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Review

Radiotherapy-induced remodeling of the tumor microenvironment by stromal cells

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ABSTRACT

Cancer cells reside amongst a complex milieu of stromal cells and structural features known as the tumor microenvironment. Often cancer cells divert and co-opt functions of stromal cells of the microenvironment to support tumor progression and treatment resistance. During therapy targeting cancer cells, the stromal cells of the microenvironment receive therapy to the same extent as cancer cells. Stromal cells therefore activate a variety of responses to the damage induced by these therapies, and some of those responses may support tumor progression and resistance. We review here the response of stromal cells to cancer therapy with a focus on radiotherapy in glioblastoma. We highlight the response of endothelial cells and the vasculature, macrophages and microglia, and astrocytes, as well as describing resulting changes in the extracellular matrix. We emphasize the complex interplay of these cellular factors in their dynamic responses. Finally, we discuss their resulting support of cancer cells in tumor progression and therapy resistance. Understanding the stromal cell response to therapy provides insight into complementary therapeutic targets to enhance tumor response to existing treatment options.

1. Background

The complex microenvironment of a tumor supports many functions of cancer cells that drive every step of tumor formation, growth, and eventual invasion, metastasis, and treatment resistance. There has been extensive research on how tumor cell intrinsic responses to therapies such as radiotherapy and chemotherapy, allow them to evade cell death (reviewed in [1–3]). There has also been much recent work on how stromal cells and the tumor microenvironment support tumor cells and therapy resistance (reviewed in [4]). However, only just begun is research attempting to understand the feedback between stromal cells and tumor cells as the stromal cells respond to therapy. We review the literature here on how therapy affects the stromal cells in ways which may feed back to promoting or repressing malignant features of the cancer cells themselves. The findings reviewed here emphasize that therapy resistance may arise not only from selection pressure on cancer cells themselves, but also from stromal cell responses to therapy. These stromal responses may provide new avenues of treatment.

The entire spectrum of cancer treatments would be difficult to address in a single review. We therefore focus on ionizing radiation in glioblastoma (GBM) with occasional observations from other therapeutic regimes in other cancers to support the discussion. (We will henceforth refer to ionizing radiation as radiation.)

Glioma is the most common primary brain tumor and GBM is its most aggressive form. GBMs differ from lower-grade gliomas and are characterized from a histological point of view by microvascular proliferation and pseudopalisading necrosis, the latter of which is accompanied by widespread areas of hypoxia. GBMs are remarkably heterogeneous and tumor cells within a given GBM tumor present with a wide range of phenotypes [5]. Tumor cells furthermore display high phenotypic

Abbreviations: AKT, Ak strain transforming; AP-1, activator protein 1; CCL11, C-C motif chemokine ligand 11; CD44v6, cluster of differentiation 44 variant isoform v6; CRP, c-reactive protein; Cx43, connexin 43 Cx43; DNA, deoxyribonucleic acid; ECM, extracellular matrix; GBM, glioblastoma; GFAP, glial fibrillary acidic protein; Gy, Gray; HIF1α, hypoxia inducible factor 1 alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloprotease; mTOR, mechanistic target of rapamycin; NF-κB, nuclear factor kappa B; PAI-1, plasminogen activator inhibitor-1; PDGFRα, platelet derived growth factor receptor alpha; PECAM-1, platelet endothelial cell adhesion molecule; pSTAT3, phosphorylated signal transducer and activator of transcription; RNA, ribonucleic acid; ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype; STAT3, signal transducer and activator of transcription; TGM2, transglutaminase 2; TNF-α, tumor necrosis factor alpha; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor.

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plasticity, meaning that cells can transition from one state to the other as a result of e.g. genetic aberrations or microenvironmental factors such as hypoxia [6]. GBM has a very poor prognosis, with a 5-year survival rate of less than 10 % (reviewed in [7]). Initial treatment consists of surgery, radiation, and sometimes temozolomide [8]. After surgery, the inclusion of radiotherapy has provided the greatest increase in GBM survival [7]. However, while radiotherapy provides significant life extension, patients almost invariably relapse and the recurred tumor has few treatment options [7]. One of the major obstacles to treatment includes preventing relapse. The recurred tumor is hypothesized to derive from a variety of potential sources, including cells that have migrated just beyond the treatment field of surgery and radiation, as well as GBM cells with intrinsic radiation resistance such as stem-like cells. Notably, relapse generally occurs in the original tumor volume and within tumor borders within the radiation field [9–11], fitting a model in which cells at the tumor margins escape therapy to repopulate the original tumor area.

Stromal cells are present both within the tumor as well as densely populating the tumor margins where recurrence originates, particularly in the case of immune cells [12] and astrocytes [13,14]. As they experience the effects of therapy at the same levels as the glioma cells, it is important to understand how stromal cell responses to therapy might affect the tumor cells, in either tumor cell migratory phenotypes or acquisition of intrinsic radioresistance.

The influence of microenvironmental factors on migration is well established, with contributions that include cytokine signaling, matrix remodeling, and cell intrinsic pathways. Stromal cells contribute to all of these pathways. Radiotherapy has been shown to stimulate migratory phenotypes in GBM [15,16] and other cancers (reviewed in [17]). Notably, recent work by Gauthier et al. links the radiation-induced migratory phenotype of GBM cells to stemness in those cells, demonstrating a loss of the pro-migratory phenotype post-irradiation once the stem-like cells are differentiated [18]. This migratory phenotype required nuclear localization of Hif1α. Interestingly, recent work by Lin et al. demonstrates hypoxic regions at the tumor edge, as opposed to the tumor core, in GBM [13], suggesting a potential for synergistic actions between radiation and tumor hypoxia at the tumor border.

