REVIEW

Addressing the Symptoms or Fixing the Problem? Developing Countermeasures against Normal Tissue Radiation Injury

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INTRODUCTION

The successful development of countermeasures for radiation-induced damage to normal tissue could potentially benefit a broad range of populations and conditions. At one end of the spectrum are patients in a therapeutic setting where prevention or mitigation of acute and delayed normal tissue effects might improve survival; an improved therapeutic ratio would offer clinicians the opportunity to raise radiation dose limitations, thereby increasing tumor cure rates. An additional benefit would be an improvement in the quality of life of survivor populations, a growing concern at the National Cancer Institute (1, 2). At the other end of the spectrum are personnel affected by a mass casualty situation such as a deliberate or accidental nuclear or radiological event (3). Unlike approaches that might be of use in the highly defined clinical setting, countermeasures that would be utilized in an emergency event require broad activity since they will be targeted to radiation injuries resulting from, in all likelihood, relatively unknown dose exposures, involving heterogeneous volumes that may have affected multiple tissues and organs. Furthermore, agents in this category must be easily deliverable with minimal (or preferably no) toxicities (4, 5). Although overall, this latter category of countermeasures may prove to have little

immediate apparent commercial value other than placement in the strategic national stockpile, however the potential does exist for crossover usage in the clinic, as well as for the equally broad need of reducing the risk of delayed or late effects such as carcinogenesis, cataracts, cardiovascular disease, etc. (6) The potential risk for late effects is not limited to acute high doses, but also prolonged low-dose exposures associated with occupational or environmental exposures (7), as well as the chronic exposures to high-linear energy transfer (LET) cosmic radiation encountered by astronauts during long-term missions (8). It remains unclear whether this spectrum of conditions will respond to similar mitigation strategies.

Given such a broad potential market, it is not surprising that many researchers have chosen normal tissue radiation biology as their field of interest. The importance of this field to advancing cancer therapy, particularly for radiation oncologists, is underscored by the number of past and current world-renowned experts in the field of radiation research who dedicated their careers to this area, including Julianna Denekamp, Rodney Withers, Elizabeth Travis and Richard Hill, to name just a few. However, to develop valid radiation countermeasures, particularly for emergency use in the U.S., there is a Food and Drug Administration (FDA) licensure requirement for a clear understanding of the biology that underlies the target condition (9, 10), i.e., knowledge of the mechanisms that lead to the development of radiation-induced normal tissue effects. Unfortunately, the random nature of ionizing radiation injury at both the cellular and tissue levels and the complexity of the resultant pathological and physiological changes have combined to confound investigators, despite decades of research, so that the development of viable radiation countermeasures for either the clinic or accidental exposure has remained relatively elusive. The resurgence of interest in this field, prompted by the events of 9/11 and, more recently in Fukushima, has opened new insights and may offer some degree of hope (11). This review offers a reflection on how

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FIG. 1. Clinicopathological course of radiation injury. [Figure modified from Rubin and Casarett (12)].

far this work has advanced of late and a proposal for a paradigm shift in thinking and approach.

In the mid 1960s, two investigators, Rubin and Casarett, took a holistic rather than a “target cell” approach to radiation-induced normal tissue effects, describing a hypothetical progression (12) that was derived from their respective observations of the clinical and pathological events after radiation therapy. In their proffered theoretical schema (Fig. 1), Rubin and Casarett suggested that the so-called latent period, defined clinically as the asymptomatic span of days to weeks seen in patients after exposure, was instead an interval of accumulating biophysical damage; they further proposed that this progressive injury could remain unrealized or be fully expressed once an unspecified threshold of tissue or organ damage is crossed. As can be surmised from Fig. 1, they also considered acute and late effects to be related and/or interdependent events. One of their overarching hypotheses was that the chronic disease observed in previously irradiated tissues was almost exclusively predicated on a radiation-induced deficit within the microcirculation, itself a consequence of early cell loss from the vascular endothelial cell population, coupled with a subsequent inability to adequately repopulate the injured volume (12, 13). This theory contrasted with the more prevalently acknowledged target cell hypothesis from which the alpha/beta model was derived (14, 15); this hypothesis presupposes that radiation-induced normal tissue effects are dependent on the kinetics of critical cell populations within the target tissue parenchyma. However, given the technical advances in molecular science since these hypotheses were first espoused, the fact that the underlying mechanisms of normal tissue effects continue to be “theoretical” is a source of ongoing frustration to many investigators within the field, and now rises to an imperative need in the arena of countermeasure development, where mechanism identification is a requirement not only for FDA licensure, but also for designing approaches and validating targets.

