



Impacts of Ionizing Radiation on the Different Compartments of the Tumor Microenvironment

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Radiotherapy (RT) is one of the most important modalities for cancer treatment. For many years, the impact of RT on cancer cells has been extensively studied. Recently, the tumor microenvironment (TME) emerged as one of the key factors in therapy resistance. RT is known to influence and modify diverse components of the TME. Hence, we intent to review data from the literature on the impact of low and high single dose, as well as fractionated RT on host cells (endothelial cells, fibroblasts, immune and inflammatory cells) and the extracellular matrix. Optimizing the schedule of RT (i.e., dose per fraction) and other treatment modalities is a current challenge. A better understanding of the cascade of events and TME remodeling following RT would be helpful to design optimal treatment combination.

Keywords: radiotherapy, tumor microenvironment, angiogenesis, hypoxia, inflammation, cancer-associated fibroblasts, treatment combination

INTRODUCTION

A human tumor is a complex tissue composed of malignant cells and stromal cells including endothelial cells, inflammatory cells, immune cells and fibroblasts-like cells embedded in an extracellular matrix (ECM). These cellular and extracellular components of the tumor microenvironment (TME) not only regulate different steps of cancer progression (Ribatti et al., 2006; Mandani et al., 2008; Hanahan and Weinberg, 2011), but also play a pivotal role in therapeutic efficacy (Klemm and Joyce, 2015). Radiotherapy (RT) is considered as a corner stone of cancer treatment, and more than 50% of cancer patients will experiment RT at least once during their treatment. RT can be applied alone in a curative intent or associated with chemotherapy and/or surgery performed before or after RT. High energy photons (X-rays) used in RT sparsely deposit their energy along their track. Due to physical properties of these ionizing radiations, direct events on the DNA (i.e., Double Strand Break) can be considered as rare. Most of the energy deposit occurs in water and the produced radiolysis ends up with free radical formation: Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). ROS and RNS will subsequently activate several cascades and cellular processes by oxidation of molecular targets including kinases, phosphatases, cell cycle regulators, cell membrane and lipids leading to cell function dysregulation (Wu, 2006; Corre et al., 2010). These free radicals also target the DNA leading to single and double strand breaks (**Figure 1**). As RT has a non-specific effect, triggering both tumor and host cells (Valerie et al., 2007), the logical consequence is that it exerts effects beyond the simple destruction of cancer cells (Feys et al., 2015).