Brain tumor therapeutic resistance is thought to be associated with the presence of cells displaying high *stemness*; cells with intrinsic resistance to radiotherapy [19], and the ability to fuel regrowth of the recurrent tumor [20] (reviewed in [21]). The influence of the microenvironment over brain tumor stem cell phenotypes was first inferred by the finding that such cells appear to locate primarily to perivascular and/or perinecrotic (hypoxic) niches, or more recently a tumor microtube niche [22–26] (reviewed in [27]). Brain tumor cells surviving radiation therapy *in vivo* are largely spatially restricted to these tumor niches in mouse models [24,28], suggesting either that tumor cells with intrinsic radioresistance are attracted to these niches, or that the microenvironment actively contributes to brain tumor radioresistance. The latter model is supported by an increasing body of literature describing what is essentially dedifferentiation of tumor cells towards stem-like states, induced by microenvironmental or cell intrinsic factors [14,29–35]. Recent efforts in single cell RNA sequencing of human GBM samples lend further support to the idea of plasticity in tumor cell phenotypes related to stemness and therapeutic resistance [36], and these and other data suggest that the resistance phenotype is largely unrelated to specific genetic aberrations [37].

1.1. Radiation and the microenvironment

It is important to note some of the well established effects of radiation. Radiation produces broad effects directly on cancer cells and stromal cells, as well as bystander effects, in which neighboring cells outside the field of radiation experience effects of the radiation (reviewed in [38]). The after effects of radiation include short term as well as long term changes to the microenvironment and affect cytokine secretion (reviewed in [39] and ECM structure (reviewed in [40]) as well as altering the types and components of stromal cells (reviewed in [39]). Some of these changes are well documented while others are only poorly understood. Given the wide variety of radiation dosing that has been investigated, it is difficult to make generalizations; however, we will summarize some of the most basic effects of radiation on tissues.

1.2. The physical effects of radiation

Radiation results in both direct and indirect physical changes to biomolecules (Fig. 1). Direct damage includes double strand breaks in DNA as well as changes to proteins, such as fragmentation, crosslinking, and other molecular changes to collagens [41–45]. Indirect damage from radiation occurs largely from the generation of reactive oxygen species (ROS). Oxidative stress can result in telomere shortening or lengthening, lipid peroxidation, protein oxidation, and DNA damage (reviewed in [46]). DNA damage directly from radiation and indirectly from ROS are main triggers of cell death among proliferating cancer cells. All of these changes are also linked to altered signaling in stromal and tumor cells.

1.3. Effects of radiation on stromal cells

Residing among cancer cells are a variety of other cell types. These include immune cells, such as neutrophils, macrophages, and brain resident microglia; fibroblasts; pericytes and endothelial cells in the vasculature; and in the brain, neurons and astrocytes. Cells in the microenvironment have varied responses to radiation which can include cell death, senescence, activation, survival, and migration (Fig. 2). While there are various other stromal cells in GBM, we focus in this review on three important cell types and their associated niches, including endothelial cells in the vasculature, macrophages and microglia, and astrocytes. We further examine potential lasting changes to the extracellular matrix (ECM) in the context of how these changes might be driven by stromal cell response to therapy. We will first introduce these cell types before entering into an in-depth discussion of their response to therapy. Fig. 2 summarizes the effects of radiation on stromal cells and the resulting downstream effects on tumor cells.

1.4. Endothelial cells in the vasculature

In the vasculature, both pericytes and endothelial cells respond to radiation damage. Endothelial cells have well documented short term responses to radiation with long term responses more poorly described. Short term responses largely involve activation of the endothelium that involve NF-κB and AP-1 pathways which lead to secretion of pro-inflammatory cytokines (reviewed in [47]). Long term responses are not well characterized, but include changes to ECM [48], cell cycle, inflammation, and cytokine secretion up to 14 days post irradiation [49–51].

1.5. Immune cells: macrophages and microglia

Immune cells, including neutrophils and macrophages, respond to radiation damage. Activated macrophages secrete a range of pro-inflammatory cytokines that induce changes to the tumor microenvironment. In the brain, two sources of macrophages are present, either brain-native microglia or those which come from peripheral blood-derived monocytes. These cells, like macrophages, become activated by tissue damage. Macrophage response to neuronal injury and brain tumors has been recently reviewed [52,53]. Microglial activation has been linked to radiation-induced neurological deficits with ties to inflammatory signaling [54]. Like in other cancers, macrophages present a diverse population that express a range of clinically beneficial and pro-tumorigenic phenotypes with immune stimulatory or immune suppressing functions. Clinically beneficial macrophages are often

