROL OF T1D COMPLICATIONS BY TREG CELLS: THE CASE FOR ATHEROSCLEROSIS

Persistent, dysregulated inflammation contributes to the development of secondary chronic disorders such as vascular or neurodegenerative disease in T1D patients. One of the most common T1D complications is atherosclerosis, an inflammatory disorder of the arterial wall caused by the retention of cholesterol in the subendothelial region of the artery. Whereas inflammation is driven by both innate and adaptive immune effector cells, recent studies suggest that Foxp3+ Treg cells control the development and progression of atherosclerosis (Veillard et al., 2004; de Boer et al., 2007). Treg cells account for 1–5% of T cell population within atherosclerotic lesions in which they produce of immunomodulatory cytokines, such as IL-10 and TGF-β (de Boer et al., 2007). Pro-atherogenic ApoE-deficient mice exhibit significantly lower numbers of Treg cells than their wild type counterparts (Mallat et al., 2003). The role of Treg cells was further elucidated via generation of low density lipoprotein receptor knock-out chimeric mice (Ait-Oufella et al., 2006). Reconstitution of these mice following irradiation with CD80/CD86 and CD28 deficient bone marrow cells resulted in a marked reduction in Treg cells and an increase in atherosclerotic lesion size compared to control mice (Ait-Oufella et al., 2006). Finally, various successful immune therapies used in atherosclerosis suggest an important role for Treg cells. Use of anti-CD3 mAB reduced plaque formation when administered prior to a high cholesterol diet and markedly decreased lesion progression in mice with established atherosclerosis (Steffens et al., 2006). Vaccine administration into atherosclerotic prone mice of pro-atherogenic auto-antigens, such as apolipoprotein B-100 or heat shock protein, led to the inhibition of atherosclerosis development. Both treatments resulted in increased production of Foxp3+ Treg cells and secretion of TGF-β and IL-10 (van Puijvelde et al., 2006; Klingenberg et al., 2010).

THE PATHO-PHYSIOLOGY OF TYPE 2 DIABETES AND ITS COMPLICATIONS

Whereas the autoimmune etiology of T1D pathogenesis is well established, T2D was historically considered a non-immune condition. However, recent work highlighting adiposity-associated chronic inflammation in T2D implicates immune mediators in metabolic dysregulation. In conjunction with adipocytes, the innate and adaptive immune system drives systemic inflammation, promoting both insulin resistance and associated complications such as diabetic nephropathy (DN). As crucial mediators of peripheral tolerance, it is not surprising that within this environment Treg cells are key regulators of adipose tissue inflammation and resultant diabetogenesis.

Excess adiposity is associated with an increase in serum C-reactive protein (CRP) and pro-inflammatory cytokines such as IL-6 in humans (Visser et al., 1999). Furthermore, insulin resistance positively correlated with the levels of these cytokines in the blood of T2D patients (Bruun et al., 2003). The mechanism by which inflammatory mediators can disrupt intracellular metabolic signaling has been elucidated in mouse models. Stimulation of the JNK and NF-κB pathways by pro-inflammatory cytokines activates negative regulators of the insulin receptor pathway. Specifically JNK and IKK-β phosphorylate insulin receptor substrate 1 (IRS1) inhibiting tyrosine phosphorylation by the insulin receptor (Arkan et al., 2005; Sabio et al., 2010). Genetic ablation of these kinases results in significant amelioration of insulin resistance. Thus cross talk between insulin receptor and inflammatory signaling cascades can disrupt cell metabolism and exacerbate insulin resistance.

In addition to the predominant adipocyte population, lean adipose tissue contains an appreciable number of leukocytes in the absence of inflammation, suggesting involvement in fat tissue homeostasis (Lumeng et al., 2007; Feuerer et al., 2009). However, profound accumulation of inflammatory immune infiltrates accompanies the accrual of lipids in the visceral adipose tissue (mVAT) of obese mice (Weisberg et al., 2003; Nishimura et al., 2009). Most notably, macrophages of the inflammatory M1 subtype predominate in obese VAT over tolerogenic M2 macrophages found in lean fat (Lumeng et al., 2007). The M1 phenotype is the major source of pro-inflammatory cytokines that promote insulin resistance in adipocytes. Though in humans a dichotomous M1/M2 paradigm is absent, macrophages nonetheless accumulate in obese fat and drive inflammation (Zeyda et al., 2007). Recently, CD8$^+$ Teff cells have been identified as key regulators of macrophage recruitment and switch to the M1 type in obese adipose tissue (Nishimura et al., 2009). CD8$^+$ cells were observed prior to macrophage populations in VAT of mice when obesity was induced via high fat diet (HFD). Furthermore, in CD8 depleted or deficient mice on HFD, VAT macrophage infiltration and phenotypic switch was repressed and indices of metabolic dysfunction significantly ameliorated. Others have proposed that CD4$^+$T$_h$1 cells coordinate adipose tissue inflammation, describing restored insulin sensitivity via T$_h$1 cell depletion in a HFD mouse model of