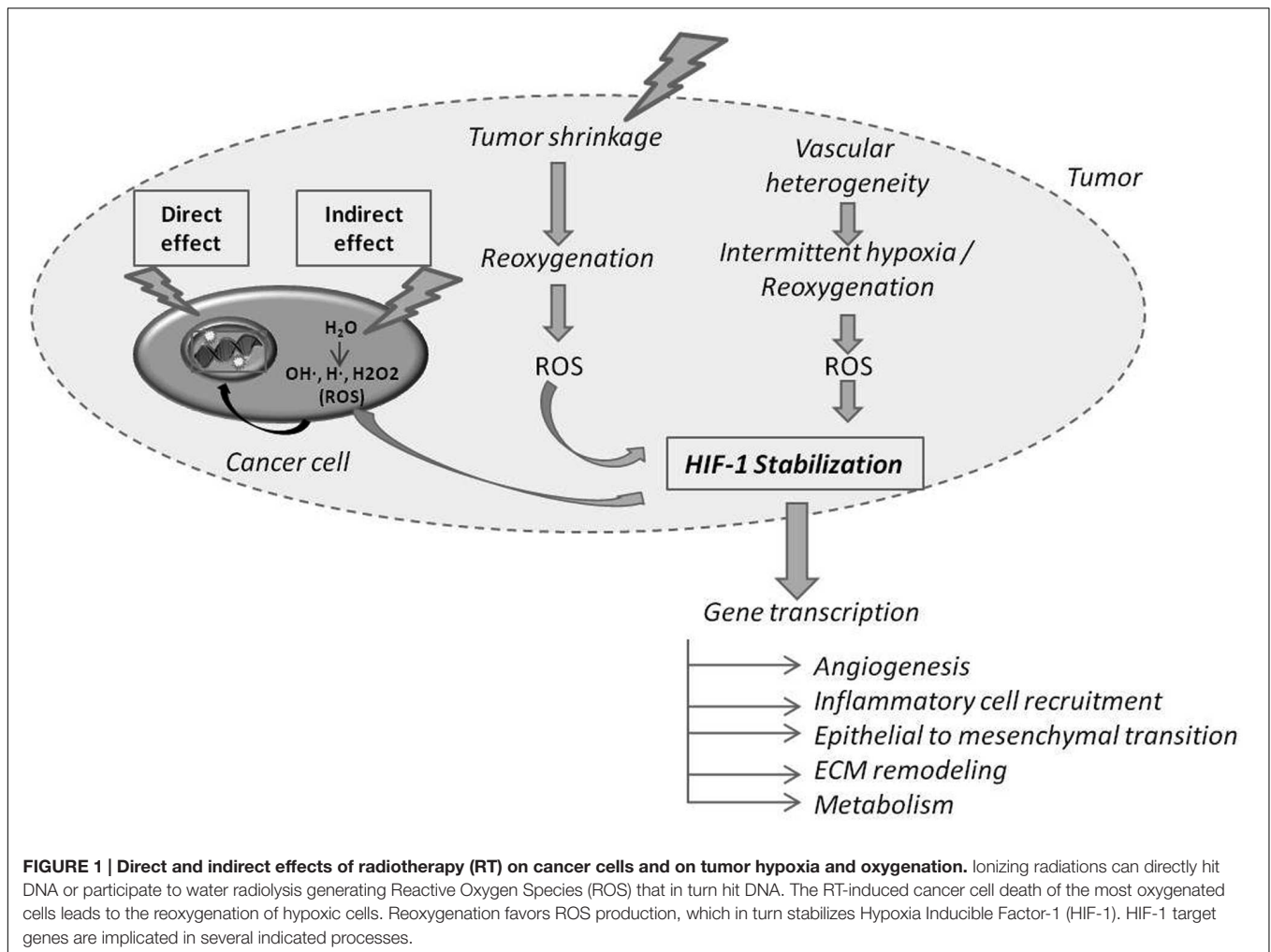


FIGURE 1 | Direct and indirect effects of radiotherapy (RT) on cancer cells and on tumor hypoxia and oxygenation. Ionizing radiations can directly hit DNA or participate to water radiolysis generating Reactive Oxygen Species (ROS) that in turn hit DNA. The RT-induced cancer cell death of the most oxygenated cells leads to the reoxygenation of hypoxic cells. Reoxygenation favors ROS production, which in turn stabilizes Hypoxia Inducible Factor-1 (HIF-1). HIF-1 target genes are implicated in several indicated processes.

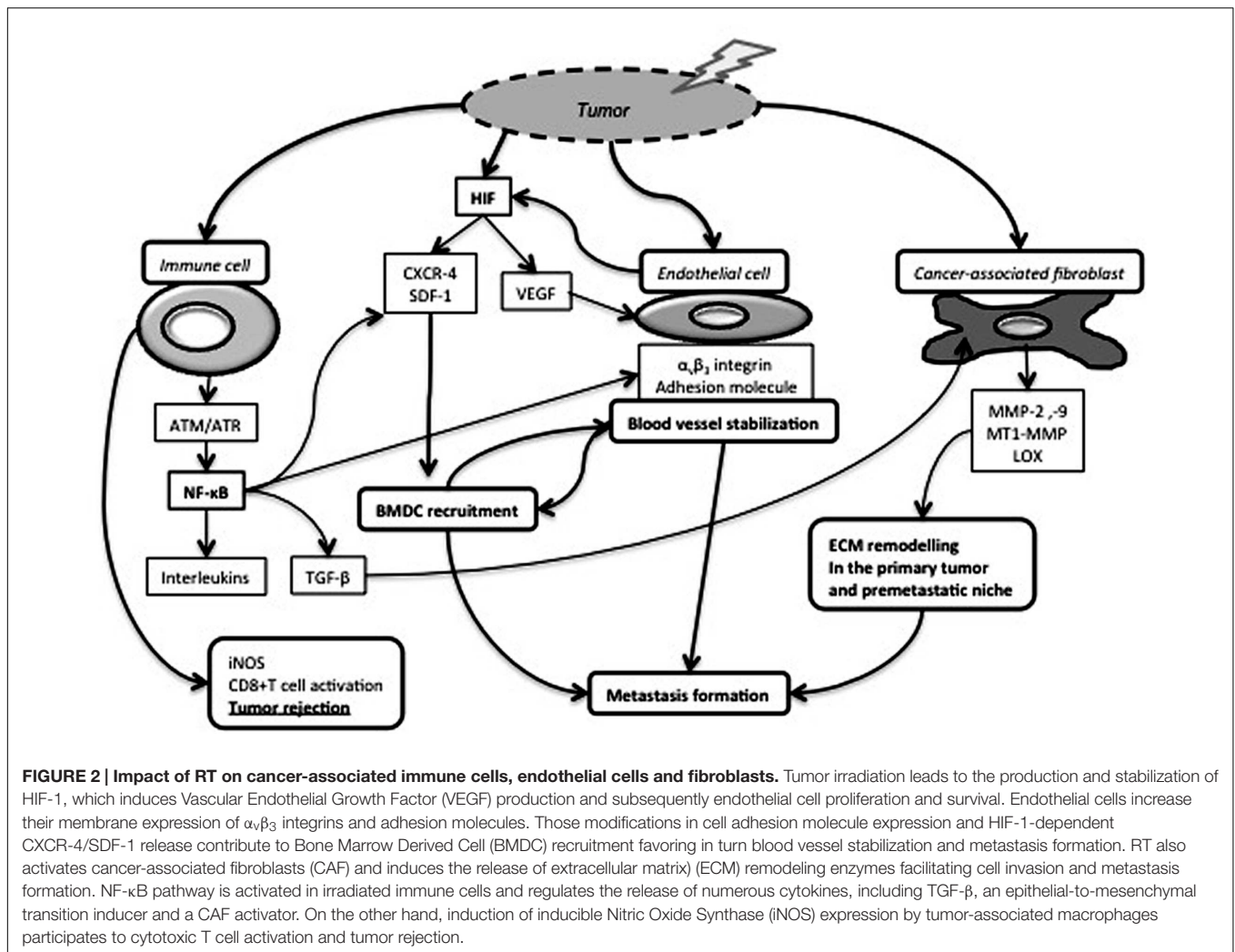
Recent understanding that distinct stromal cell types might have tumor-promoting or tumor-suppressing capabilities (Özdemir et al., 2014) led to an even more complex picture of the tumor ecosystem and its putative impact on therapy outcome. Moreover, intriguing clinical and experimental observations reveal that the timing of surgery treatment after RT influences metastasis occurrence and patient overall survival, suggesting the implication of TME remodeling in treatment efficacy (Coucke et al., 2006; Pajonk et al., 2010; Marie-Egyptienne et al., 2013; Leroi et al., 2015). In this review, we will focus on how RT affects TME components such as the ECM, blood vessels, inflammatory and immune cells (Figure 2).

RECIPROCAL DYNAMICS BETWEEN RADIOTHERAPY AND TUMOR VESSELS

Tumor blood vessels are recognized as major actors in tumor development at least through an active and passive exchange of nutrients, waste and gas (oxygen and CO_2) between the blood stream and tumor compartment. Therefore, any modifications of these exchanges can profoundly impact the tumor phenotype.

RT can affect endothelial cells directly or indirectly by inducing several cascades of events through ROS or RNS productions. RT can also indirectly impact tumor blood vessel homeostasis through the release and modification of several messengers by the tumor, which secondarily modify endothelial cell phenotype.

Garcia-Barros et al. (2003) first highlighted that microvascular radiosensitivity also influences tumor response. Membrane signaling, and especially acid sphingomyelinase/ceramide pathway, are strongly implicated in endothelial cell apoptosis after high dose RT (Corre et al., 2013). Proangiogenic factors, such as bFGF and VEGF, rapidly repress IR-induced ceramide generation, and subsequently endothelial apoptosis. Thus, combining anti-angiogenic drugs and RT would be relevant (Rao et al., 2014). On the other hand, RT leads to rapid phosphorylation of several signaling proteins (i.e., Akt and ERK) and VEGFR2, responsible of endothelial cell survival and migration (Gorski et al., 1999; Gille et al., 2001; Sofia Vala et al., 2010; Yu et al., 2012). RT also participates to endothelial activation through up-regulation of $\alpha_v\beta_3$ integrin (Abdollahi et al., 2005) and adhesion molecule expression (i.e., E-selectin, P-selectin, I-CAM, V-CAM). It is also worth mentioning that



RT promotes bone marrow-derived cell recruitment (**Figure 2**) (Mihaescu et al., 2007). These cells can trans-differentiate into pericytes associated to tumor blood vessels and contribute to endothelial cell radioresistance to fractionated RT (Zong et al., 2008; Lerman et al., 2010). Through vasculogenesis, CD11+ cells also participate to post-RT vasculature recovery (**Figure 2**) (Martin, 2013). RT promotes endothelial nitric oxide synthase (eNOS) expression and activation leading to NO production and finally angiogenesis and increased tumor blood flow (Sonveaux et al., 2003). Increased eNOS mRNA levels are observed after RT in human head and neck squamous cell carcinomas (Sonveaux et al., 2003). These post-RT changes of tumor vasculature are worth considering to enhance drug delivery and design treatment modalities (Sonveaux et al., 2007).

The effect on endothelial cells depends on the dose per fraction. At a clinical single dose of 2Gy, endothelial cell survival is favored through miRNA (miR-189 and miR-20a) upregulation (Wagner-Ecker et al., 2010). High doses (above 10Gy) are more likely to induce endothelial cell apoptosis and tumor vessel collapse (Park et al., 2012; Song et al., 2015). This could explain

the clinical efficacy of Stereotactic Body Radiotherapy Treatments (SBRT) using high fractional dose. With intermediate doses (5-10Gy), tumor vessel normalization and dilatation are observed and associated with reduced vascular leakage and increased tumor oxygenation (Sonveaux et al., 2002; Crockart et al., 2005a).

Radiotherapy is prompt to kill the most oxygenated tumor cells thereby inducing tumor shrinkage and subsequently the perfusion and reoxygenation of initial hypoxic tumor areas (**Figure 1**) (Crockart et al., 2005a; Dewhirst et al., 2008). The reoxygenation phase following RT participates to transcriptional regulation and stabilization of HIF-1 α through ROS (Kedersha et al., 1999; Moeller et al., 2004, 2005; Dewhirst et al., 2008). One direct consequence of HIF-1 and downstream target activation (i.e., PI3K/Akt, MEK/ERK and NF- κ B pathways) by RT is the release of endothelial cell-derived radioprotective growth factors (VEGF and bFGF) minimizing vascular damages (Gorski et al., 1999; Kedersha et al., 1999; Moeller et al., 2004, 2005; Dewhirst et al., 2008; Sofia Vala et al., 2010; Yu et al., 2012). Interestingly, the cascade of reperfusion/reoxygenation following RT displays some similarities with intermittent hypoxia, which is a source

of resistance to treatments (Martinive et al., 2006, 2009) (Figure 1). Moreover, RT-induced tumor cell death promotes post-irradiation angiogenesis through a caspase 3-dependent mechanism (Feng et al., 2015).

Although RT impact on tumor blood vessels is extensively studied, little is known with discrepancy results about its effect on lymphatic endothelial cells. *In vitro*, VEGF-C radiosensitizes lymphatic endothelial cells (Kesler et al., 2014). A single dose of 20Gy does not seem to alter lymphatic vessels (Pastouret et al., 2014). However, a single dose irradiation (14Gy) of murine lung tissue impairs lymphatic vasculature, progressively leading to lung fibrosis (Cui et al., 2014). In skin biopsies from irradiated breast cancer patients, similar numbers of lymphatic vessels were detected in irradiated and non-irradiated sites (Russell et al., 2015). These observations suggest a differential RT effect on blood and lymphatic endothelial cells that warrant further investigation.

RADIOTHERAPY AND INFLAMMATORY SIGNALS

The link between RT and immunity is elegantly described in recent reviews (Frey et al., 2014; Barker et al., 2015; Derer et al., 2015), which highlight the importance of the chronology between RT and immunotherapy. Here, we will focus on post-RT inflammatory in the TME.

By immuno-modulatory effects, low doses RT (<1Gy) can be used as an anti-inflammatory treatment. Following low dose RT, the secretion of transforming growth factor β 1 (TGF- β 1), the local induction of apoptosis rather than necrosis, the decreased E-selectin expression on endothelial cell surface and the proteolytic shedding of L-selectin, altogether hamper peripheral blood mononuclear cell (PBMC) adhesion to the endothelium and subsequently inflammation. Moreover, decreased expression of interleukin- 1β (IL- 1β), tumor necrosis factor- α (TNF- α) and inducible nitric oxide synthase (iNOS) activity in stimulated macrophages maintain an anti-inflammatory microenvironment (Rodel et al., 2012). In contrary, clinical irradiation doses (\geq 2Gy) are known to activate inflammatory pathways in different cell types, including endothelial, immune cells and senescent fibroblasts (Mantovani et al., 2008). Furthermore, RT-induced cell death has also immunological consequences through macrophage and dendritic cell activation (Lauber et al., 2012).

Radiotherapy can initiate inflammatory cascades by two main pathways: the nuclear and cytoplasmic pathways. The first one refers to signaling events consecutive to RT-induced DNA damage. The two main effectors of DNA damage repair pathways are ataxia-telangiectasia mutated (ATM) and ATR (ATM and RAD3-related) kinases. Activated ATM can trigger NF- κ B dimer activation and nuclear translocation (Wu and Miyamoto, 2007; Lavin, 2008). The cytoplasmic pathway refers to ROS-induced inactivation of phosphatases leading to the activation of Ras-Raf-MAPK and PI3K/Akt cascades. These latter also induce the expression of many genes implicated

in inflammation including interleukins (IL- 1α and β , IL-6, TNF α , TGF- β), adhesion molecules (I-CAM, V-CAM, E-selectin), chemokines [CCL-5, SDF1 (CXCL12)/CXCR-4] and anti-apoptotic factors (Bax and Bcl-2) (Criswell et al., 2003; Zong et al., 2008).

Bone marrow-derived cell recruitment (especially CD11b⁺ cells) following RT is largely reported in different *in vivo* models and cancer types (Vatner and Formenti, 2015). It involves mainly SDF-1/CXCR-4 (Kioi et al., 2010) and CSF-1/CSF-1R (Xu et al., 2013) pathways. The inhibition of CD11b⁺ cell recruitment through different approaches (i.e., CXCR-4 or SDF-1 inhibition) impairs tumor regrowth after single dose or fractionated RT in rat glioblastoma model and in murine prostate cancer model (Chen et al., 2013; Liu et al., 2014). CD11b⁺ cells can differentiate into endothelial cells but are also an important source of macrophages. Accordingly, SDF-1/CXCR-4 inhibition prevents macrophage infiltration and tumor regrowth after RT (Kozin et al., 2010). Macrophages are the main inflammatory cells infiltrating tumor and their role in tumor growth and dissemination depends on their polarization (M1 vs. M2) (Condeelis and Pollard, 2006). Briefly, M1 macrophages are pro-inflammatory, have a high level of iNOS production and are considered to exert anti-tumor effects. In contrast, the M2 phenotype is described as anti-inflammatory, pro-angiogenic and pro-metastatic (Hanada et al., 2000; Mantovani et al., 2002). While the TME is recognized to affect macrophage differentiation (Weigert and Brune, 2008), the RT impact on macrophage differentiation is not well understood and is still controversial (Lambert and Paulnock, 1987; Shan et al., 2007). M2-like macrophages are preferentially attracted in hypoxic areas (Movahedi et al., 2010), in which M2 macrophage activity is fine-tuned (Laoui et al., 2014). Single high dose or fractionated doses seem to favor M2 phenotype in astrocytoma, glioma and prostate cancer models (Tsai et al., 2007; Chiang et al., 2012). On the other hand, conventional daily irradiation dose of 2Gy has been shown to convert M2-like to M1-like TAMs in melanoma xenograft model and in human pancreatic cancers. The resulting iNOS expression is responsible for vascular normalization, T cell recruitment and activation and finally tumor rejection (Klug et al., 2013). The *in vitro* exposure of THP-1 monocyte-derived macrophages to low RT doses increases IL- 1β secretion in a NF- κ B dependent manner, leading to an anti-inflammatory cascade (Lödermann et al., 2012). Macrophages are important NO homeostasis regulators by their differential expression of HIF- α isoforms (Takeda et al., 2010). In the presence of activated macrophages, NO is a powerful radiosensitizer for hypoxic tumor cells by inhibiting cellular respiration, which leads to oxygen sparing (De Ridder et al., 2003, 2004, 2006, 2008; Jiang et al., 2010).

NK cell mobilization following neoadjuvant RT appears crucial (Leroi et al., 2015). Indeed, TME remodeling and NK cell mobilization occurring between RT and surgery impacts the metastatic spreading. These data are in line with previous clinical data reporting that the timing of surgery following RT influences patient overall survival (Coucke et al., 2006). Interestingly, combining RT with an immunotherapy approach

that triggers NK cells appears relevant, but only when RT is applied before immunotherapy (Rekers et al., 2015).

In total-body irradiation model, langerhans cells, antigen presenting cells, resist to high dose of RT (Merad et al., 2002) and induce regulatory T cell infiltration in tumors resulting in anti-tumor immunity suppression (Price et al., 2015). Moreover, in esophageal cancer, the accumulation of tumor-infiltrating regulatory T cells after neoadjuvant radiochemotherapy is associated with a worst prognosis (Vacchelli et al., 2015).

RADIOTHERAPY AND EXTRACELLULAR MATRIX REMODELING

Fibroblasts are the most important producers of ECM. Normal fibroblasts are well known to resist to high radiation dose (up to 50Gy) (Tachiiri et al., 2006). Cancer-associated fibroblasts (CAF) actively contribute to cancer aggressiveness by modulating different processes (angiogenesis, inflammation and ECM remodeling) and to treatment resistance (Straussman et al., 2012; Augsten, 2014; Hirata et al., 2015). The *in vitro* crosstalk between CAF and cervical cancer cells appears to enhance cancer cell survival and proliferation following RT (Chu et al., 2014). *In vitro*, CAF isolated from lung cancer patients display similar immunosuppressive abilities following high dose RT (>5Gy) compared to non-irradiated CAF (Gorchs et al., 2015). Furthermore, the ratio between α -SMA positive (myofibroblasts) and neoplastic epithelial areas was higher after neoadjuvant RT in human rectal cancers, and was an adverse prognostic factor regarding recurrence-free survival (Verset et al., 2015). CAF presence is often viewed as a bad prognostic marker in colon (Tsujino et al., 2007), pancreatic (Erkan et al., 2008) and breast (Yamashita et al., 2012) cancers. However, a recent *in vivo* study using a murine genetic model of pancreatic ductal adenocarcinoma sheds light on an unexpected protective function of proliferating CAF (Özdemir et al., 2014). Altogether these observations suggest that different subsets of CAF can exert opposite effects on cancer progression and that RT has a propensity to induce CAF pro-tumor activity.

An intense ECM remodeling is associated with cancer progression and relies on the activity of several proteases that can be modulated by irradiation. Matrix proteolysis leads to the release of active molecules stored in the ECM, such as growth factors, angiogenic factors and active fragments of matrix components. In physiological conditions, proteolysis is tightly controlled by an appropriate balance between Matrix Metalloproteases (MMPs) and Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) (Egeblad and Werb, 2002). The alteration of protease activity in tumor cells after irradiation is documented both *in vitro* and *in vivo*. MMP-2 is up-regulated following different irradiation protocols in various tumor types such as glioblastoma (Kargiotis et al., 2008), pancreatic (Qian et al., 2002), lung (Chetty et al., 2009) and colorectal cancers (Speake et al., 2005), leading to increased tumor invasion. MMP-2 inhibition before RT enhances the radiosensitivity of lung cancer cells *in vitro*. It is worth noting that proinvasive factors can be released *in vitro* from a reconstituted basement

membrane (Matrigel) subjected to RT. Breast cancer cells seeded on irradiated Matrigel have increased invasion capacity with an increased expression of MT1-MMP and TIMP-2, both involved in MMP-2 activation (Paquette et al., 2007). In murine breast carcinomas, MT1-MMP blockade with a neutralizing antibody enhances the response to RT (3x6Gy) via a shift in macrophage phenotype toward anti-tumor M1-like cells associated with increased iNOS expression and tumor perfusion (Ager et al., 2015). MMP-9 expression and activity are also altered after RT in hepatocellular carcinoma cells through the PI3K/Akt/NF-KappaB cascade (Cheng et al., 2006). In non-small cell lung carcinoma cells, after 2Gy irradiation, SDF-1/CXCR-4 pathway induces MMP expression, via Pi3K/Akt and MAPK activation, leading to increased cell invasiveness *in vitro* and *in vivo* (Gu et al., 2015).

Lysyl oxidase (LOX) is an enzyme implied in collagen and elastin fiber crosslinking, which increases ECM soluble deposition and tensile strength (Kagan and Li, 2003). The link between extracellular LOX, hypoxia and metastases is clearly demonstrated in breast cancers (Erler and Giaccia, 2006; Erler et al., 2006). LOX plays an obvious role in the premetastatic niche formation by modifying the basement membrane at the premetastatic site and thereby allowing CD11b⁺ myeloid cell recruitment (Erler et al., 2009). *In vitro*, RT increases LOX secretion in a dose-dependent manner in several tumor cell types (lung adenocarcinoma, colon carcinoma, glioma, vulva cancer, breast adenocarcinoma), which in turn promotes cancer cell invasion. Increased LOX secretion after RT was also observed *in vivo* in a lung adenocarcinoma xenograft model (Shen et al., 2014). It is worth noting that, while extracellular LOX is associated with tumor progression, intracellular LOX could be a tumor suppressor (Erler and Giaccia, 2006). Indeed, LOX propeptide inhibits prostate cancer cell growth *in vitro* and xenograft growth *in vivo* by direct interaction with DNA repair proteins leading to subsequent radio-sensitization (Bais et al., 2014). Altogether these data show that RT-induced protease release and activation varies according to the tumor type, the dose and the model.

CONCLUSION

During the last decade, the initial cancer cell-centered view of tumors has greatly evolved to an integrated vision of tumor biology taking into account the key contribution of the TME. Obviously, the different compartments of TME are closely related and contribute not only to tumor progression, but also to its response to treatments. Importantly, the TME evolves over time during the different steps of cancer development and is also affected by different therapeutic modalities. Although, improvements have been achieved regarding RT delivery to the primary tumor, ionizing radiation also target non-tumor cells that influence tumor growth and metastatic dissemination. Different approaches have been proposed to overcome the radioresistance of cancer cells. The TME-mediated radioresistance is now the object of researches, which has been elegantly reviewed recently by Barker et al. (2015) and several

articles pointed out the importance of treatments that modify the TME and likely radiosensitize tumor (Ansiaux et al., 2005; Crockart et al., 2005b; Frérart et al., 2008).

However, the impact of anti-cancer treatments on the TME and consequently on the tumor phenotype, response to treatment and metastases, is often neglected. Here we pointed out the impact of RT on the TME. Recent findings emphasize the interest to optimize RT (i.e., dose per fraction) and timing of surgery (Leroi et al., 2015; Surace et al., 2015) in order to prevent metastatic spreading. The future challenge in RT will be to define the most appropriate combinations between RT, and other therapeutic modalities with the optimal sequence and timing of treatments. In this context, investigation of the TME-related acquired resistance will be essential and will provide important innovative data.

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AUTHOR CONTRIBUTIONS

NL wrote the manuscript including figures and gathered manuscript modifications from the authors. AN and PM designed and wrote the manuscript. FL contributed to figures and reviewed the manuscript. PC reviewed the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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