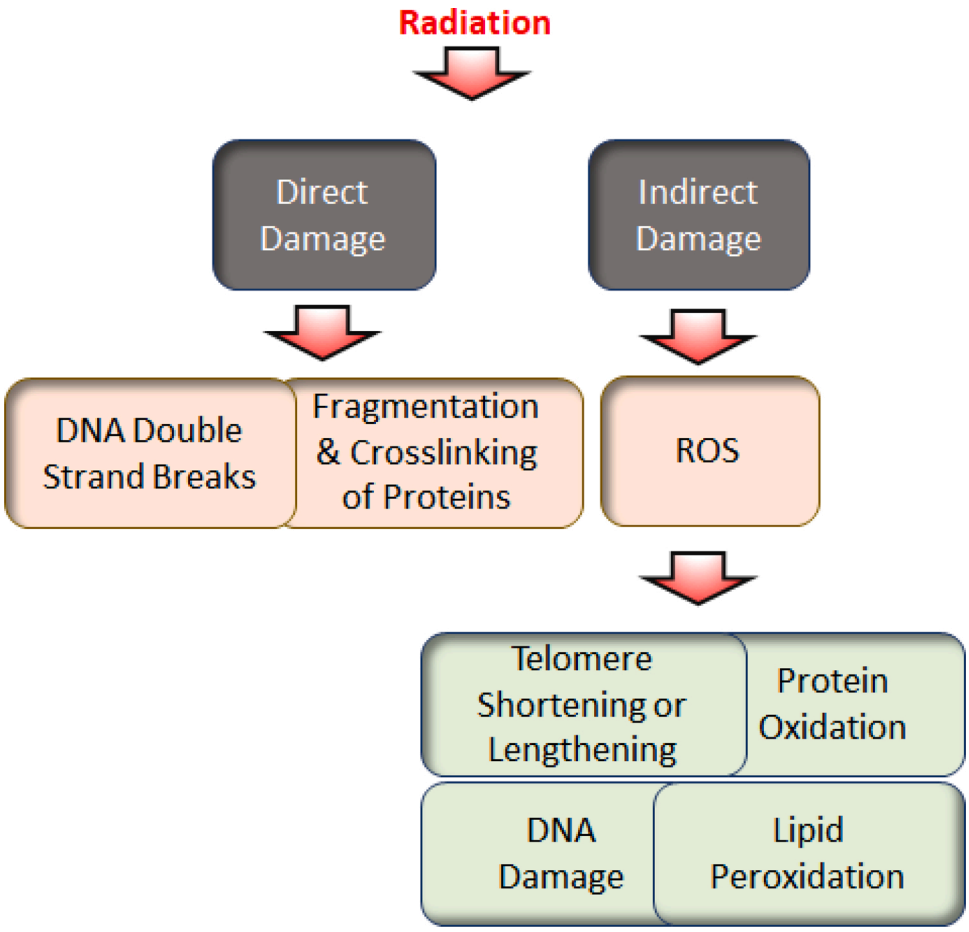


Fig. 1. Pathways to cell death post irradiation. Radiation induced direct damage to proteins and DNA. Radiation also increases ROS, which creates a cascade of events that leads to other damage to cells. These two processes each lead to cell death or senescence post-irradiation.

characterized as M1 and pro-tumorigenic as M2, though these designations are increasingly debated as a wider ranges of macrophages with less clearly defined functional boundaries are characterized (reviewed in [53,55]).

1.6. Fibroblasts and astrocytes

Outside of the brain, fibroblasts induce structural changes in response to irradiation. Injury, including radiation, stimulates activation of fibroblasts to myofibroblasts. Myofibroblasts have an altered pattern of cytokine and ECM secretion which has been strongly linked to the pathological radiation-induced fibrosis that is often a dose-limiting side effect of radiation (reviewed in [56]). A similar response in the brain occurs with astrocytes, which respond to radiation damage among other types of injury and disease by undergoing reactive gliosis, which alters contacts with other cell types, secretion of cytokines, and ECM [57,58] (reviewed in [59]). Astrocyte response to injury is diverse and varies depending on brain region. Terminology surrounding astrocytes and their response to injury and disease varies, but we will use the terms suggested in the recent consensus review by Escartin et al. [60].

1.7. Senescence associated secretory phenotype (SASP)

In all of these cell types, in addition to their active response to radiation, a subset may become senescent, thus inducing the senescence-associated secretory phenotype (SASP). SASP is a broad descriptor of secretory changes in senescent cells that has been a widely explored response of both stromal and tumor cells to radiation and has been recently reviewed [61]. While the many important results of senescence-induced changes on the tumor microenvironment are still being elucidated, we focus in this review on changes to the stroma that go beyond SASP and other senescence phenotypes.

2. Vascular and endothelial response to radiation

Radiotherapy results in a range of responses in the normal and tumor vasculature, leading to endothelial senescence [62], increased inflammation and immune cell recruitment [47,49,63,64], fibrosis [48], and re-vascularization of the tumor as a result of hypoxia-induced HIF1 and VEGF signaling from GBM cells [65], endothelial cells [66] and astrocytes [67]. Therefore the influence of radiotherapy on the vasculature results in a complicated picture for tumor response that encompasses the effects of loss of vasculature on oxygen levels as well as the complex signaling pathways invoked in response to hypoxia and endothelial cell radiation response.

On the one hand, GBM is a particularly vascularized tumor and radiation severely disrupts the vasculature. Inhibiting angiogenesis, on the surface, sounds like a promising result therapeutically. However, in the context of radiotherapy, a plethora of negative outcomes can also result from the loss of vasculature. One of the mechanisms of tumor cell death in radiotherapy is through the generation of ROS. In the absence of oxygen, ROS generation can be severely depleted, resulting in reduced efficacy of radiotherapy [68]. Increasing oxygenation of tumors, such as in hyperbaric chamber therapy, is suggested to improve response to radiotherapy but is not easily implemented clinically [69,70]. In addition to reduced tumor cell damage by ROS, the resulting hypoxic regions can lead to increased HIF signaling in tumor cells, which is linked to increased stemness and radio-resistance [71]; reviewed in [27].