Despite this ongoing deficit in our mechanistic understanding of the initiation and progression of normal tissue effects, particularly those defined as delayed or late, there are a number of common themes or events that appear to be probable contributing factors. First, from the predominant use of pre- or early postirradiation administration of investigative agents, it appears that most researchers assume that the immediate events after radiation injury are critical, not only to the initiation of the acute normal tissue effects, but also to the late outcomes, and therefore provide logical targets for countermeasure intervention. Second, given that ionizing radiation imposes a physical injury, albeit at the molecular rather than macro level, it is reasonable to assume that it creates a “wound” within the irradiated tissue volume; thus factors associated with repair, both at the cellular and tissue level, may be considered as primary contenders for investigation, especially when there is ample evidence of radiation-induced compromised healing (16, 17). A number of reviewers have compared and contrasted the events seen downstream of radiation injury with those pathways associated with canonical wound healing (18–20).

In one of the frequently cited examples of such a review, Bentzen describes normal wound repair in terms of an initial inflammatory response regulated through cytokine and chemokine expression (Fig. 2) (20). He illustrates that, where there is physical cell loss, the inflammatory phase will be followed shortly thereafter by a proliferative phase involving locally surviving clonogens, cells migrating in from the periphery of the injured volume or recruited mesenchymal stem cells. Vascular damage leads to the onset of angiogenesis and, finally, wound healing culminates in scar formation, involving collagen deposition and tissue remodeling. Importantly, under normal conditions, the entire process is self-regulating so that, through various feedback loops, this final phase will terminate naturally (20). However, after a radiation injury, the wound healing process is disrupted in a fashion that is almost unique to

FIG. 2. Comparison of events seen following radiation injury with normal wound repair. [Figure modified from Bentzen (20)].

radiation exposure. Indeed, at every phase, ionizing radiation introduces a level of uncertainty that makes outcomes difficult to predict. For example, even with respect to cell loss, the magnitude of cell death is not only dependent on the radiation dose/volume, but also on the extent and integrity of DNA repair, the tissue architecture and phenotype of the various affected cell populations within the exposed tissue (21). As a result, even end points that are solely consequent to cell loss alone will vary, not only with respect to known and/or predictable radiation parameters (quality, volume, dose rate, etc.), but also from organ to organ and person to person. Furthermore, due to the random nature of ionizing events within each cell, the process of radiation-induced cell death itself can occur through multiple pathways in both normal and tumor tissues (21, 22). Fortunately for researchers, in the majority of normal tissues, death will occur through mitotic catastrophe (23, 24); as a result, the temporal appearance of cell death will be dependent on the cell's position within the cell cycle at the time of injury, as well as the overall population kinetics and, therefore, will occur at times ranging from hours to weeks, if not months, after exposure.

In addition to cell loss as a factor in normal tissue outcomes, the more interesting (and, likely, more confounding) events occur in the surviving cells. After irradiation, the cellular physiological outcomes include not only complete repair, but also a residue of cells with nonrepetitive, heterogeneous damage consequent to either the direct effects of radiation itself or inadequate or misrepair (25, 26). It is these damaged cells that likely play a critical role in downstream progression, either directly through autocrine signals or radiation-induced dysfunction, or indirectly by affecting other cells through paracrine or, possibly, bystander signaling. For example, although the expression of pro-inflammatory signals seen immediately after

injury is a recognized component of canonical wound repair, a recognized feature of the progression to delayed radiation-induced effects is a failure to self-limit the cytokine expression (19). Instead, many irradiated tissues appear subject to cyclical waves of cytokine and chemokine expression (27–29), with their attendant influxes of inflammatory cells and antagonistic pro-fibrotic responses. Although it is unclear whether the infiltration of immune and inflammatory cells (versus the signals themselves) represents the chicken or the egg in the normal tissue effects story, it is nonetheless possible that recognition of chronic cellular damage by recruited macrophages would result in a cascade of secondary and tertiary inflammatory events. Alternatively, radiation-induced dysfunction in the wound repair apparatus of damaged tissues may explain recurrent signaling due to a persistent failure to either complete repair or clear the injured cells. Whatever the biological explanation, this phenomenon, sometimes described as a “perpetual cytokine cascade” (27), is believed by many to be a critical player in normal tissue late effects, particularly with respect to radiation-induced fibrogenesis.