obesity (Winer et al., 2009). Collectively, these studies posit that T lymphocytes drive adipose tissue macrophage (ATM) recruitment and differentiation and consequent chronic inflammation in T2D.

In contrast, T lymphocytes can also exact essential regulatory function in adipose tissue. In both mouse and human, Foxp3$^+$ Treg cells have been found in both VAT and subcutaneous fat (SAT) (Feuerer et al., 2009; Eller et al., 2011; Zeyda et al., 2011). Indeed Treg cells comprised more than half of the total CD4 compartment in VAT of healthy C57BL/6 mice (Feuerer et al., 2009). These Treg cells produce high quantities of the anti-inflammatory cytokine IL-10 and uniquely express nuclear receptor PPAR-γ, which is necessary for their homeostasis and regulatory function in VAT (Feuerer et al., 2009; Cipolletta et al., 2012). Gene expression profiling indicates PPAR-γ and Foxp3 may coordinately regulate VAT Treg transcriptional programs necessary to suppress adipose-associated inflammation. This could be akin to how recently identified Treg subsets are phenotypically specified to suppress T_h1, T_h2, or T_h17 responses (Campbell and Koch, 2011). While in both genetic and diet-induced mouse models a waning of VAT Treg cells accompanies increasing adiposity, the effect of obesity on VAT Treg cell numbers in humans remains controversial (Feuerer et al., 2009). Whereas some groups have found FOXP3 expression to negatively correlate with BMI, others report no change or an increase in FOXP3 in the fat of obese individuals compared to normal BMI controls (Feuerer et al., 2009; Eller et al., 2011; Zeyda et al., 2011). However, these studies indirectly enumerate Treg cells via qPCR rather than flow cytometry and thus do not discriminate between FOXP3 expression by *bona fide* Treg cells, and transient FOXP3 upregulation by Teff cells upon activation. Nevertheless, such findings highlight the risk in literal application of established mouse models to human T2D.

Thus T cells direct adipose-induced systemic inflammation and therefore may indirectly promote T2D complications via metabolic disruption. Further evidence suggests T cells could mediate immunopathology directly in the target organ. For instance, profound infiltration of the kidneys by activated T cells is associated with the development of DN in T2D patients (Moon et al., 2012). This is accompanied by macrophage accumulation and production of pro-inflammatory cytokines such as IFN-γ and IL-1β (Galkina and Ley, 2006). However the relative contribution of inflammatory verses metabolic and hemodynamic factors to the initiation and progression of renal lesions remains unclear. A study of DN in db/db mice suggests Treg cells may dampen kidney immunopathology (Eller et al., 2011). Treg depletion via administration of anti-CD25 antibodies exacerbated nephropathy, renal dysfunction and leukocyte infiltration of the kidneys. Furthermore, adoptive transfer of Treg cells into db/db mice improved kidney function and ameliorated DN. Thus, Treg cells suppress inflammation both at the primary and secondary sites of T2D pathogenesis. Like in T1D, Treg cells may represent a key homeostatic checkpoint that, if breached, results in the breakdown of peripheral tolerance and progression of autoreactive responses.

IMMUNOTHERAPEUTIC STRATEGIES: CURRENT AND FUTURE AVENUES

Type 1 diabetes susceptibility and pathogenesis results from a complex interplay between genetic, environmental, and immunological factors. Therefore, a multi-faceted solution is likely necessary to effectively treat T1D. An ideal immunotherapy would simultaneously shut down pathogenic T cells and enhance regulatory mechanisms, while also promoting β-cell regeneration or neogenesis. All three therapeutic goals have been proposed to occur through anti-CD3 mAB therapy. Anti-CD3 works as an immune suppressant, promotes antigen-specific Treg cells and both increases and preserves β-cell mass. Anti-CD3 mAB causes internalization of the CD3-TCR complex and prevents Teff cells from recognizing antigen. Furthermore it affects TCR-mediated signal transduction and provokes apoptosis and anergy of Teff cells (Chatenoud et al., 1982, 1994). Beyond modulation of effectors cell pools, anti-CD3 has recently been shown to promote induction and stabilization of Treg cells (You et al., 2007; Penaranda et al., 2011). Two independent clinical trials using either Teplizumab (United States) or Otelixizumab (Europe) led to the sustained preservation of insulin production. In NOD mice, anti-CD3 therapy permanently reversed diabetes and in humans C-peptide levels were sustained from 1 to 5 years, demonstrating long term protection could be obtained (Herold et al., 2002, 2009; Keymeulen et al., 2005).

Can antigen-specific therapies prevent the immune-driven pathology in disease? In T1D, several studies had focused on the use of insulin and GAD65 as a primary targets for antigen-specific therapies as they are proposed to be key initiating auto-antigens in NOD mice and major auto-antigens in human. The most prominent clinical trial so far involves oral insulin administration in first-degree relatives of T1D patients with high levels of insulin autoantibodies. This regimen modulates diabetogenic immune responses and consequently delays diabetes onset by as much as 5 years (Skyler et al., 2005). In addition, a single injection of the GAD-alum vaccine, the most successful antigen-specific therapy to date, delayed the loss of C-peptide production in new onset T1D children and adolescents (Ludvigsson et al., 2008). Administration of agents such as gastrin that stimulate beta cell neogenesis without increasing proliferation, can minimize antigen spread and prevent β-cell loss (Rooman et al., 2002). Lastly, modulation of local tissue or systemic metabolism, possibly by targeting the PPAR-γ pathway, may impact the generation of adipose-related Treg cells and suppress local inflammatory responses.

CONCLUSION

Thus in T1D and T2D, inflammation in the target tissue and at secondary sites drives disease progression. Whereas in T1D this response is quintessentially autoimmune, the factors that initiate adipose tissue inflammation in T2D have yet to be elucidated. However, in both conditions innate and adaptive leukocyte infiltration and local tissue destruction instigate chronic systemic inflammation. This promotes diabetogenic complications including autoimmune responses at secondary tissues and metabolic perturbation in T2D. Treg cells, potent suppressors of autoimmunity in the periphery, can dampen immune effector cell responses in the β-islets. Furthermore, an important role for this subset in inflamed adipose tissue has recently been characterized. Thus, enhancing the activity of Treg cells may present a therapeutic avenue to limit type 1 and type 2 diabetes pathogenesis and its complications.

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Incretin hormones as immunomodulators of atherosclerosis

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Atherosclerosis results from endothelial cell dysfunction and inflammatory processes affecting both macro- and microvasculature which are involved in vascular diabetic complications. Glucagon-like peptide-1 (GLP-1) is an incretin hormone responsible for amplification of insulin secretion when nutrients are given orally as opposed to intravenously and it retains its insulinotropic activity in patients with type 2 diabetes mellitus (T2D). GLP-1 based therapies, such as GLP-1 receptor (GLP-1R) agonists and inhibitors of dipeptidyl peptidase-4, an enzyme that degrades endogenous GLP-1 are routinely used to treat patients with T2D. Recent experimental model studies have established that GLP-1R mRNA is widely expressed in several immune cells. Moreover, its activation contributes to the regulation of both thymocyte and peripheral T cells proliferation and is involved in the maintenance of peripheral regulatory T cells. GLP-1R is also expressed in endothelial and smooth muscle cells. The effect of incretin hormones on atherosclerogenesis have recently been studied in animal models of apolipoprotein E-deficient mice (apoE$^{-/-}$). These studies have demonstrated that treatment with incretin hormones or related compounds suppresses the progression of atherosclerosis and macrophage infiltration in the arterial wall as well as a marked anti-oxidative and anti-inflammatory effect on endothelial cells. This effect may have a major impact on the attenuation of atherosclerosis and may help in the design of new therapies for cardiovascular disease in patients with type 2 diabetes.

Keywords: atherosclerosis, diabetes, GLP-1, incretins, GIP

INTRODUCTION

Diabetes is a global health problem with a prevalence of more than 285 million cases worldwide and an incidence that continues to increase. The vast majority of diabetic patients (∼90–95%) suffer from type 2 diabetes (T2D), whereas type 1 diabetes (T1D), accounts for 5–10% and rare forms (i.e., genetic forms of diabetes, diabetes secondary to pancreatic diseases or surgery, as well as gestational diabetes) constitute the remaining subtypes (International Diabetes Federation, 2009). Cardiovascular complications represent the primary source of morbidity and mortality in diabetic subjects (Mazzone et al., 2008) and it is well known that diabetic milieu *per se* accelerates the course of atherosclerosis (Nogi et al., 2012). It is also well established that T2D is caused by a combination of insulin resistance in skeletal muscle, liver, and adipose tissues and impaired insulin secretion from the pancreatic islets (Stumvoll et al., 2005). Insulin resistance is the main feature of metabolic syndrome, which refers to the clustering of cardiovascular risk factors that include diabetes, obesity, dyslipidemia, and hypertension (Bajaj and Defronzo, 2003). In relation to insulin resistance, the mechanisms that can promote both atherogenesis and advanced plaque progression likely involve both systemic factors that promote these processes, particularly dyslipidemia, but also hypertension and a proinflammatory state as well as the effect of perturbed insulin signaling at the level of the intimal cells that participate in atherosclerosis (Bornfeldt and Tabas, 2011). There is extensive evidence indicating that insulin resistance increases the risk of coronary artery disease (CAD) even in the absence of hyperglycemia (DeFronzo, 2010). *In vivo* studies have provided data showing that insulin resistance in macrophages and endothelial cells may promote atherogenesis and clinical progression of advanced plaques (Rask-Madsen et al., 2010). Data from human and animal studies supporting a direct pro-atherogenic role of hyperglycemia in vascular cells are not as strong as for insulin resistance but there is suggestive evidence that high glucose is atherogenic, particularly at the level of the arterial endothelium by promoting early stages of lesion formation (Vikramadithyan et al., 2005). However, it is possible that hyperglycemia acts also synergistically with other cardiovascular risk factors and even insulin resistance itself in advanced lesions in the setting of T2D (Bornfeldt and Tabas, 2011). For example, glucotoxicity may contribute to insulin resistance, and treatment of hyperglycemia in T2D improves insulin resistance in some tissues (Henry, 1996).

Two novel classes of glucose-lowering agents for the treatment of T2D have been introduced in the market in the last years: glucagon-like peptide-1 receptor (GLP-1R) agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors or incretin enhancers (Tahrani et al., 2011). The mechanism of action of these drugs is based on the enhancement of the incretin effect. The well-documented phenomenon of oral glucose eliciting a higher insulin response than intravenous glucose at identical plasma levels of

glucose is known as the incretin effect (McIntyre et al., 1964). The incretin effect has been found to be mediated mainly by two gut-derived hormones: glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP; Baggio and Drucker, 2007). These incretins control blood glucose by stimulating insulin release from the β cells of the pancreatic islet, decreasing glucagon secretion and slowing gastric emptying (Nauck et al., 2011). However, both GLP-1 and GIP are rapidly inactivated *in vivo* by circulating peptidases, mainly DPP-4/CD26. Thus, the administration of DPP-4 inhibitors prevents the degradation and inactivation of GLP-1 and GIP. In addition to their antidiabetic action through the aforementioned mechanisms, recent experimental and clinical studies have demonstrated that incretin therapies have several effects on cardiovascular function (Grieve et al., 2009). These effects are possibly mediated at least in part by mechanisms independent of their glucose-lowering activity and include: changes in blood pressure (Brown, 2012), endothelial function (Irace et al., 2012), body weight (Vilsbøll et al., 2012), cardiac metabolism (Nielsen et al., 2012), lipid metabolism (Farr and Adeli, 2012), left ventricular function (Poornima et al., 2008), and the response to ischemia-reperfusion injury (Chinda et al., 2012).

It is now widely accepted that inflammation and immunity play important roles in the pathogenesis of atherosclerosis (Hansson and Libby, 2006). Recently, several studies have shown that DPP-4 inhibitors and GLP-1R agonists exert also a potent anti-inflammatory effect and thus, may potentially contribute to the prevention of atherosclerosis (Chaudhuri et al., 2012; Makdissi et al., 2012).

ATHEROSCLEROSIS AS AN IMMUNE-MEDIATED DISORDER

Studies over the last decade strongly support the idea that atherosclerosis results from endothelial cell dysfunction followed by lipid accumulation and an inflammatory process affecting both macro- and microvasculature (Lusis, 2000). But also, atherosclerosis is now universally recognized as having an inflammatory immune-mediated component in disease development in which both the monocyte and the macrophage are central to this inflammation (Ross, 1999). Macrophage infiltration is one of the driving factors for plaque development and during the first stages of atherosclerosis, they are actively recruited from the circulation and the *vasa vasorum* into the intimal lining of blood vessels. On the other hand, there is now solid evidence that T cell are involved in atherogenesis. The available data suggest that T cell-mediated responses contribute to both the development and the progression of atherosclerosis. The majority of pathogenic T cells involved in atherosclerosis are of Th1 profile producing high levels of INF-γ among other cytokines which are known to activate macrophages and dendritic cells, leading to the perpetuation of this pathogenic Th1 response (Hansson, 2001). In addition, IFN-γ may inhibit vascular smooth muscle cell (SMC) proliferation and reduces local collagen production. Matrix metalloproteinases are also up-regulated, thereby contributing to the thinning of the fibrous cap (Tedgui and Mallat, 2006). Deficiency of IFN-γ receptor or IFN-γ significantly reduces lesion development and enhances collagen content of the plaque, whereas exogenous administration of IFN-γ stimulates lesion development (Whitman et al., 2000).

In relation to Th2 cells, Th2-biased responses have been proposed to antagonize pro-atherogenic Th1 effects and thereby confer atheroprotection. However, the role of Th2 pathway in the development of atherosclerosis remains controversial depending on the stage and/or site of the lesion, as well as on the experimental model used (Mallat et al., 2009). In mouse models that are relatively resistant to atherosclerosis, a Th2-bias has been shown to protect against early fatty streak development (Huber et al., 2001). However, in other models using LDLR$^{-/-}$ mice, deficiency in IL-4, the prototypic Th2-related cytokine, had no substantial effect on lesion development al least in one study (King et al., 2007). However, while initial studies focused more on the pathogenic arm of the immune system, recent work clearly suggests an important role for several subsets of regulatory T cells (Treg) in the protection against lesion development (Wigren et al., 2011). These cells home to peripheral tissues to maintain self-tolerance and prevent autoimmunity by inhibiting pathogenic lymphocytes (Sakaguchi et al., 2009). Data gathered from the literature indicate that several populations of Tregs "tune down" the inflammatory response within the atherosclerotic lesion in transgenic atherosclerosis-prone mice which points to a protective role of Tregs in this process (Mallat et al., 2003).

INCRETIN HORMONES AND ATHEROSCLEROSIS

Two main incretin hormones have been fully characterized to date: GLP-1 and GIP. GLP-1 stimulates insulin and inhibits glucagon secretion in a glucose-dependent manner. It also inhibits gastric emptying and reduces appetite, actions that contribute to improved glycemic control in T2D patients (Kazakos, 2011). It is synthesized and secreted by enteroendocrine L cells distributed through the small and large intestine; however, the majority of intestinal GLP-1 content has been localized to the distal small bowel and colon (Brubaker, 2010). The GLP-1R was originally identified in islet β cells but is widely expressed in extrapancreatic tissues, including the lung, kidney, central nervous system, enteric and peripheral nervous system, lymphocytes, macrophages (Bullock et al., 1996), human coronary endothelial cells (Erdogdu et al., 2012), human umbilical vein endothelial cells (HUVECs; Ding and Zhang, 2012), and heart (Wei and Mojsov, 1995). GIP is synthesized in and secreted from enteroendocrine K cells localized to the proximal small bowel (Ussher and Drucker, 2012). GIP receptor (GIP-R) are widely expressed in extrapancreatic tissues, including the gastrointestinal tract, adipose tissue, heart, pituitary, adrenal cortex, and multiple regions of the central nervous system (Usdin et al., 1993).

The mechanisms by which incretin modulation might be associated with cardiovascular benefits are multiple. Considerable evidence incriminates the dysfunctional adipocyte and excess ectopic adiposity – in the liver, around visceral organs, and/or in the skeletal musculature – as culprits in both T2D and its atherosclerotic complications (Bays, 2011). Adipose tissue is an important inflammatory source in obesity and T2D, not only because of cytokines produced from the adipocyte itself, but also because of infiltration by proinflammatory macrophages (Shoelson and Goldfine, 2009). Thus, weight loss associated with GLP-1R agonists decreases cardiovascular risk in successfully

treated subjects and may have a positive influence in cardiovascular endpoints. In relation to lipid metabolism, a direct role for GLP-1 in the control of chylomicron secretion has been suggested. Indeed, treatment with GLP-1 as well as GLP-1R agonists in animals models reduces triacylglycerol (TAG) absorption, decreases intestinal lymph flow, and reduces intestinal B-48 apolipoprotein production (Qin et al., 2005). In T2D patients, treatments with GLP-1R agonists and DPP-4 inhibitors have demonstrated favorable effects on postprandial dyslipidemia (TAG, Apo B-48, and FFA; Hsieh et al., 2010).

On the other hand, GLP-1R expression in endothelial cells, vascular SMCs, monocytes, macrophages, and lymphocytes also raises the prospect for direct effects on atherosclerosis and inflammation (**Figure 1**). However, little information exists on the effects of GLP-1 on atherogenesis itself. Elevated tumor necrosis factor-alpha (TNF-α) levels and hyperglycemia are implicated in diabetes-associated endothelial cell dysfunction and may be causal in premature atherosclerosis (Iwasaki et al., 2008). Indeed, TNF-α and hyperglycemia have been shown to induce plasminogen activator inhibitor-1 (PAI-1) and vascular cell adhesion molecules (VCAM-1 and ICAM-1) expression in human vascular endothelial cells (Morigi et al., 1998). Recently, the protective properties in endothelial cells of GLP-1 and liraglutide, a GLP-1R agonist, have been demonstrated by several investigators. This treatment reduces TNF-α-mediated expression of PAI-1, ICAM-1, and VCAM-1 expression in human vascular endothelial cells (Liu et al., 2009) as well as TNF-α induced oxidative stress (Shiraki et al., 2012). In relation to the atherosclerotic lesion, recent experimental studies have shown that GLP-1R activation by the administration of exendin-4 significantly reduces the accumulation of monocytes/macrophages in the vascular wall of C57BL/6 and ApoE$^{-/-}$ mice. This effect seems to be mediated, at least in part, by suppressing the inflammatory response in macrophages through the activation of the cAMP/PKa pathway which inhibits the expression of TNF-α and the monocyte chemoattractant protein-1 (MCP-1) in activated macrophages (Arakawa et al., 2010). Administration of active forms of native incretins (GLP-1 and GIP) has also been described to be associated with a suppression of atherosclerotic lesions and macrophage infiltration in the vascular wall in ApoE$^{-/-}$ mice. This treatment directly suppresses the expression of MCP-1, VCAM-1, ICAM-1, and PAI-1 in aortic endothelial cells, as well as suppresses aortic SMCs proliferation and macrophage foam cell formation associated with the down-regulation of CD36, a type A scavenger receptor that takes up modified LDLs, and acyl-coenzyme A: cholesterol acyltransferase-1 (ACAT-1), the enzyme that promotes cholesteryl ester accumulation in macrophages (Nagashima et al., 2011).

Glucagon-like peptide-1 receptor protein expression is not only found in endothelial cells and macrophages but also in murine SMCs suggesting that GLP-1R agonists may have direct effects on SMCs during neointimal formation. The effects of exendin-4, a GLP-1R agonist, on intimal thickening after vascular injury have been investigated in a recent study. The results obtained have shown that treatment with a GLP-1R agonist is associated with a reduced intimal thickening after vascular injury. This effect seems

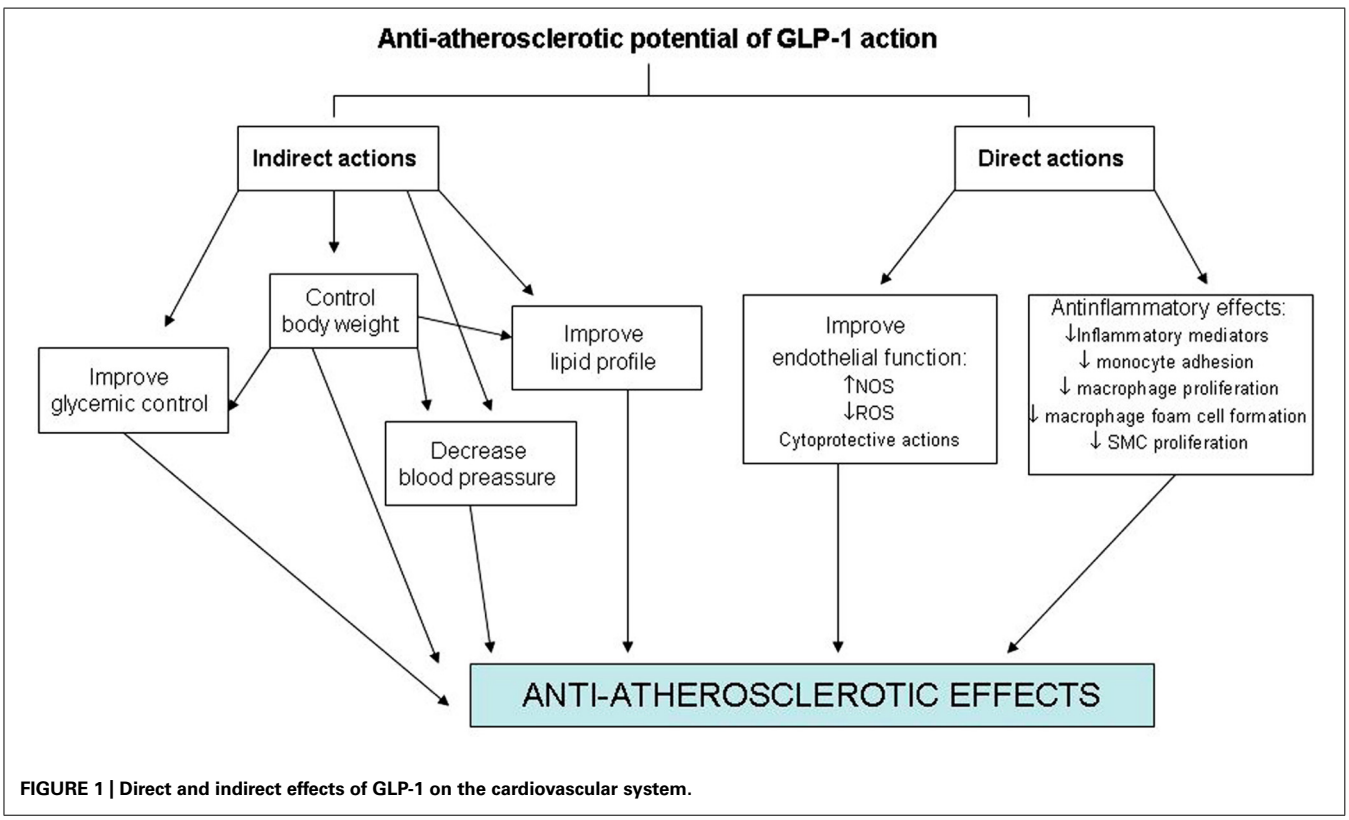

FIGURE 1 | Direct and indirect effects of GLP-1 on the cardiovascular system.

to be mediated through a reduced proliferation of SMC induced by platelet-derived growth factor (PDGF) which seems to be independent of the canonical (cAMP) GLP-1R signal pathway suggesting a direct action of GLP-1 on SMCs (Goto et al., 2011).

The truncated metabolite of GLP-1 (9–36) amide has also been described to exert some cardioprotective effects, increasing basal myocardial glucose uptake, and improving left ventricular function in animal models with cardiomyopathy (Nikolaidis et al., 2005; Ban et al., 2008).

Since its description in 1966, DPP-4 has been considered to be a unique peptidase that cleaves dipeptides from peptides and proteins containing proline in the penultimate position. However, proteolysis is only one of the multiple functions that this protein executes. Other functions attributed to this glycoprotein include the regulation of T cell activation, DNA synthesis, cell proliferation, cytokine production, and signaling activation (Hegen et al., 1997). In addition to the action of CD26/DPP-4 on GLP-1 and GIP, CD26/DDP-4 directly activates a number of proteins such as mitogen-activated protein kinases (MAPKs) which are involved, in particular the extracellular signal-regulated kinase (ERK), in cell proliferation. In a recent *in vitro* study it has been demonstrated that the inhibition of DPP-4/CD26 by alogliptin suppresses Toll-like receptor (TLR)-4-mediated ERK activation and ERK-dependent matrix metalloproteinases expression by histiocytes (Ta et al., 2010). The ERK pathway is an important signaling cascade involved in many physiological and pathophysiological processes including cell proliferation, apoptosis, angiogenesis, and inflammation (McCubrey et al., 2007). Thus, results of this study suggest that DPP-4/CD26 may play an important role in macrophage-mediated inflammation response and tissue remodeling since matrix metalloproteinases are crucially involved in atherosclerosis. The inhibition of DPP-4 has also demonstrated a reduction of atherosclerotic lesions in diabetic apolipoprotein E-deficient mice. Moreover, *ex vivo* studies have shown that DPP-4 inhibition attenuates diabetes-augmented IL-6 and IL-1β expression in atherosclerotic plaques (Ta et al., 2011) and reduces plaque macrophages infiltration and monocyte migration to the aorta of male LDLR$^{-/-}$ mice (Shah et al., 2011).

Two recent clinical studies have evaluated the anti-inflammatory effect of a GLP-1R analog (exenatide) and a DPP-4 inhibitor (sitagliptin) in a group of patients with T2D. Results of these studies have demonstrated that both treatments have a potent and rapid anti-inflammatory effect with a significant reduction in reactive oxygen species generation and the mRNA expression of several inflammatory mediators (TNF-α, JNK-1, TLR-2, TLR-4, IL-1β, and SOCS-3) in mononuclear cells, which might potentially contribute to the inhibition of atherosclerosis. Remarkably, these anti-inflammatory effects occurred at an earlier phase of treatment and were independent of weight loss (Chaudhuri et al., 2012; Makdissi et al., 2012).

On the other hand, DPP-4 or CD26 cleaves other multiple peptide substrates, many of which have direct actions on the heart and blood vessels. Among them are included the stromal cell-derived factor-1α (SDF-1α), the neuropeptide Y (NPY), the peptide Y (PYY), the B-type (brain) natriuretic peptide (BNP), and the GLP-2 (Ussher and Drucker, 2012). In relation to SDF-1α, a chemokine that promotes homing of endothelial progenitor cells to sites of cellular injury, considerable evidence supports a role for SDF-1α as a cardioactive DPP-4 substrate. Indeed, DPP-4 inhibitors have been used, mainly in combination with granulocyte-colony-stimulating factor (G-CSF), to increase stem cell number in both preclinical and clinical studies of cardiovascular injury (Zaruba et al., 2009). The therapeutic use of SDF-1 has also been studied in an animal model to treat the peripheral artery disease. The study shows that SDF-1 engineered to be resistant to DPP-4 improves the blood flow (Segers et al., 2011).

Little has been published on GIP and atherosclerosis. GIP has a potent stimulatory effect on insulin release from the pancreas, but several experimental studies have demonstrated that GIP looses this action in diabetes because GIP-R in pancreatic islets are substantially down-regulated in a hyperglycemic state (Lynn et al., 2001, 2003). This may explain the inability of GIP to induce insulin secretion in diabetes. Recently, Nogi et al. (2012) have described that chronic administration of GIP at a level several-fold higher remarkably suppresses the progression of atherosclerosis in STZ-induced diabetic ApoE$^{-/-}$ mice. In this study, GIP infusion significantly suppressed macrophage-driven atherosclerotic lesions and reduced foam cell formation in macrophages, even though GIP-R expression in macrophages was partially down-regulated in the diabetic state. Recently a published experimental study suggests that GIP could block the signal pathways of advanced glycation end products (AGEs) in HUVECs, which play a crucial role in vascular damage in diabetes (Ojima et al., 2012). The same protective mechanism has been described with GLP-1 (Zhan et al., 2012).

Finally, the majority of GLP-1R agonists and DPP-4 inhibitors are undergoing assessment of cardiovascular outcomes in large, multicenter clinical trials of cardiovascular outcomes. To date, results of randomized trials do not suggest any detrimental effect of GLP-1 receptor agonists on cardiovascular events. However, specifically designed longer-term trials are needed for verifying the possibility of a beneficial effect (Monami et al., 2011a). In relation to DPP-4 inhibitors a recent meta-analysis suggests a possible protection from cardiovascular events, although results should be interpreted with caution, as those events were not the primary endpoint, the trial duration was short, and the characteristics of patients included could be different from routine clinical practice (Monami et al., 2011b).

CONCLUSION

In conclusion, there is now overwhelming evidence that the macrophage has a crucial role in the initiation and progression of atherosclerotic plaque and thus has emerged as a novel therapeutic target for the treatment of atherosclerosis. Recent studies suggest that incretin agents seem to have direct effects on macrophages and endothelial cells which are both involved in the progression of atherosclerosis. Despite the intriguing findings in animals, data on the long-term effects of incretin-based therapy on atherosclerosis-associated outcomes in diabetic humans are not yet available. The critical issue of whether the anti-atherogenic action of incretin agents can be translated into improved cardiovascular outcomes for diabetic patients remains to be elucidated with prospective, large-scale clinical trials.

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