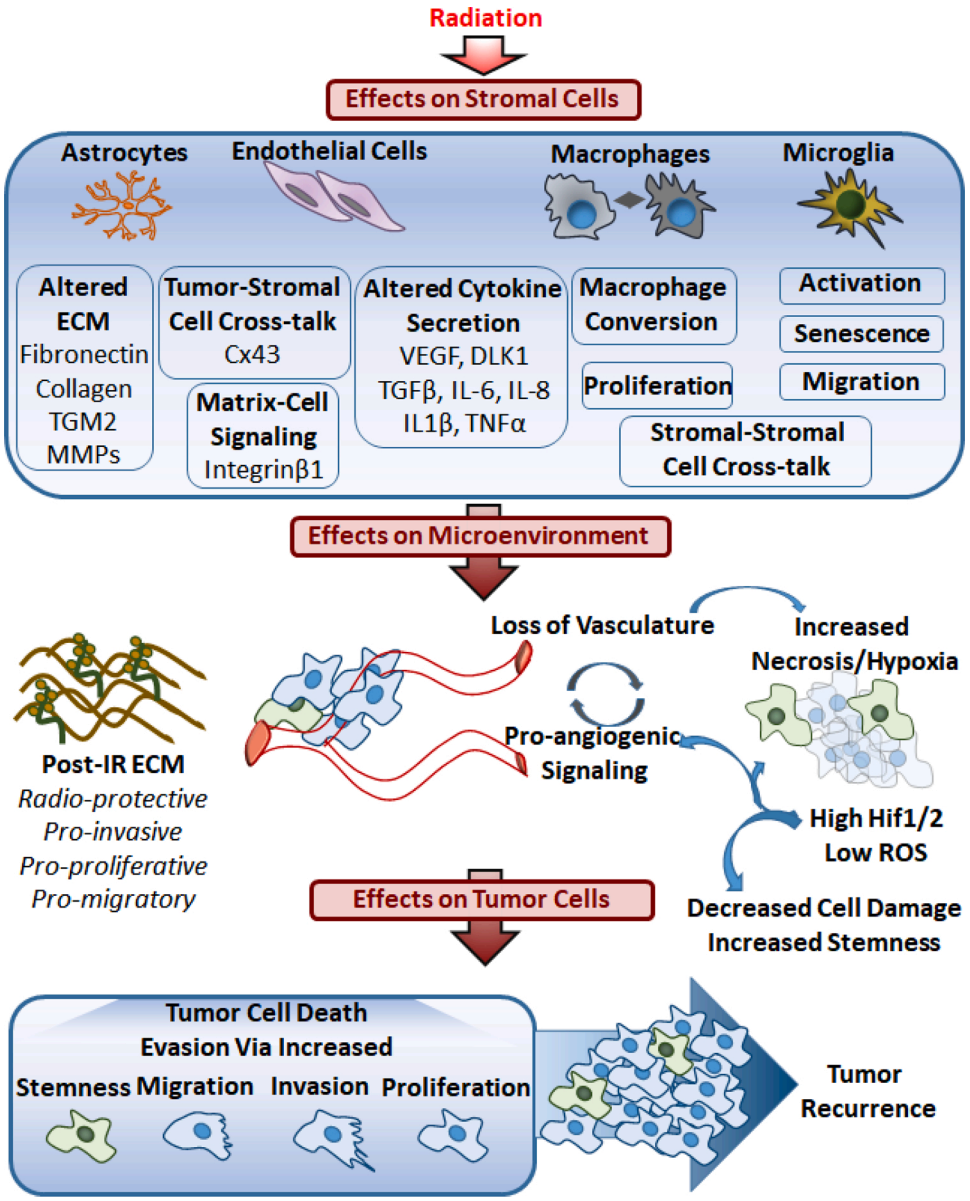


Fig. 2. Stromal cell response to therapy promotes protection of cancer cells through microenvironmental changes. Stromal cells of the tumor microenvironment receive radiotherapy concurrent with cancer cells. These cells respond by secreting soluble and insoluble factors as well as altering cell intrinsic signaling pathways and cell-cell contacts with cancer cells and other stromal cells. These responses lead to changes in the tumor microenvironment such as a post-irradiation ECM which is radioprotective and supports tumor progression. The tumor vasculature also responds, initially with loss of vasculature which leads to hypoxia, which in turn reduces ROS, a necessary element for radiation-induced cell death. Additionally, the hypoxic environment promotes stem-like features of glioma cells. Hypoxia also, in turn, stimulates pro-angiogenic signaling which promotes re-vascularization to provide support for tumor re-growth. These stromal cell responses lead to tumor cells evading the effects of radiation through increased stemness, migratory, and invasive phenotypes. These cells can then repopulate the tumor in a pro-proliferative, radioprotective microenvironment, leading to tumor recurrence.

A recent study examining an inhibitor of endothelial proliferation in glioma found that, when used alone, it severely reduced vessel formation at the tumor core and increased overall survival [72]. Strikingly, however, when combined with radiotherapy, the survival benefit of radiotherapy was entirely lost [72]. Perhaps it is unsurprising then that anti-angiogenic therapies in GBM have had no effect on overall survival (reviewed in [73,74]). The negative effects of hypoxia may at least partially explain the poor performance of these drugs in clinical trials. However, understanding the underlying changes to the tumor microenvironment resulting from radiotherapy may help bypass such adverse effects while still utilizing the therapeutic potential.

Radiation effects on endothelial cells are not limited to their function in maintaining the vasculature. In lung cancer, endothelial-to-mesenchymal transition stimulated by radiotherapy prompted proliferation of lung cancer cells bearing the stemness marker CD44v6, a process linked to increased osteopontin secretion [75]. The downstream effects of radiation on endothelial cells in this model was not limited to tumor cells, as an increase in M2 polarization of tumor associated macrophages was also seen [75]. In liver cancer, irradiation of endothelial cells has been shown to promote the invasion potential of liver cancer cells. This resulted from changes in cytokine secretion and alterations to the ECM by endothelial cells in response to radiation [76,77]. Notably, only a single dose of 2 Gy, and not the higher single dose of 6 Gy nor the lower dose of fractionated radiation caused a similar response, suggesting treatment might be tuned to maximize benefits of radiation by minimizing negative impacts derived from the stromal response [76,77]. Radiotherapy has also been shown to stimulate increased drug efflux capacity in endothelial cells, presenting potential avenues for drug resistance with drugs targeting the blood brain barrier [78].

Radiotherapy increases pro-inflammatory signaling from endothelial cells as well. Boström et al. demonstrated that 24 h and 7 days post-irradiation of P14 postnatal mice, endothelial cell proportions in the hippocampus and cerebellum increased and had elevated expression of the pro-inflammatory gene for CCL11([63]). Ramadan et al., using coronary artery and microvascular endothelial cells, demonstrated increased ROS and inflammatory markers, including IL-6, MCP-1, PECAM-1, IL-1β, TNF-α, CRP, VCAM-1, E-Selectin, Endothelin-1, IL-8, and PAI-1, after a single dose of 5 Gy irradiation. These pro-inflammatory signals can have direct effects on the tumor cells as

well as indirect effects by influencing the types of immune cells recruited to tumors [79].

3. Immune cells: macrophages and microglia

In the brain, there are both brain resident microglia and monocyte-derived macrophages from the circulatory system. A recent study by Ott et al. has emphasized the importance of the radiation-induced immune response [80]. They combined whole brain radiotherapy with a STAT3 inhibitor in a mouse glioma model, which increased the number of long term survivors but only in immunocompetent mice. They observed increased migration of dendritic cells to gliomas upon irradiation, but the ability of the dendritic cells to stimulate an immune response was suppressed in the absence of the STAT3 inhibition. These findings emphasize that stimulating a beneficial immune response and suppressing the pro-tumorigenic response is essential to developing effective GBM therapy and that radiotherapy-altered cross-talk between tumor and stromal cells will influence treatment strategies.

It is well established in GBM and in many other cancers that macrophages can be pro-tumorigenic or clinically beneficial. In GBM, macrophages which accumulate at the tumor border secrete factors which promote stemness features of GBM cells [12], which is strongly linked to therapy resistance and recurrence [19,20] (reviewed in [21]). It has recently been shown that a subset of tumor associated macrophages can promote a mesenchymal-like phenotype, a feature associated with stemness, and that their enrichment is associated with worse outcome [81]. However, how radiotherapy might tip the balance between pro- and anti-tumorigenic macrophages is not yet well understood.

In the lung, studying radiation induced fibrosis, it was shown that radiation triggers lung epithelial cells to undergo an epithelial to mesenchymal transition as well as a acquiring a secretory phenotype which attracts M2 macrophages [82]. The M2 macrophages in turn feed back to the cancer cells to promote further epithelial to mesenchymal transition [82]. This suggests that migratory effects and chemoattraction may be important factors in balancing the macrophage population and that this process is influenced by radiation.

In the brain, growing evidence suggests that the environment likely plays a critical role in modulating the macrophage population. An examination of metastatic lung cancer revealed that whole brain irradiation did not stimulate peripheral immune cells to migrate to the brain except in the presence of metastatic lung cancer cells [83]. Notably, brain resident microglia and circulating macrophages had differing responses to whole brain radiation, potentially due to the fact that the entire microglial population was exposed to radiation while the circulating monocyte derived macrophages were replenished by cells that had not received radiation [83]. Changes to those cells might be attributed to environmental factors.

Indeed, in a study of GBM, Akkari et al. examined the response of monocyte-derived macrophages and microglia to radiation in two different genetic models of glioma [84]. While they did not observe changes in the proportions of monocyte-derived macrophages to microglia at either 5 or 21 days post irradiation, the recurrent tumor had increased ratios of monocyte-derived macrophages. Dual inhibition of the monocyte-derived macrophage and microglial populations with BLZ945, a CSF-1R inhibitor, in combination with radiotherapy resulted in remarkable increases in survival of glioma-bearing mice, but notably, only when the inhibitor was given continuously. Acute treatment resulted in much poorer outcomes. Throughout the therapy regime, microglia and monocyte-derived macrophages maintained a core gene expression signature related to their origins. However, in the recurrent tumor, they developed an additional common signature. Much of the common signature in the monocyte-derived macrophage population was driving it to a signature more similar to treatment naïve microglia [84].

Notably, if monocyte-derived macrophages are migrating from outside the brain, then is it unlikely that this is a carryover effect from initial irradiation on the macrophages themselves. Instead, these are entirely treatment naïve cells which are interacting with the recurrent tumor environment; therefore, it is likely that it is the environment of the recurrent tumor that is driving the changes to monocyte-derived macrophages. Indeed, Lund et al. showed that depletion of microglia led to repopulation by circulating macrophages that, while maintaining a distinct genetic expression profile, acquired a methylation signature much like microglia, and concluded that macrophages are highly influenced by their environment [85].

The requirement for continuous BLZ945 treatment to maintain the survival effect on mice suggests a durable and persistent change to the tumor microenvironment in the recurrent tumor, capable of re-educating the monocyte-derived macrophages once there is a release from BLZ945 inhibition. It is difficult to say from this study which cell types may be responsible for these lasting changes, and possibly multiple cell types and signaling feedback pathways are involved. However, recent work from our lab suggests that astrocytes, one of the most abundant cell types in the brain, persist in the previous tumor location after the tumor recedes post-irradiation [14]. These irradiated astrocytes are reactive and alter the microenvironment by secreting the matrix-modifying protein transglutaminase 2 (TGM2), resulting in a radio-protective environment. Understanding how that radio-protective or otherwise altered environment might influence macrophage tumor education in the recurrent tumor may offer avenues of treatment that do not require continuous treatment.

4. Astrocyte response to radiotherapy

Given the abundance of astrocytes in the brain and their role in damage response, astrocyte function in GBM is an active field of study. Astrocytes have recently been shown to promote the proliferation of glioma cells [86], and a subset of PDGFRα-positive, GFAP-positive astrocytes at the tumor periphery have been associated with worse survival [87]. Their roll in GBM has been recently reviewed [88]. However, the effects of radiotherapy on astrocytes, and how those effects feedback to cross-talk with GBM cells or other stromal cells, is still poorly understood. Changes in soluble and insoluble secreted factors as well as direct cell-cell communication are all potential consequences of radiotherapy on astrocytes.

4.1. Changes in direct cell-cell communication

Therapy has the potential to shift the patterns of communication between stromal cells and tumor cells through direct cell-cell contacts. One notable change in this pattern is gap junction protein connexin 43 (Cx43). Several studies have linked Cx43 expression to GBM invasion, a phenomena that occurs along both the vasculature and astrocyte processes [89–91]. Levels of stromal Cx43 changing in response to therapy may be important aspects of this Cx43 signaling. Sin et al. reported that reactive astrocytes upregulate Cx43 when they undergo reactive gliosis resulting from contact with glioma cells, thereby promoting invasion [90]. Intriguingly, the gap junction activity or hemichannel activity differs based on the trigger of the reactive gliosis, with glioma cells triggering gap junction activity [90] and bacteria, hemichannel activity [92]. Sin et al. also observed that inhibition of gap junction activity inhibits the reactive gliosis that resulted from this contact [90]. It has since been found that both radiation [93] and TMZ [91,94] increase Cx43 activity and expression, and it is the connection between astrocytes and glioma cells which results in therapy resistance [91].

In the study of stroke, it has been found that preconditioning with sublethal insults of hypoxia upregulates Cx43 expression and hemichannel activity to protect against further hypoxic insult [95]. The concept of preconditioning is not new, but it is intriguing to speculate that contact with glioma cells may prime astrocytes in a type of pseudo-preconditioning to respond to therapy-related insults, allowing for more rapid protective responses from astrocytes in tumor-bearing areas (Fig. 3). Disruption of this rapid response potential may provide

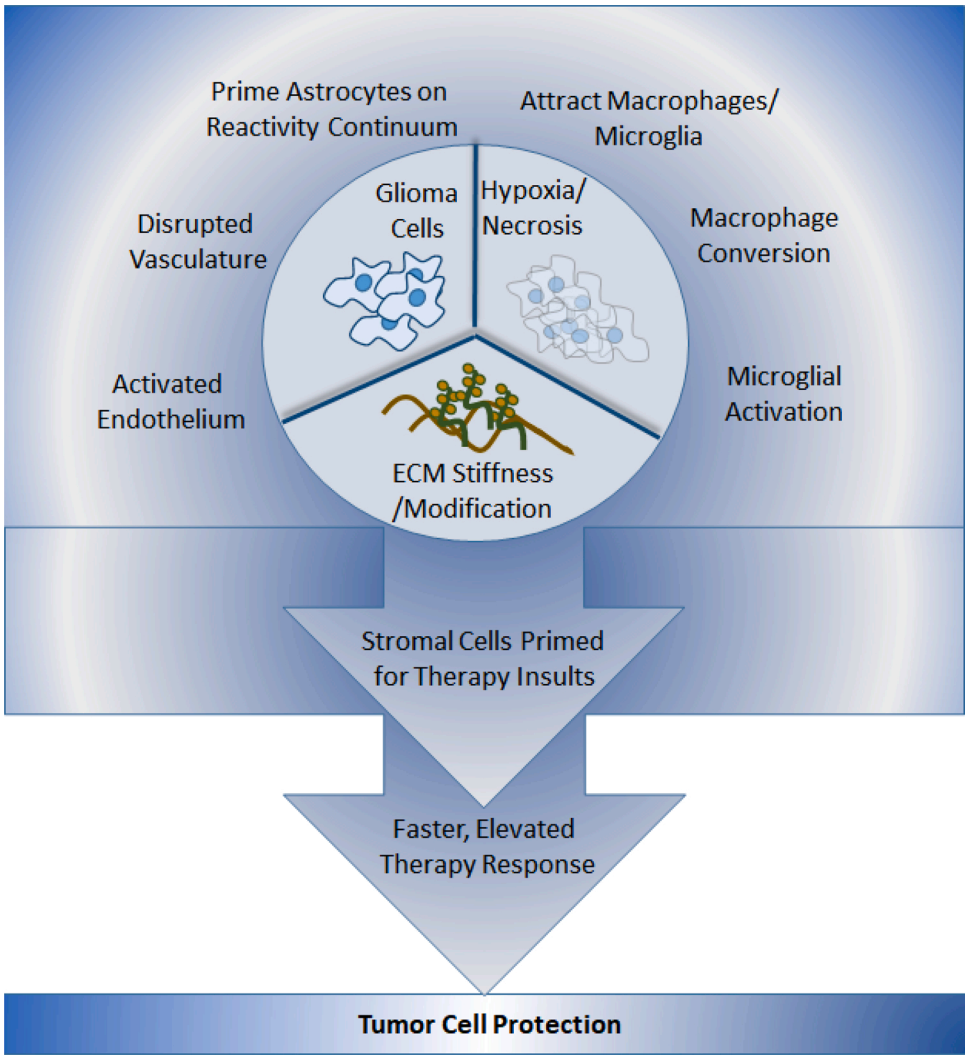


Fig. 3. Microenvironmental Priming of Stromal Cells Prepares them for Rapid, Elevated Response to Therapy. Prior to therapy, stromal cells exist in a disrupted microenvironment. The presence of glioma cells, a modified and stiffer matrix, and regions of hypoxia and necrosis alter stromal cell behavior. This may put stromal cells into a state of early or pre-activation or place them more firmly on the continuum of reactivity. While these are responses to the presence of the tumor, it may prime these stromal cells for a more rapid or exaggerated response to therapy. This can in turn provide cancer cells with protection during the earliest, critical stages of therapy.

a promising avenue for preventing therapy resistance.

Notably, Cx43 upregulation in response to radiation is not limited to astrocytes. In both cardiac fibroblasts and endothelial cells, Cx43 is upregulated after irradiation [96]. A study of increases in atherosclerotic plaques after radiation treatment of breast and thoracic cancers revealed increased expression of Cx43 in endothelial cells [79]. Targeting Cx43 in GBM led to reduced invasion and better survival, particularly in combination with radiation [89]. While Yusubalieva et al. noted that increased effectiveness of Cx43 inhibition in GBM in combination with radiation may be the result of increased blood brain barrier permeability, it is also possible that radiation-induced Cx43 activity and expression increases the reliance of cells on Cx43 for survival, further sensitizing cells to the drug. This emphasizes the importance of understanding stromal responses as well as tumor cell responses to therapy and the resulting shifts in interactions. Focusing solely on the tumor cells will not fully explain responses to therapies and understanding both responses may allow us to fine tune therapies to avoid the support mechanisms that stromal cells may provide.

4.2. Long term changes to the GBM microenvironment by astrocytes

Stromal cells have the potential to modify the microenvironment in lasting ways. Duan et al. and Fletcher-Sananikone et al. recently demonstrated that the irradiated microenvironment experiences lasting changes that can support glioma growth by implanting glioma cells weeks post-irradiation [97,136]. The pre-irradiated mice died faster and developed larger tumors with disrupted vasculature like that seen in human GBM. Recent research from our lab also showed increased vasculature and more proliferative tumors in mice pre-irradiated 72 h before implantation with glioma cells [14]. What factors might be involved in this radiation-induced "priming" is still unclear, but astrocytes provide one potential source of this lasting change [14,136].

Astrocytes undergo a process of reactive astrogliosis in reponse to brain injury. The changes seen in astrocytes undergoing this process vary depending on brain location and injury type (reviewed in [59,60]). Reactive astrogliosis has been detected both in the early response to radiation 6−24 hours post irradiation with 15 Gy [98] and in the brains of rats 18–21 weeks after irradiation with 7, 5 Gy fractions [99]. Our group and others have shown that cultured astrocytes survive radiation of single doses of 10−15 Gy [14,100], and Bylicky et al. showed that these astrocytes resume proliferation after 6 days [100]. Together, this suggests that astrocytes survive radiotherapy and their potential radiation response could cause lasting effects long after therapy. Furthermore, it introduces the question of whether various signaling pathways shown to be stimulated by reactive astrocytes at shorter time points after irradiation might persist within the previous tumor microenvironment and support regrowth of the tumor.

Reactive astrocytes alter secretion of both soluble and insoluble proteins after irradiation. Soluble factors include VEGF [67], DLK1 [35], IL6 [14,101], IL8 [14], MMP2 [102] and interleukin 1β [101]. They also

increase STAT3 signaling [67]. The longer term secretion of these proteins may have effects on invasion [91], proliferation [35], or treatment resistance of glioma cells [35], or potentially become involved in feedback cycles between other stromal cell types or tumor cells. Feedback pathways between stromal cell is an exciting new field of research (reviewed in [103,104]). It is less clear how radiotherapy influences crosstalk of stromal cells and how that in turn affects the tumor cells and microenvironment. However, many cytokines released by stromal cells have been implicated in fibrosis (reviewed in [105,106]), and some of those have been shown to increase in response to radiotherapy, such as TNFα from both microglia and astrocytes [107,108]. Table 1 summarizes the soluble factors released by stromal cells into the tumor microenvironment after irradiation.

Likewise, astrocytes with nuclear phosphorylated STAT3 (pSTAT3) have been shown to surround brain metastases from multiple primary tumor types and promote an immunoprotective, pro-proliferative environment for metastatic cell maintenance [109]. In light of elevated activation of STAT3 identified by Zhou et al. [67] in astrocytes after irradiation, and the findings by Ott et al. that STAT3 inhibition in combination with irradiation is only effective in immune-competent mice [80], it appears radiation may enhance astrocyte-immune cell cross talk in ways that protect tumors from therapy.

Astrocytes also modify the ECM, including increased secretion or modification of fibronectin [110–113], which has been strongly implicated in invasion and metastasis (reviewed in [114]). In breast cancer [115,116] and GBM [14], radiation induces upregulation of β1-integrin, one of the major binding partners of fibronectin, suggesting cross-talk between these molecules might be uniquely amplified with radiotherapy. Indeed, β1-integrin is associated with poor response to radiotherapy in head and neck cancers [117] and in the long term response to radiotherapy in prostate cancers [118].

Recent work from our lab demonstrates increased secretion of TGM2 by astrocytes after irradiation, a process which promotes stemness and radio-resistance of GBM cells [14,119]. This increased secretion persists after tumor regression and elevated TGM2 is still detectable in the recurrent tumor. TGM2 is a matrix-modifying protein that increases fibronectin bundling [112,120]. Changes to fibronectin and other ECM proteins play important roles in proliferation, invasion, and treatment resistance in multiple cancers and lasting changes to these proteins have potentially important consequences which will be discussed in the final section.

4.3. Stromal-mediated, radiation-induced changes to ECM

Fibronectin is an important mediator of signaling. In many cases, fibronectin secretion by stromal cells in response to therapy is examined in the context of clinical side effects, which can be devastating to the patient (reviewed in [121]). In radiotherapy, neurological deficits are common (reviewed in [122]), and in general, a phenomena known as radiation-induced fibrosis can cause crippling side effects in almost any tissue (reviewed in [121]). In some tissues, such as the lung and heart, fibrosis can result in death. Understanding side effects such as these in their own right is essential to successful cancer therapy. However, in addition to detrimental clinical side effects, fibrosis or increased tissue stiffness that results from treatment may have effects even if it does not result in a pathological response.

Non-pathological fibrosis in response to treatments such as radiation is not well characterized, but increased fibrosis is strongly linked to a

Table 1
Stromal secreted molecules detected post irradiation.

Cell Type	Molecule	Tissue/Tumor/Cell Source	Reference	Radiation Dose	Time Post IR
Astrocytes	DLK1	primary human astrocytes	[31]	10 Gy	2 d
	IL6	primary human astrocytes	[11]	10 Gy	Up to 10 Days
	IL6	primary rat astrocytes	[97]	10 Gy	7 h
	IL8	primary human astrocytes	[11]	10 Gy	Up to 10 Days
	IL-1β	primary rat astrocytes	[97]	10 Gy	1 h
	MMP2	human SVG p12 astrocytes	[98]	4–10 Gy	24–48 h
	STAT3	primary rat astrocytes	[63]	20 Gy	4–48 h
	TGM2	mouse and human GBM; primary human astrocytes	[11]	10 Gy, 3 Gy, 5 × 2 Gy	72 h, tumor recurrence
	TNFα	primary murine astrocytes	[104]	2 Gy, 4 Gy, 2 × 2 Gy	2–8 hr
	VEGF	primary rat astrocytes	[63]	20 Gy	4–48 h
Endothelial	CCL11	Neural	[59]	10 Gy	6 h- 7 d
	CRP	TIME/TICAE*	[75]	5 Gy	24 h-7 d
	Endothelin-1	TIME/TICAE	[75]	5 Gy	24 h-7 d
	E-Selectin	TIME/TICAE	[75]	5 Gy	24 h-7 d
	IL-1β	TIME/TICAE	[75]	5 Gy	24 h-7 d
	IL4	liver/hepatic carcinoma	[72]	2 Gy	24 h
	IL-6	TIME/TICAE	[75]	5 Gy	24 h-7 d
	IL-8	TIME/TICAE	[75]	5 Gy	24 h-7 d
	MCP-1	TIME/TICAE	[75]	5 Gy	24 h-7 d
	Osteopontin	lung/lung carcinoma	[71]	10 Gy	48 h
	PAI-1	TIME/TICAE	[75]	5 Gy	24 h-7 d
	PECAM-1	TIME/TICAE	[75]	5 Gy	24 h-7 d
	TNF-α	TIME/TICAE	[75]	5 Gy	24 h-7 d
	VCAM-1	TIME/TICAE	[75]	5 Gy	24 h-7 d
	VEGF	rectal adenocarcinoma (peritumoral area-clinical samples)	[62]	Daily 0.18–0.72 Gy (2% of therapeutic dose)	8 wk
Microglia	TNFα	mouse brain	[103]	35 Gy	4–24 h
	TNFα	primary mouse microglia	[103]	25 Gy	4–24 h
Undetermined	Arg-1	mouse lung tissue	[78]	14 Gy	1–6 mo
	CCL2	bronchoalvelolar lavage	[78]	14 Gy	2–24 wks
	CD206	mouse lung tissue	[78]	14 Gy	1–6 mo
	IGF-1	mouse lung tissue	[78]	14 Gy	2–24 wk
	IL-10	bronchoalvelolar lavage	[78]	14 Gy	2 wk
	IL-1β	bronchoalvelolar lavage	[78]	14 Gy	2 wk
	IL-4	bronchoalvelolar lavage	[78]	14 Gy	2 wk
	TGF-β	bronchoalvelolar lavage	[78]	14 Gy	2–24 wk

* Telomerase Immortalized human Coronary Artery Endothelial cells (TICAE); Telomerase Immortalized human Microvascular Endothelial cells (TIME).

variety of phenomena, including sustained proliferative signaling and increased invasion and migration (reviewed in [105]). While the immediate damage caused by radiotherapy may recede along with the tumor, if the resulting fibrosis is maintained, it leaves a pro-invasive, pro-proliferative microenvironment intact, increasing the difficulty of treating surviving cells.

Increased tissue stiffness instigated by increased secretion and bundling of ECM proteins such as collagens and fibronectin is strongly linked with increased proliferation, invasion, and stemness in GBM [123]. Fibronectin signals through integrin family proteins to promote proliferation through multiple pathways, including Ras, AKT, and mTOR, in various cancers including breast, ovarian, gastric, and lung cancers (reviewed in [124]). The large family of collagens are also affected by radiation, with changes in expression and cross-linking [44,125] which can be measured as early as 1 day after irradiation [44]. Members of the collagen family have been shown to affect proliferation and invasion of diverse cancers, including, gastric [126], lung [127], pancreatic (reviewed in [128], and breast (reviewed in [129]). In GBM, collagens have been linked to increased invasion [130] and stemness [131], two key factors in GBM therapy resistance. While long term radiation-induced fibrosis is well characterized, the early response to radiation of these pro-fibrotic factors is less well understood, yet it can clearly influence important factors in disease progression and therapy resistance

As one example of the potentially important and complex interplay of stromal cells, tumor cells, signaling, and ECM, we can examine integrin and fibronectin signaling. Blandin et al. showed that α5-integrin promotes cell-cell adhesion in a low-fibronectin environment but cell dispersion in a high fibronectin environment, emphasizing that outcomes of cancer intrinsic functions of glioma cells are dependent on microenvironmental factors that might be mediated by stromal cells [132]. Indeed, it has been shown in a rat xenograft model injecting human glioma spheroids, that fibronectin, laminin, and collagen type IV were produced by the host [133], further supporting the importance of stromal cells in this signaling pathway. Belkin et al. showed that M1 matrix metalloprotease secreted by cancer cells could degrade TGM2, but only in low fibronectin environments, suggesting that stromal cells and cancer cells can enter important feedback cycles regulating the matrix [134]. As matrix proteins like fibronectin, its modifiers like TGM2, as well as its binding partners, like integrin-family protiens, are all affected by radiotherapy in both the stromal and tumor cells [14] (reviewed in [40,56] signaling networks such as these paint an increasingly complex picture which requires close attention to overcome therapy resistance.

5. Conclusions

It has become increasingly important to understand the complex environment that makes up a tumor to develop effective therapies. The ability of tumors to evolve and adapt, while previously attributed largely to the rapid changes within the diverse population of tumor cells, can now be appreciated to take place in an adaptable ecosystem that changes and evolves based on intrinsic changes in stromal cells as well as tumor cells. A notable example of this is the 2010 study by Gilbert and Hemann, showing that doxorubicin-induced DNA damage causes IL6 release from the thymus, which in turn creates a pro-survival environment for tumor cells [135]. Notably, this release preceded the appearance of SASP markers and did not promote proliferation but only survival. IL6 release is essential for thymic re-growth after genotoxic injury, and it is notable to consider that tumor cells take advantage of protections initiated by normal tissue to recover from damage and evade cell death [135]. Our work in GBM, showing that astrocytes respond to radiotherapy by secreting the radio-protective TGM2 [14], suggests that this may be a relevant process in multiple cancer types. This review further highlights that complex interactions both among differing stromal cell types and between tumor and stromal cells can establish feedback and signaling networks which must be understood to develop effective therapies. Examining the response of normal tissue to therapy and how that might in turn feed back to protect and support tumors is an important avenue of research that may lead to new drug targets as well as potential targets to enhance existing therapies.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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