Another theorized outcome of persistent cellular damage, found specifically within the mitochondrial apparatus, is the downstream generation of reactive oxygen species leading to chronic oxidative stress (30, 31). Alterations in the redox state may arise consequent to, or independent of, tissue hypoxia within the irradiated volume (32, 33), itself hypothesized as being the result of microcirculatory injury (34). Furthermore, increased free radical production and changes in tissue antioxidant capacity as a result of the interactions between inflammatory cells and damaged cells within the parenchyma (35) may also contribute to persistent (chronic) oxidative stress.

For years, each of these factors (cell loss, chronic cellular damage, altered redox state, dysregulated signaling) have

provided researchers with potential targets for countermeasures with varying degrees of success (26). Thus, given the recent upsurge in interest, can any of these factors continue to serve as potential critical target(s) in countermeasure development or should we look beyond the empirical conclusions?

COUNTERMEASURE EFFORTS: TARGETS/ APPROACHES/ALTERNATIVES

Targeting Cell Death/Loss

Since radiation effectively induces cell death, a large cadre of investigators has proposed altering outcome by interfering with the associated pathways, with many in the therapeutic realm looking at mechanisms associated with apoptosis (36, 37) and more recently, autophagy (38, 39). However, the majority of these approaches have focused on increasing cell kill in the tumor, rather than its reduction in normal tissues. Only in the field of radiation protection has preventing cell death represented a “holy grail”, with a major focus on protecting the critical normal tissues associated with the acute radiation syndromes, i.e., the bone marrow, gastrointestinal tract and skin. These tissues have an architecture that is largely dependent on stem and progenitor cell populations and therefore, appear acutely radiosensitive due to their proliferative phenotype (26, 40). However, importantly, cell loss also can affect function in organs dominated by post-mitotic, fully differentiated cells, such as the heart, lung and kidney, which are characterized as late responding organs (41–43). This observation led Withers to propose that it is a deficit in mature parenchymal cells, rather than in the microcirculation, that forms the basic lesion underlying radiation late effects (44, 45). Since this hypothesis was put forward, chronic cell loss associated with dysfunction in late responding tissues and organs has been observed by many investigators; for example, recent findings have suggested that radiation-induced loss of neurogenic stem cells may culminate in long-term deficits in brain function, posing a potential risk to astronauts during space travel (46). Although a direct correlation between acute stem cell loss from the hippocampus and delayed radiation effects in the brain has yet to be shown, catastrophic decrements in cognitive function have been seen in pediatric cancer survivors who were treated with whole-brain irradiation (47, 48), with IQ losses as great as 20 points observed in the youngest patients (49). Recent studies have also indicated a risk of cognitive dysfunction in adult cancer survivors (50), suggesting that this outcome may not simply be a consequence of a radiation effect on brain development; this finding has led to increased efforts by clinicians to shield the hippocampus in all patients (51, 52).

Not surprisingly, given their role in overall tissue maintenance and homeostasis, delayed effects subsequent to acute radiation-induced stem or progenitor cell loss are not limited to the brain. For example, studies from Booth *et al.* indicate evidence of chronic changes in the gastrointes-

tinal crypts after high-dose irradiation, and the authors equated these changes to accelerated aging within the surviving clonogenic stem cell pool (53). Stem cell-dependent effects also have been observed in organs less recognized for their hierarchical nature. For example, Johnston *et al.* has described the progressive loss of a putative stem cell population, the Club cell, from the airways of irradiated mouse lungs (54). This dose- and volume-dependent deficit was associated with an increased susceptibility to subsequent aerosol infections, such as flu (55), with a differential response noted between adult and pediatric models (55, 56).

Since radiation-induced cell loss may explain elements of both acute and delayed normal tissue effects, an obvious countermeasure strategy is to prevent the lethal injury occurring in the first place (i.e., radioprotection) through, for example, the administration of free radical scavengers. However, to maintain a positive therapeutic ratio, the use of such an agent in the clinic requires differential protection/uptake levels between normal tissues and tumors. This necessity likely explains the limited number of agents developed with this mode of action after more than 50 years of investigation. Indeed, despite the herculean efforts made in the 1950s–1980s, the free radical scavenger, amifostine (ethiol/WR-2721), remains one of only two radioprotection agents that have made it into clinical practice, and then in only limited practice (57, 58). However, when considering protection measures for the general population, where there is no definitive need for a differential tumor/normal tissue response, a broader range of agents has been assessed. While many of the potential countermeasures in this category are partly or wholly antioxidant in nature, such as the vitamin-derived tocopherols and tocotrienols (59), it has been suggested that the free radical scavenging mechanisms associated with such compounds will unlikely be sufficiently reactive to enable efficient removal of the primary and secondary radicals associated with high doses of radiation (60). Therefore, any observed success more likely comes from alternative or additional modes of action, such as effects on cytokine expression (61). However, it is worth noting that in scenarios where protection strategies are needed against relatively low, albeit chronic, exposures, such as the space environment, the low toxicity risk associated with purely antioxidant approaches could prove to be of considerable benefit. The Kennedy group has demonstrated a reduction in the incidence of radiation-induced cataracts, a consequence of cell loss from the germinal zone of the eye lens, through the use of an antioxidant diet; this approach also showed evidence of reducing carcinogenic risk (62).

An alternative source of intracellular free radicals are the mitochondria, organelles that are also intimately involved with the cytosolic apparatus inherent in their control, leading some investigators to focus on mitochondria and their associated detoxification pathways as potential sources of radioprotection (63, 64). For example, the Pittsburgh

Center for Medical Countermeasures against Radiation (CMCR) has developed a series of novel TEMPOL-derived mitochondria-targeted GS-nitroxides (65) that inhibit cytochrome c peroxidase activity, blocking acute apoptosis and reducing early lethality after cytotoxic doses of both chemotherapy and radiation. Of note, the most promising results published to date for the lead candidate, JP4-039, when administered alone, were seen when the agent was delivered in close temporal proximity to irradiation [within minutes to hours either pre- or postirradiation (66)], limiting its utility after a large accidental or deliberate radiological event.

Moving beyond radioprotection *per se*, one approach that has had some success in the clinic, particularly with respect to countering cell loss in the hematopoietic compartment, has been the employment of cell population expansion techniques through the administration of colony-stimulating factors. Interestingly, this strategy was at one time proposed as a form of radioprotection since administration at a sufficient interval prior to exposure would increase the number of potential radiation targets. In the late 1990s, it was shown that the administration of keratinocyte growth factor (KGF) improved acute survival when given prior to either cytotoxic chemotherapy or irradiation (67), and has subsequently been approved for use as a mitigator of delayed development of oral mucositis (68). More commonly, however, this approach is used as treatment for therapy-induced cytopenias, particularly those induced by the older chemotherapeutic agents. Indeed, as a result of their success with respect to the treatment of chemotherapy-induced neutropenia, granulocyte colony-stimulating factor (G-CSF/filgrastim) and PEGylated G-CSF (pegfilgrastim) have been added to the strategic national stockpile as countermeasures to myelosuppression for use in the aftermath of a large-scale radiation event (69) and recently received FDA approval for treatment of hematopoietic syndrome (70, 71).

Unfortunately, although preclinical data (72, 73) supports the use of these countermeasures to treat patients at risk of developing or suffering from acute radiation syndrome (74), there is little to no evidence that this approach mitigates radiation-induced delayed or long-term effects. Instead, there is a growing amount of preclinical (75, 76) and clinical evidence from the bone marrow transplant field (77, 78), as well as the published finding from radiation accidents such as Tokai-mura (79), that despite the early normalization of circulating cell numbers, measures that solely target recovery of the bone marrow stem cell population numbers fail to restore a fully functional hematopoietic compartment and do not protect against downstream systemic late radiation-induced effects.

One possible explanation for this failure is that strategies that target stem cells alone do not address the injury to the hematopoietic microenvironment, which may be an equally critical lesion; this hypothesis has led some investigators to investigate countermeasures targeting the entire niche. For

example, pleiotrophin (PTN) is a neurokinin secreted by bone marrow endothelial cells, and studies have suggested that PTN promotes retention and self-renewal of long-term hematopoietic stem cells (LT-HSCs) (80). One CMCR group has recently shown that systemic administration of PTN, even when given at 48 or 96 h postirradiation, not only reduced acute mortality after a lethal dose of total-body irradiation (TBI), but also improved outcome after bone marrow transplant (81). Their investigation has indicated that the underlying mechanism for this efficacy was through the RAS/MEK/ERK pathway, with additional intriguing evidence suggesting an alternative mechanism related to PTN's role in promoting retention of LT-HSCs in a quiescent phase, i.e., allowing more time for DNA repair. Similarly promising results, again targeting the hematopoietic microenvironment, have come from a number of groups using parathyroid hormone (82, 83), an agent known to expand the osteoblastic lineage (84), they have demonstrated not only an overall survival benefit after lethal TBI, but also improved long-term cellularity in mouse femurs (82).

While another logical approach to correcting a radiation-induced cellular deficit is the use of cell transplantation, enthusiasm for this is limited due to frequently observed long-term risks, such as nephropathy and pneumonitis, in the bone marrow transplant population (85, 86), as well as the somewhat ambiguous results after use of this strategy in Chernobyl (87). Nonetheless, with the growing appreciation for the role of stem and/or stem-like cells in normal tissue maintenance, there is also evidence that end points associated with cell loss from tissues other than the hematopoietic compartment may benefit from such an approach (88). For example, the Limoli group have shown that at one month after 10 Gy irradiation to the head only, rats clearly showed less interest in, and possible aversion to, a novel versus familiar location compared to unirradiated animals when using the novel placement recognition assay as a means of assessing hippocampal stem cell integrity. Importantly for this review, transplantation with human neural stem cells at time points from 2 days to 4 weeks postirradiation mitigated this deficit (89). However, significant safety issues arise when contemplating the use of stem cells, including the heightened risk of teratoma formation, GVHD and genomic stability, along with considerable technical issues with respect to compatibility testing and the requisite scale-up that would be needed for clinical use (88).

Some of the problems associated with pluripotent stem cells may be mitigated through the use of mesenchymal stem cells (MSCs), which are capable of migrating to injury sites and, once there, differentiating into fully functional mature tissues (90). Alternatively, MSCs may provide a scaffold for wound repair, rather than serving as replacement cells (91), and have the added advantage of modulating both pro-angiogenic and anti-inflammatory characteristics (90, 92). Work from the Guha group has provided positive preclinical support for such an approach, using stromal cell transplant after gastrointestinal ablative

FIG. 3. Irradiated mouse lung at 10 weeks after 12.5 Gy whole-thorax irradiation, comparing the response between C57BL/6J (panel A) and IL-1R1 (panel B) knockout female mice. Lungs were stained with F480 and hematoxylin. The lungs from the IL-1R^{-/-} animals showed a robust infiltration of macrophages (black arrows), thickened septa and significant presence of edema (pink staining) compared to the irradiated C57BL/6J wild-type (20× magnification).

doses of radiation (93). Interestingly, their work indicated the need for both CD11b-positive and -negative populations, with a mixture of MSCs, macrophages and endothelial progenitor cells providing the best results, further supporting the need for strategies that target multiple cell types. The therapeutic potential of MSCs has certainly stirred the imagination of clinicians and researchers alike (94, 95), with early work from France indicating their successful use in combination with surgery for treating radiation burns (96). The administration of MSCs has demonstrated success in the mitigation of ongoing cardiovascular disease, albeit in a single patient (97), a possibly important observation given the significant risk of radiation-associated cardiovascular disease seen, for example, in Japanese A-bomb survivors (6).

Targeting Inflammation

Despite the decades-long discussions of whether or not the early inflammation and delayed fibrosis seen in many late-responding organs are a continuum or two distinct and independent phases, chronic inflammation is nevertheless a characteristic component of many radiation-associated diseases. Thus, many investigators consider anti-inflammatory approaches to be “low-hanging fruit”. Certainly, there have been a plethora of preclinical (98, 99) and clinical investigations (100, 101) determining the efficacy of using anti-inflammatory agents as mitigators of acute and/or chronic radiation-induced outcomes, although to date, the results from many clinical trials have been mixed. The use of steroids has shown considerable success, particularly in the treatment of radiation-induced lung end points (102, 103), although their long-term administration can lead to

multiple issues, most notably being the rebound effect that can occur after withdrawal. Nonsteroidals similarly have produced mixed results, both preclinically (104, 105) and clinically (105, 106). At the preclinical level, an anti-inflammatory approach continues to be a popular strategy investigated by many groups, buoyed by relatively positive results with respect to studies examining the efficacy of isoflavones such as genistein (107, 108), as well as agents deemed to have secondary anti-inflammatory activities such as statins (109–111). However, the broad-spectrum activities of many, if not all, of these agents suggests that, like the antioxidants, their observed efficacy may not necessarily be through an inflammation pathway alone.

One possible explanation for the variability in outcome with this class of agents may be the dosing schedules employed in the various studies, since administration too soon after exposure could impact the beneficial role played by early inflammation. Indeed, the recruitment of inflammatory cells into an injured volume is one of the first and, possibly, most critical steps towards normal wound repair (112, 113), and there is evidence that this is also true after irradiation. For example, Wang *et al.* have shown that radiation enteropathy is exacerbated in the absence of mast cells (114), and our group similarly has demonstrated an increased radiation-induced pneumonitic response in the lungs of a knockout mouse model deficient in interleukin-1, a key pro-inflammatory signal (Fig. 3). Nonetheless, interference with the delayed recruitment of inflammatory cells, using, for example, clodronate, a bisphosphonate with anti-inflammatory properties, has led to significant reductions in late therapy-related diseases, such as pulmonary fibrosis, neuropathy and osteoradionecrosis (115, 116).

Taken as a whole, these data suggest that when anti-inflammatory therapies are used to reduce or mitigate acute or chronic radiation-induced inflammation consideration should be given to the temporal sequence of events that make up normal wound healing to prevent further disruption of the dysregulated environment created by radiation damage *per se*. As has been suggested for other conditions where acute injury precedes interstitial inflammation and gliosis/fibrosis, such as traumatic brain injury or renal ischemia (117), it is unlikely to be the prompt appearance of inflammatory cells that disrupts the wound healing process, but instead, the failure to remove those cells once regeneration is complete (113). Furthermore, the differential roles played in the response to radiation injury by subgroups within many of the recruited immune and inflammatory cell populations (118) and their differential responses to both intrinsic and extrinsic signals from the disrupted microenvironment comprise an exciting and evolving field of investigation; future insights in this area will likely significantly impact our understanding of the postirradiation process. Anticipating such knowledge, a more fruitful “anti-inflammatory” approach may therefore be to investigate the mechanisms underlying the localization and kinetics of the delayed mononuclear infiltrate (monocytes/macrophages and T cells) (118, 119), potentially leading to the development of strategies that target their response to, rather than their effect on, the radiation-altered microenvironment. Significantly, the contextual inflammatory and immune responses of tumor versus the surrounding normal tissues to radiation therapy during pre-, peri- and post-therapeutic periods will be of increasing clinical importance with the growing interest in, and use of, immunotherapy (120, 121).

Targeting Chronic Oxidative Stress

Given the abundance of evidence indicating a temporal disconnect between acute inflammatory signaling and the delayed recruitment/infiltration of inflammatory cells, the development of chronic inflammation may not necessarily be a direct consequence of dysregulation in the acute wound healing mechanisms. Instead, some investigators have proposed that it is the perpetuation and accumulation of radiation-induced mitochondrial damage, the result of less efficient repair mechanisms inherent in these organelles (122), that is the major factor contributing to chronic oxidative stress levels (30, 35). There is accumulating clinical evidence that polymorphic variants of genes associated with the oxidative stress response, e.g., GSTP1, GSTA1 and eNOS, are associated with the risk of developing downstream radiation disease, such as fibrosis (123). