Key features of the intragraft microenvironment that determine long-term survival following transplantation

*Sarah Bruneau[1,2], Craig Bryan Woda[1,2], Kevin Patrick Daly[1,2], Leonard Boneschansker[1,2], Namrata Gargee Jain[1,2], Nora Kochupurakkal[1,2], Alan Gabriel Contreras[1,2], Tatsuichiro Seto[1,2] and David Michael Briscoe[1,2] ***

[1] The Division of Nephrology, Transplantation Research Center, Children's Hospital Boston, Boston, MA, USA
[2] Department of Pediatrics, Harvard Medical School, Boston, MA, USA

In this review, we discuss how changes in the intragraft microenvironment serve to promote or sustain the development of chronic allograft rejection. We propose two key elements within the microenvironment that contribute to the rejection process. The first is endothelial cell proliferation and angiogenesis that serve to create abnormal microvascular blood flow patterns as well as local tissue hypoxia, and precedes endothelial-to-mesenchymal transition. The second is the overexpression of local cytokines and growth factors that serve to sustain inflammation and, in turn, function to promote a leukocyte-induced angiogenesis reaction. Central to both events is overexpression of vascular endothelial growth factor (VEGF), which is both pro-inflammatory and pro-angiogenic, and thus drives progression of the chronic rejection microenvironment. In our discussion, we focus on how inflammation results in angiogenesis and how leukocyte-induced angiogenesis is pathological. We also discuss how VEGF is a master control factor that fosters the development of the chronic rejection microenvironment. Overall, this review provides insight into the intragraft microenvironment as an important paradigm for future direction in the field.

Keywords: endothelial cell, microvascular injury, angiogenesis, vascular endothelial growth factor, hypoxia, allograft rejection, chronic allograft rejection, allograft vasculopathy

GENERAL OVERVIEW

In an endothelial cell (EC)-based model, the initiation of inflammation within an allograft results from the activation of donor ECs responding to pro-inflammatory cytokines released from resident macrophages in response to hypoxia (Cotran, 1994; Briscoe et al., 1998; Denton et al., 2000; Pober and Sessa, 2007; Ingulli et al., 2009). Graft EC also respond to cytokines and growth factors produced in association with alloimmune cellular and humoral targeting of the graft (Pober et al., 1996; Valujskikh and Heeger, 2003; Zhang and Reed, 2009; Halloran et al., 2010; Sis and Halloran, 2010), as well as by factors produced by infiltrating mononuclear cells that are characteristic of chronic rejection (Libby and Pober, 2001). The induced expression of adhesion molecules and chemokines by donor EC results in the recruitment of leukocytes into the graft, whereas the expression of MHC class I and II molecules on donor EC is critical for the local presentation of alloantigen to infiltrating effector/memory lymphocytes (Briscoe and Sayegh, 2002; Kreisel et al., 2002; Pober and Sessa, 2007). These events set the stage for an intragraft microenvironment that sustains donor-directed alloimmune inflammation and the development of chronic rejection.

In this review, we focus on how the integrity of the vascular endothelium is critical for a microenvironment that sustains allograft function. In vascularized solid organ allografts, such as the kidney, early ischemia–reperfusion results in profound injury to the microvasculature (Bishop et al., 1989; Vos and Briscoe, 2002; Woywodt et al., 2003; Reinders et al., 2006; Aydin et al., 2007; Contreras and Briscoe, 2007; Rabelink et al., 2007; Mayer, 2011). Furthermore, the degree of injury and microvascular EC loss at early times post transplantation can be predictive of long-term graft survival (Bishop et al., 1989; Choi et al., 2000; Fine and Norman, 2008; Mayer, 2011; Steegh et al., 2011). Indeed, it is reported that the sequential loss of peritubular capillaries, starting as early as 3 months post renal transplantation predicts the development of interstitial fibrosis and tubular atrophy (IFTA) and later chronic rejection (Steegh et al., 2011). It is proposed that the loss of the intrarenal microvasculature results in impaired delivery of oxygen and nutrients to the renal tubules, which in turn contributes to local tissue ischemia, tubular dropout, and cell death (Kang et al., 2002; Reinders et al., 2006; Contreras and Briscoe, 2007; Rabelink et al., 2007; Fine and Norman, 2008; Mayer, 2011). Thus, the initial loss of microvessels/peritubular capillaries may be a primary factor in the development of fibrosis and chronic renal disease (Kang et al., 2002; Reinders and Briscoe, 2002; Contreras and Briscoe, 2007; Mayer, 2011). Pharmacologic therapy which can protect microvascular integrity at early times post transplantation has potential to improve long-term graft survival (Johnson et al., 2006; Nakao et al., 2006; Aydin et al., 2007; Rabelink et al., 2007; Briscoe and Pal, 2008; Leonard et al., 2008; Hanto et al., 2010). If early protection and repair is not accomplished, then ongoing local ischemia will result in cellular atrophy, and chronic allograft disease will be inevitable.

However, it is underappreciated that inflammatory infiltrates also cause EC proliferation, a process called leukocyte-induced angiogenesis (Auerbach and Sidky, 1979; Cotran, 1994). In addition, the binding of alloantibodies to the graft vascular endothelium can result in EC activation and a proliferative response (Zhang and Reed, 2009). As will be discussed below, this abnormal or pathological angiogenesis response may be associated with local tissue hypoxia, and thus precedes later hypoxic tissue injury (Babu et al., 2007; Contreras and Briscoe, 2007; Goel et al., 2011). If EC proliferation occurs at later times post transplantation in association with pericyte loss, microvessels become disorganized and endothelial-to-mesenchymal transition (EndMT) may occur. This results in collagen deposition and tissue fibrosis (Schor et al., 1995; Humphreys et al., 2010; Medici et al., 2010). Central to all these events is the expression of *vascular endothelial growth factor* (VEGF), which is both pro-angiogenic and pro-inflammatory, and thus drives progression of the chronic rejection microenvironment (Reinders et al., 2006). Here, we discuss a new paradigm, whereby local tissue hypoxia and overexpressed intragraft VEGF are key features of a microenvironment that determine the development of chronic rejection. Cartoons illustrating this paradigm are shown in **Figures 1** and **2**.

MICROVASCULAR PATTERNING AND THE INTRAGRAFT MICROENVIRONMENT

Angiogenesis, the generation of new blood vessels from pre-existing ones, is a complex process involving the degradation of the vascular basement membrane and surrounding extracellular matrix as well as EC proliferation and migration (Cotran, 1994; Folkman, 1995a,b; Brown et al., 1997; Carmeliet and Jain, 2000; Ferrara and Kerbel, 2005; Goel et al., 2011). The creation of new blood vessels is critical for normal organ growth and development and it is a requirement for normal wound healing and tissue repair (Cotran, 1994; Majno, 1998). In all of these biological conditions, angiogenesis is tightly regulated in a tissue specific manner, such that the microvascular bed provides tissues with their nutritive and oxygen demands in a manner that is sufficient for normal physiological processes. However, angiogenesis is also characteristic of many disease states, and is well-established to occur in association with cell-mediated immune responses (Cotran, 1994) and chronic inflammatory diseases (Folkman and Brem, 1992; Ferrara and Alitalo, 1999; Ezaki et al., 2001), notably inflammatory bowel disease (Kanazawa et al., 2001), arthritis (Walsh and Pearson, 2001), chronic asthma (Detoraki et al., 2010), and chronic allograft rejection (Tanaka et al., 1994; Moulton et al., 1999; Rein-
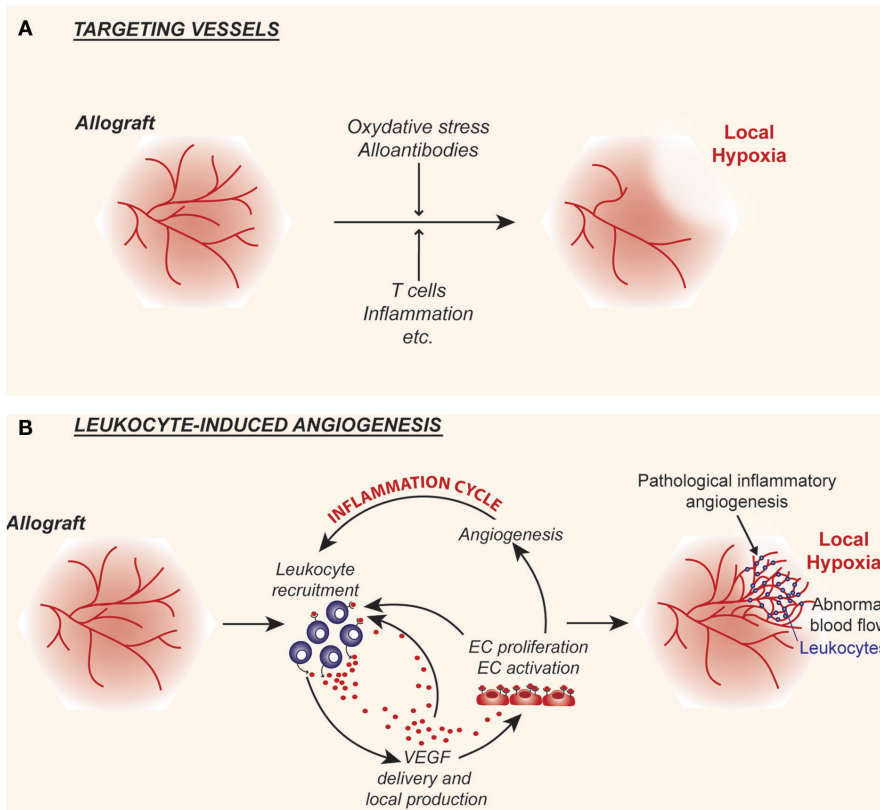

FIGURE 1 | Cartoon illustrating the interplay between alloimmunity and the intragraft microvasculature. (A) Following transplantation, alloimmune inflammatory responses target the graft vascular endothelium resulting in the destruction of microvessels, which in turn leads to local hypoxia and tissue injury. **(B)** Alloimmune inflammatory responses may also stimulate endothelial cell proliferation and promote leukocyte-induced angiogenesis within allografts. The local delivery of the pro-inflammatory and pro-angiogenic factor vascular endothelial growth factor (VEGF) is central to this response. Pathological leukocyte-induced angiogenesis results in the formation of abnormal networks of capillaries that lead to chaotic blood flow patterns and paradoxically results in local hypoxia. Thus, local tissue hypoxia is the end result of acute events and direct targeting of the graft endothelial cells **(A)** as well as inflammation and the associated leukocyte-induced angiogenesis **(B)**.
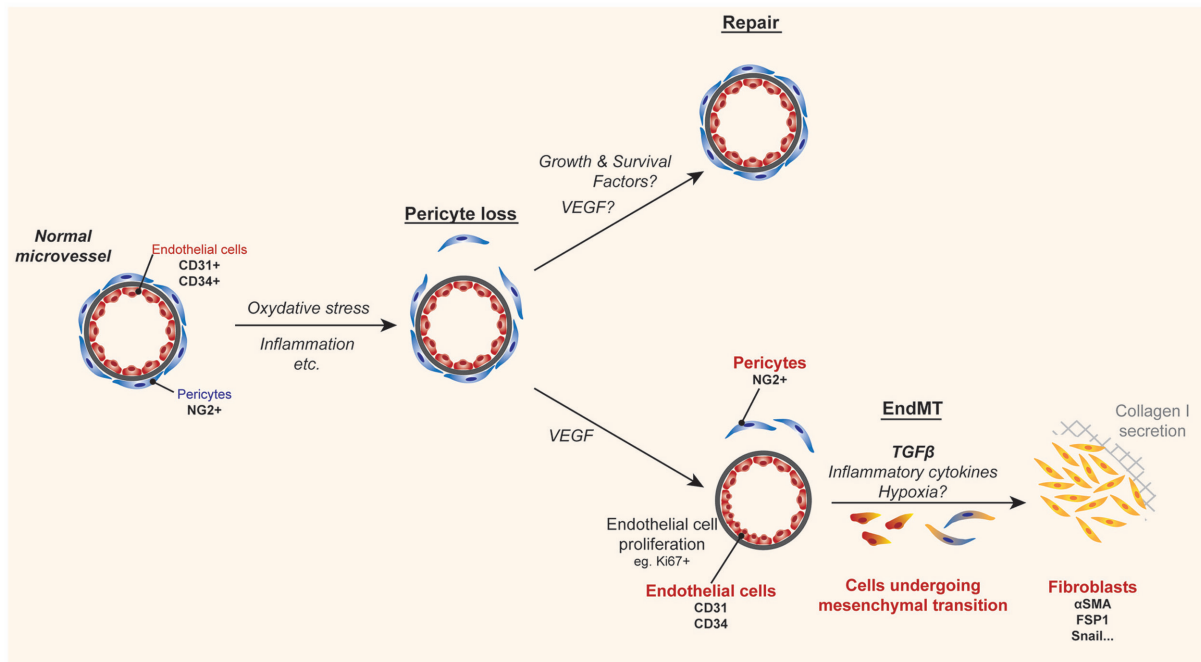
FIGURE 2 | Cartoon illustrating a mechanism of tissue fibrosis associated with allograft rejection. During inflammation, pathological angiogenesis, and/or local hypoxia can lead to pericyte loss. Under normal conditions homeostatic repair occurs under the influence of protective growth and survival factors. In contrast, when the inflammatory microenvironment is sustained, the loss of pericytes serves as a precedent for endothelial-to-mesenchymal transition (EndMT), where endothelial cells become denuded from their basement membrane and migrate along with pericytes into the surrounding tissue. Although still under debate, it is reported that the presence of TGFβ, inflammatory cytokines, and hypoxia enables dissociated pericytes and/or endothelial cells to dedifferentiate into collagen-secreting fibroblasts, which in turn results in fibrosis and scarring.

ders and Briscoe, 2002; Denton et al., 2004; Babu et al., 2007). In disease processes, the neoangiogenesis response occurs in an abnormal and disorganized manner. In some chronic disease conditions, such as arthritis and chronic asthma, it can be uncontrolled (Walsh and Pearson, 2001; Detoraki et al., 2010). Solid tumors are a prototype example where angiogenesis can be abnormal and pathological (Fukumura and Jain, 2007; Jain, 2008; Goel et al., 2011). In this disease, local tissue hypoxia drives the production of angiogenesis factors, notably VEGF. This in turn elicits a powerful neovascular response (Brown et al., 1997; Goel et al., 2011). Since newly formed vessels are irregular in size with random branching patterns, the associated blood flow within the entire microvascular tree becomes abnormal and shunting occurs throughout the tissue (Jain, 2008; Goel et al., 2011). In this manner, some areas of the tissue have potential for increased blood flow and have adequate oxygenation. In contrast, other areas have decreased or aberrant blood flow which results in local tissue hypoxia. This latter event further drives the expression of hypoxia-inducible angiogenesis factors, including VEGF, such that the cyclical process is sustained. It is proposed that these events may also occur in association with chronic kidney disease as well as within kidney allografts in association with chronic rejection (Choi et al., 2000; Reinders et al., 2006; Contreras and Briscoe, 2007; Mayer, 2011; **Figure 1**). Therefore, local tissue hypoxia may occur as a result of both initial targeting and loss of microvessels (**Figure 1A**), or as a result of leukocyte-induced angiogenesis (**Figure 1B**). We propose

that once an abnormal pattern of blood vessels develop within the intragraft microenvironment, it likely serves to elicit local tissue hypoxia as well as to induce VEGF expression, analogous to that described in tumors (Goel et al., 2011). We hypothesize that these events sustain inflammation and the progression of chronic rejection.

LEUKOCYTE-INDUCED ANGIOGENESIS: A PATHOLOGICAL RESPONSE THAT SUSTAINS INTRAGRAFT INJURY

Leukocyte-induced angiogenesis was initially described following the local injection of spleen cells into the skin of nude mice (Sidky and Auerbach, 1975; Auerbach and Sidky, 1979). In these original studies, it was noted that the reaction did not occur following the injection of syngeneic spleen cells, but it was dose-dependent and reproducible following the intradermal injection of allogeneic cells (Auerbach and Sidky, 1979). Subsequently, it was demonstrated that this leukocyte-induced angiogenesis reaction was mediated by CD4^{+} T lymphocytes (Kaminski and Auerbach, 1988), which we now understand to be critical for the initiation of the alloimmune inflammatory response (Ingulli et al., 2009). Thus, while not the intention of these studies, this model clearly indicates that alloactivated leukocytes are potent for the production of angiogenesis factors. Indeed, it is now known that both monocyte/macrophages (Koch et al., 1986; Leibovich and Wiseman, 1988; Polverini, 1997) and activated T cells (Freeman et al., 1995; Melter et al., 2000; Mor et al., 2004) secrete angiogenesis factors, including VEGF, which is

a central mediator of the leukocyte-induced reaction (Leibovich et al., 1987; Giraudo et al., 1998; Reinders et al., 2006). Other factors including TNF-α (63), TGF-β, and nitric oxide (Wiseman et al., 1988; Leibovich et al., 1994) may also elicit the response. Therefore, angiogenesis may result from the local production of distinct factors, or via cytokine- and cell-mediated responses that increase local concentrations of VEGF. This interplay between cell-mediated immune reactions and the local delivery of TGF-β and VEGF by monocyte/macrophages have resulted in the development of paradigms to explain how angiogenesis and fibrosis are characteristic of chronic inflammatory disease states (Cotran, 1994; Brown et al., 1995; Freeman et al., 1995; Sharma et al., 1996; Inoue et al., 1998; Majno, 1998; Pilmore et al., 1999; Jain et al., 2000, 2002; Ezaki et al., 2001; Kanazawa et al., 2001; Mannon, 2006; Booth and Bishop, 2010).

In our own studies, we evaluated recipient angiogenesis using a humanized SCID mouse (huSCID) model of rejection (Moulton et al., 1999). Human foreskin was transplanted onto SCID mice and was found to engraft after 4–6 weeks. Functional vessels and vascular networks within the healed human skin were derived from both human and mouse EC (Briscoe et al., 1999). The subsequent adoptive transfer of human peripheral blood leukocytes into the mouse resulted in human leukocytic infiltrates within the engrafted human skin but not within the adjacent mouse skin, as evaluated by videomicroscopy and by immunohistochemistry (Briscoe et al., 1999; Moulton et al., 1999). After 2–3 days, infiltrates were present within grafts by videomicroscopy, and by 7 days, infiltrates were notable by immunohistochemistry; by day 14, cellular infiltrates were profound within grafts. In our analyses, we also found a notable angiogenesis response that was spatially associated with leukocytic infiltrates within the human skins. The angiogenesis response occurred at early time points, typically on day 3–5 by videomicroscopy and on day 7 by immunohistochemistry (Moulton et al., 1999). Notably, the response preceded the development of marked infiltrates, which were ultimately associated with microvascular destruction. Therefore, we propose that local tissue hypoxia was not the primary stimulus for the initiation of the angiogenesis response within these allografts. Rather, we believe that the angiogenesis reaction was initiated by factors produced by infiltrating leukocytes in a similar manner as previously noted by Auerbach and Sidky (1979) in their model of leukocyte-induced angiogenesis.

More recently, Babu et al. (2007) found a similar neovascularization reaction in their analysis of rejecting tracheal allografts. Although they did not characterize this response in great detail, their studies illustrated prominent angiogenesis in grafts on days 4-, 6-, and 8-post transplantation. Similar to our studies (Moulton et al., 1999; Contreras and Briscoe, 2007), they noted that it did not persist and completely disappeared by day 10–12 following transplantation in association with fulminant rejection. However, these authors also evaluated tissue oxygenation within the graft. Surprisingly, rather than finding that oxygenation was normal at sites of neovascularization, they observed that tissue pO_2 decreased in day 4–6 allografts when the leukocyte-induced angiogenesis reaction was prominent (Babu et al., 2007). Their observations indicate that, contrary to expectations, hypoxia

occurs within allografts *prior to* microvascular destruction, and it is associated with the presence of leukocyte-induced angiogenesis. This observation is consistent with extensive studies by Rakesh Jains group demonstrating that pathological neoangiogenesis and its association with abnormal blood flow patterns within a tumor is ineffective to support tissue oxygenation (Goel et al., 2011).

Angiogenesis has been demonstrated to occur in allografts with evidence of chronic rejection in association with allograft vasculopathy (Atkinson et al., 2005). Similar to our studies in the huSCID (discussed above), as well as those reported by Babu et al. (2007), increased capillary density within the parenchyma of cardiac allografts has been found to be associated with T cell and monocyte infiltrates (Tanaka et al., 1994). Also, neovessels within the intima of large vessels with vasculopathy lesions have been found to be spatially associated with inflammatory infiltrates (Tanaka et al., 1994; Denton et al., 2004). These neovessels within allografts are activated, in as much as they express cell surface adhesion molecules and MHC class II. Thus, the angiogenesis reaction itself may be pro-inflammatory in as much as it has potential to mediate the recruitment and the activation of local infiltrates (Atkinson et al., 2005).

Collectively, these findings illustrate that leukocyte-induced EC proliferation/angiogenesis occurs at different sites within allografts. We propose that the abnormal angiogenic microvasculature may be causative of disease, and that local tissue hypoxia is the result, rather than the primary stimulus of the response (Jain, 2005; **Figure 1**). This paradigm explains in part how the intragraft microenvironment functions to initiate and sustain the development of chronic allograft rejection. Other major issues relate to the production of cytokines and growth factors that initiate EndMT, illustrated in **Figure 2**. This will be discussed in more detail below.

OVERLAPPING NATURE OF INFLAMMATION AND ANGIOGENESIS AND THE DEVELOPMENT OF CHRONIC REJECTION

During inflammation, the leukocyte-induced angiogenesis reaction may be associated with local areas of tissue hypoxia and tissue injury (illustrated in **Figure 1**). To this end, it is important to note that angiogenesis factors such as VEGF have been reported to be overexpressed in *all* models of chronic inflammation, including models of chronic rejection, and their expression has been found to be associated with disease progression (Leibovich et al., 1987; Folkman and Brem, 1992; Cotran, 1994; Majno, 1998; Ezaki et al., 2001; Ferrara, 2005). Consistent with this possibility, blockade of individual angiogenesis factors, including VEGF–VEGFR interactions, in animal models has been found to attenuate the progression of the chronic rejection disease process (Lemstrom et al., 2002; Nykanen et al., 2003; Reinders et al., 2003a; Denton et al., 2004; Sho et al., 2005; Malmstrom et al., 2008).

On the other hand, immune inflammation and the angiogenesis response can be antagonistic. For instance, some angiogenesis factors such as fibroblast growth factor (FGF) may inhibit adhesion molecule expression and have anti-inflammatory effects (Jain et al., 1996; Melder et al., 1996). Some inflammatory mediators such as IFNγ or the IFNγ-inducible chemokine CXCL10/IP-10 can be anti-angiogenic (Strieter et al., 1995a; Boulday et al., 2006).

Moreover, the competitive binding of chemokines to their receptors (and vice versa) results in competition for angiogenesis and inflammation (Strieter et al., 1995b). So, how is it possible that the evolution of the leukocyte-induced angiogenesis response can be associated with pro-inflammation? Neovessels at sites of angiogenesis express adhesion molecules and chemokines and can facilitate the recruitment of leukocytes in part via enhanced leukocyte–endothelial adhesion events (Melder et al., 1996; Detmar et al., 1998; Kim et al., 2001; Reinders et al., 2003a). In addition, mediators of the leukocyte-induced angiogenesis reaction, such as VEGF, induce the expression of EC adhesion molecules [including E-selectin, ICAM-1, and VCAM-1 (Melder et al., 1996; Kim et al., 2001)] and pro-inflammatory chemokines [such as CXCL10/IP-10 and MCP-1 (Marumo et al., 1999; Reinders et al., 2003a; Boulday et al., 2006)]. Also, as will be discussed below, VEGF can serve as a potent leukocyte chemoattractant via direct interactions with its receptors expressed on subsets of monocyte/macrophages and T cells (Barleon et al., 1996; Shin et al., 2009; Basu et al., 2010; Suzuki et al., 2010). Therefore, once established within allografts, the initial EC activation response that results in proliferation and angiogenesis also facilitates inflammation. In contrast, inflammatory mediators can both stimulate and inhibit angiogenesis. Thus, the balance between the relative production of pro- versus anti-angiogenic factors in the course of the immune response will determine the inducible neovascular response, resulting in vascular repair or injury, and this process may be a key determinant of the outcome of rejection.

Collectively, these observations suggest that the overlapping nature of inflammation and angiogenesis create an environment that is critical to shape the rejection process. They also imply that pharmacologic manipulation of the EC response to injury or leukocyte-induced angiogenesis will target the pathological intragraft microenvironment and interrupt chronic rejection. Indeed, in animal models, several angiogenesis inhibitors have been reported to slow the progression of chronic rejection (Lemstrom et al., 2002; Denton et al., 2004; Reinders et al., 2006; Malmstrom et al., 2008). PTK787, a selective VEGFR protein tyrosine kinase angiogenesis inhibitor was found to attenuate the development of interstitial fibrosis and allograft vasculopathy in well-established rat cardiac and renal transplantation models (Lemstrom et al., 2002; Malmstrom et al., 2008). Also, TNP-470, a synthetic fumagillin derivative and a well-established angiogenesis inhibitor was found to interrupt the progression of inflammation, intragraft fibrosis, and the degree of allograft vasculopathy in the Fischer 344 into Lewis rat cardiac allograft model (Denton et al., 2004). Furthermore, we find that endostatin, another well-established angiogenesis inhibitor prevents the progression of allograft vasculopathy in the MHC class II mismatched B6.C-H2^{bm12} into C57BL/6 mouse model of chronic rejection (Contreras and Briscoe, unpublished observations). Therefore, it appears that transient interruption therapy with angiogenesis inhibitors has potential to normalize the vasculature and inhibit the progression of chronic rejection.

To this end, it is important to note that the mTOR kinase and its associated signaling pathway has profound effects on EC proliferation *in vitro* and *in vivo* (Dormond et al., 2007; Contreras et al., 2008). We believe that mTOR inhibitors represent the first-in-kind angiogenesis inhibitor agents that are currently being used therapeutically in humans following transplantation. Furthermore, we suggest that this biological effect may account for some of their ability to retard the progression of chronic rejection (Contreras et al., 2008). Several reports have indicated that a switch from calcineurin inhibitor therapy to an mTOR inhibitor based regimen (interruption protocol) in humans slows the progression of chronic rejection (Oberbauer et al., 2005; Schena et al., 2009; Arora et al., 2011). These observations support the possibility that angiogenesis inhibitors have potential as therapeutics in the future.

ENDOTHELIAL CELL TO MESENCHYMAL TRANSITION: POSSIBLE FINAL END RESULT OF LEUKOCYTE-INDUCED OR PATHOLOGICAL ANGIOGENESIS

The Kalluri group demonstrated that EC within cardiac allografts appear to undergo a process of mesenchymal transition to fibroblasts in association with inflammation and chronic rejection (Zeisberg et al., 2007a). This process, called EndMT by several laboratories results from the dedifferentiation of EC, such that they lose their endothelial phenotype and gain the expression of mesenchymal markers (illustrated in **Figure 2**). In this manner, the process of EndMT is characterized by the loss of well-established EC molecules including CD31 and CD34, and the gain in expression of fibroblast-specific protein 1 (FSP1), alpha-smooth muscle actin (Zeisberg et al., 2007a,b), as well as other non-EC molecules (Kokudo et al., 2008). *In vitro*, it is reported, that EndMT occurs in response to both TGFβ1 (Arciniegas et al., 1992) and TGFβ2 (Medici et al., 2010), but the stimuli for mesenchymal transition *in vivo* are poorly understood. The process of EndMT occurs in association with pericyte loss, when EC are denuded from their basement membrane. Denuded EC and pericytes migrate into the surrounding tissue, where they are exposed to TGFβ that may be produced locally by multiple cell types and/or delivered into the graft by monocyte/macrophages (Jain et al., 2002; Booth and Bishop, 2010), thus leading to their differentiation into fibroblasts (illustrated in **Figure 2**).

Endothelial-to-mesenchymal transition is well-established to occur during embryonic development of the heart, in normal wound healing and in several different cancers (Markwald et al., 1975; Zeisberg et al., 2007b). It has also been implicated in chronic fibrotic disease states including atherosclerosis, pulmonary hypertension, cardiac fibrosis, and diabetic nephropathy (Zeisberg et al., 2007a; Li et al., 2009; Hashimoto et al., 2010). In cancer, EndMT has been reported to account for up to 40% of cancer-associated fibroblasts, and the process has been found to alter the microenvironment in several ways (Zeisberg et al., 2007b). EC that have undergone EndMT produce collagen, deposit extracellular matrix molecules, and secrete pro-fibrotic factors including TGFβ, leading to a self-perpetuating cycle of events. In contrast, its role in allograft rejection has not been well characterized, and it remains controversial whether EndMT is primarily related to pericyte loss and their migration into the local tissue, or whether it is truly related to EC dedifferentiation (Humphreys et al., 2010). Regardless of whether the pericyte and/or the EC dedifferentiates into the collagen-producing cell, the process, and long-term consequence of microvascular destruction is fibrosis.

Since the intragraft microenvironment has similarities to the tumor microenvironment (i.e., abnormal microvessels, local hypoxia, TGFβ, high VEGF, mononuclear cell infiltrates), we have speculated that pathological and/or leukocyte-induced angiogenesis will precede EndMT within allografts (illustrated in **Figure 2**). Consistent with this possibility, proliferating EC have been shown to be associated with alloimmune targeting and chronic rejection (Sis et al., 2009; Osasan et al., 2011). In addition, we have found that proliferating EC are most susceptible to EndMT *in vitro* in the presence of inflammatory cytokines, TGFβ, and hypoxia. In contrast, we find that EC that have not undergone multiple rounds of proliferation are not as susceptible to EndMT (Woda and Briscoe, unpublished observations). It will be important to understand if intragraft cytokines or growth factors that activate EC can initiate this pathological process and how VEGF functions in the EndMT process. An alternate hypothesis is that the intragraft cytokine milieu and/or hypoxia within allografts at times of leukocyte-induced angiogenesis alters pericyte biology which becomes a central determinant of both the loss of the microvasculature and differentiation into fibroblasts. Thus, there is a need for research in this area as it is a potential target of future therapeutics.

VASCULAR ENDOTHELIAL GROWTH FACTOR: A CENTRAL PLAYER IN THE CHRONIC REJECTION INTRAGRAFT MICROENVIRONMENT

By definition, the development of acute allograft rejection involves a marked inflammatory reaction, characterized by the recruitment of leukocytes and an intense cellular and humoral attack on the graft (Ingulli et al., 2009). By contrast, chronic rejection is a more insidious process involving delayed type hypersensitivity mechanisms and is characterized by mononuclear cell infiltration, an obliterative vasculopathy, and progressive fibrosis (Libby and Pober, 2001; Ingulli et al., 2009). Over the past 10–15 years, VEGF has emerged as an important player in the rejection process, and its expression has been reported by several groups to be associated with both acute and chronic allograft rejection (Torry et al., 1995; Pilmore et al., 1999; Lemstrom et al., 2002; Reinders et al., 2003b, 2006; Malmstrom et al., 2008). In one study (Pilmore et al., 1999), VEGF expression was found to be most striking in the interstitium of human renal allografts in association with CD68$^+$ monocyte/macrophage infiltrates and evidence of chronic rejection. In another study (Torry et al., 1995), the expression of VEGF was found to be associated with fibrin deposition, and was confined to areas with monocyte/macrophage infiltrates. In our own analyses, we observed intense VEGF expression within human cardiac allografts localized to both inflammatory cell infiltrates as well as to vascular EC in association with acute and chronic rejection (Reinders et al., 2003b). Moreover, we found that persistent intragraft VEGF overexpression identified patients at high risk for the development of chronic allograft vasculopathy/chronic rejection (Reinders et al., 2003b). Furthermore, human transplant recipients with genotypes encoding high VEGF production are at increased risk for the development of rejection (Shahbazi et al., 2002; Girnita et al., 2008). Levels of VEGF increase significantly in the serum and urine of patients in association with cardiac and renal allograft rejection

respectively, and in most cases return to baseline after effective treatment of the rejection episode (Abramson et al., 2002; Peng et al., 2008). It is thus possible that serum levels of VEGF may serve as a reliable biomarker of the development of allograft vasculopathy following human cardiac transplantation (Daly et al., 2011).

VEGF may be delivered into allografts in the course of rejection by infiltrating monocytes and by activated T cells (Leibovich et al., 1987; Leibovich and Wiseman, 1988; Freeman et al., 1995; Melter et al., 2000). Alternatively, VEGF may be induced locally within the allograft as a result of cellular interactions among activated platelets and EC (Chiodoni et al., 2006; Dormond et al., 2008). Nevertheless, the local overexpression of VEGF within allografts results in the development of chronic rejection and allograft vasculopathy (Lemstrom et al., 2002). Taken together, there is extensive data to support the hypothesis that VEGF is mechanistic in the process of chronic allograft rejection.

The major stimulus for VEGF expression is hypoxia (Shweiki et al., 1992; Mukhopadhyay et al., 1995; Goel et al., 2011), but other factors that can upregulate local tissue VEGF production include paracrine effects of hormones, glucose and prostaglandins, as well as cytokine/growth factor modulators of protein kinase C, nitric oxide, and stimulators of adenylate cyclase (Brown et al., 1997). Several cytokines, including IL-1, TNF, and IL-6, have been found to induce the expression of VEGF and/or VEGF receptors (Leibovich et al., 1987; Giraudo et al., 1998; Amano et al., 2004; Huang et al., 2004). In addition, we have demonstrated that the ligation of CD40 by CD154 (CD40 ligand, expressed by activated platelets and T cells) is potent for local tissue induction of VEGF expression (Melter et al., 2000; Dormond et al., 2008). In this manner, it is not surprising that cell-mediated immune inflammation is associated with VEGF–VEGFR biological responses. Furthermore, since multiple VEGF-inducing factors are present within allografts at different times post transplantation, one might conclude that VEGF expression, and VEGF-dependent biological responses should be characteristic features of both the initiation and the maintenance of chronic rejection.

BIOLOGY OF VEGF–VEGFR INTERACTIONS

As its name suggests, VEGF classically functions as a potent angiogenesis factor, and as such it was originally proposed to facilitate microvascular repair following ischemic injury (Kang et al., 2001a,b; Mayer, 2011) as well as injury following inflammatory insults (Choi et al., 2000; Reinders et al., 2006). VEGF is a 45-kDa protein produced by most cell types including cells of the immune system such as monocyte/macrophages and activated T cell subsets (Freeman et al., 1995; Brown et al., 1997; Polverini, 1997; Melter et al., 2000; Basu et al., 2010). It is a heparin-binding homodimeric glycoprotein with several protein variants of 206, 189, 164, 145, and 121 amino acids that arise from the alternative splicing of a single gene (Leung et al., 1989; Tischer et al., 1991). Intense research beyond the scope of this review, has clarified the function of VEGF in EC, where it mediates migration, growth, survival as well as activation responses including the expression of adhesion molecules and chemokines (Alon et al., 1995; Klagsbrun and D'Amore, 1996; Melder et al., 1996; Brown et al., 1997; Gerber

et al., 1999; Kim et al., 2001; Ferrara, 2005; Boulday et al., 2006; Lee et al., 2007).

The biological activities of VEGF are mediated via interactions with its receptors, Flt-1 (VEGF receptor 1), KDR (VEGF receptor 2), and neuropilin-1 (Shalaby et al., 1995; Klagsbrun and D'Amore, 1996; Brown et al., 1997; Miao and Klagsbrun, 2000; Ferrara, 2005; Takahashi and Shibuya, 2005; Matsumoto and Mugishima, 2006). Several studies have shown that signaling mediated via KDR is critical for the VEGF-induced response (Zeng et al., 2001). For instance, many of the biological properties of VEGF can be inhibited by neutralization of KDR; and inhibition of KDR has similar effects as neutralization of VEGF *in vivo* in inflammatory diseases (Brown et al., 1997; Watanabe et al., 2004, 2005; Ferrara, 2005), including allograft rejection (Reinders et al., 2003a; Sho et al., 2005). Also, knockout of either VEGF or KDR results in embryonic lethality due to inhibition of angioblast differentiation and vasculogenesis (Shalaby et al., 1995; Ferrara et al., 1996). Classically, it is thought that neuropilin-1 serves as an accessory co-receptor for KDR to bind VEGF and mediate crosslinking to KDR (Klagsbrun and D'Amore, 1996; Matsumoto and Mugishima, 2006). However, some studies have suggested that neuropilin-1 might also mediate signaling directly in response to the semaphorin 3 family of proteins (Wang et al., 2003; Catalano et al., 2006; Mizui et al., 2009; Suzuki et al., 2008). VEGF also signals through Flt-1 to initiate a direct signaling response, but its interaction with Flt-1 may inhibit KDR-induced responses in EC (Zeng et al., 2002). Thus, VEGF inducible responses in different cells can be determined according to the profile of expression of its receptors and thus, the select VEGFR-dependent signal.

All VEGF receptors are expressed by EC, but individual receptors are also expressed by different leukocyte subsets indicating that VEGF may have direct effects on the immune response (Barleon et al., 1996; Bruder et al., 2004; Sarris et al., 2008; Shin et al., 2009; Basu et al., 2010; Suzuki et al., 2010). Flt-1 and neuropilin-1 are expressed by human monocytes and APCs (Barleon et al., 1996; Romeo et al., 2002; Bourbie-Vaudaine et al., 2006; Chapoval et al., 2009), and VEGF is known to induce activation responses and chemotactic activity in monocytes in part via interactions with Flt-1 (Barleon et al., 1996; Clauss et al., 1996; Laxmanan et al., 2005; Chapoval et al., 2009). VEGF–VEGFR interactions also promote the differentiation of monocytes into pro-inflammatory (Chapoval et al., 2009) or immunoregulatory (Gabrilovich et al., 1998; Laxmanan et al., 2005) APCs, but its effect is likely dependent on individual VEGFR(s) expressed by the APC (Laxmanan et al., 2005; Chapoval et al., 2009).

Moreover, several recent studies have indicated that T cell subsets can express VEGFRs, including Flt-1, KDR, and neuropilin-1 (Dias et al., 2000; Tordjman et al., 2002; Mor et al., 2004; Sarris et al., 2008; Basu et al., 2010; Edelbauer et al., 2010; Ziogas et al., 2011). In our studies (Basu et al., 2010; Edelbauer et al., 2010), we find low negligible levels of expression of all VEGFRs on freshly isolated populations of unactivated $CD4^+$ and $CD8^+$ T cells. However, we have observed that the expression of Flt-1 and KDR increase following mitogen-dependent activation. In addition, we have found high levels of KDR on memory $CD45RO^+$ populations of $CD4^+$ T cells (Basu et al., 2010). In general, KDR and Flt-1 are reported to be the dominant VEGFRs expressed on

T effector cells (Basu et al., 2010; Edelbauer et al., 2010; Zhang et al., 2010; Ziogas et al., 2011). In contrast, it is reported that neuropilin-1 is selectively expressed at high levels on populations of $CD4^+CD25^+FoxP3^+$ T regulatory cells (Bruder et al., 2004; Sarris et al., 2008). One study indicated that T regulatory cells may also express KDR (Suzuki et al., 2010). Neuropilin-1 has also been reported to be expressed on populations of human naïve T cells, where it functions to support the initiation of T cell activation in primary immune responses (Tordjman et al., 2002).

Collectively, these findings support the possibility that intragraft VEGF may interact with different T cell subsets in the course of the rejection process. VEGF may function as a potent chemoattractant for effector and memory $CD4^+$ and $CD8^+$ T cells, and may thus promote inflammation in association with acute and chronic allograft rejection. Consistent with this possibility, the blockade of VEGF or VEGFR interactions with T cells inhibits intragraft lymphocyte trafficking (Reinders et al., 2003a; Sho et al., 2005; Edelbauer et al., 2010; Zhang et al., 2010) as well as reactivation responses (Basu et al., 2010) in human model systems. In experimental animal models, the local overexpression of VEGF, and VEGF–VEGFR interactions also facilitate lymphocyte trafficking (Reinders et al., 2003a; Lee et al., 2004; Kim et al., 2009; Edelbauer et al., 2010; Zhang et al., 2010) and activation including the augmentation of Th1 (Chapoval et al., 2009), Th2 (Lee et al., 2004), and Th17 (Kim et al., 2009) effector responses. The mechanism(s) by which local tissue VEGF elicits signals for migration and activation/reactivation responses is the subject of ongoing investigations.

PRO-INFLAMMATORY EFFECTS OF VEGF–VEGF RECEPTOR INTERACTIONS

As discussed above, VEGF may act as a pro-inflammatory cytokine *in vivo* in several chronic diseases (Fava et al., 1994; Koch et al., 1994; Duh and Aiello, 1999; Ferrara and Alitalo, 1999; Griga et al., 1999; Hoshino et al., 2001; Kanazawa et al., 2001; McDonald, 2001; Walsh and Pearson, 2001; Kim et al., 2009; Detoraki et al., 2010), and its ability to function as a pro-inflammatory cytokine in part relates to its interactions with its receptors expressed on leukocytes (Barleon et al., 1996; Shin et al., 2009; Basu et al., 2010; Edelbauer et al., 2010; Suzuki et al., 2010; Zhang et al., 2010). Its ability to interact directly with monocytes (via Flt-1), NK T cells, and $CD3^+$ T cells (via Flt-1 and KDR) to facilitate chemotactic activity is likely of great pathophysiological importance in rejection (Edelbauer et al., 2010; Zhang et al., 2010). In addition, VEGF induces the expression of adhesion molecules and chemokines in EC (Melder et al., 1996; Marumo et al., 1999; Kim et al., 2001; Reinders et al., 2003a; Boulday et al., 2006), and classically enhances vascular permeability (Brown et al., 1997; Basu et al., 2001). All of these events are characteristic of acute and chronic inflammation (Cotran, 1994).

In models of chronic rejection, it was demonstrated that overexpression of VEGF mobilizes bone marrow derived monocyte/macrophages and accelerates the development of allograft vasculopathy (Lemstrom et al., 2002; Zhao et al., 2002). Further, it was found that blockade of VEGF receptor 2 (KDR) signaling decreases monocyte recruitment into vascular lesions and attenuates the development of graft arteriosclerosis (Lemstrom et al.,

2002; Zhao et al., 2002). We found that KDR is expressed on CD3$^+$ infiltrates within rejecting human allografts *in vivo*, and that both anti-VEGF and anti-KDR antibodies inhibit the transmigration of CD4$^+$ and CD8$^+$ T cells across activated EC using an *in vitro* live-time transmigration model (Edelbauer et al., 2010). Using a huSCID mouse model of lymphocyte trafficking, we also demonstrated that KDR-expressing lymphocytes migrate into human skin *in vivo*, and that migration is reduced in mice treated with a blocking anti-VEGF antibody (Edelbauer et al., 2010). Collectively, these observations demonstrate that induced expression of KDR on subsets of T cells, and locally expressed VEGF facilitate lymphocyte chemotaxis. They support a model whereby intragraft VEGF mediates the localization of T cells in association with chronic rejection.

In another recent report, Zhang et al. (2010) used an anti-human VEGF antibody to study the effect of VEGF blockade on the development of allograft vasculopathy in a humanized model in SCID mice. They found that anti-VEGF inhibited intragraft accumulation of T cells without affecting T cell activation. In addition, they observed that anti-VEGF treatment inhibited neointimal formation within human coronary artery grafts in the humanized mouse. The authors suggested that the T cell chemoattractive effect of VEGF was mediated in part via interactions with a subpopulation of Flt-1-expressing CD3$^+$ T cells. Thus, intragraft VEGF may also contribute to vascular remodeling and allograft vasculopathy by enhancing T cell recruitment into the intima of large vessels.

Collectively, these observations indicate that VEGF has potent pro-inflammatory properties under pathological conditions in association with the development of chronic rejection and allograft vasculopathy.

OVERALL SUMMARY AND PERSPECTIVE

In this review, we have discussed a paradigm where leukocyte-induced angiogenesis, local tissue hypoxia, and the overexpression of VEGF sustain an intragraft microenvironment that fosters the development of chronic allograft rejection. We have also defined EC-based events within the allograft microenvironment that are associated with chronic rejection. The first are changes in the microcirculation resulting from destruction of the microvasculature. The second are changes in the microcirculation resulting from EC proliferation and leukocyte-induced angiogenesis. Both events are likely to disrupt normal blood flow patterns within the graft and result in local areas of tissue hypoxia. In addition,

EC undergoing proliferation express adhesion molecules and chemokines that support pro-inflammation, providing another mechanism whereby the angiogenesis reaction may sustain ongoing tissue injury. A third intragraft determinant of chronic disease is the development of EndMT. While the mechanism underlying EndMT is controversial, there is sufficient evidence to suggest that inflammation and EC proliferation is associated with pericyte loss, which likely precedes microvascular capillary loss and the development of fibrosis. Whether the pericyte or the EC, or both, ultimately dedifferentiate into collagen-producing cells is being debated. Nevertheless, it has been reported that cytokines and growth factors, including TGFβ (Booth and Bishop, 2010), can accelerate this fibrotic reaction. Although beyond the scope of this review, TGFβ may also be a key aspect of the chronic rejection intragraft microenvironment (Jain et al., 2000). Finally, we propose that a fourth and key determinant of chronic rejection is intragraft overexpression of VEGF, which functions to facilitate both inflammation and pathological EC proliferation/angiogenesis. While VEGF may be delivered into allografts by inflammatory infiltrates, such as monocytes and activated T cells, the immune response can also elicit the local overproduction of VEGF within the graft. Overall, in this review we provide insight into novel aspects of the intragraft microenvironment that contribute to the development of chronic rejection and long-term attrition of allografts following transplantation. The importance of this paradigm is that it identifies key areas for future therapeutic targeting to prevent the progression of chronic rejection following solid organ transplantation.

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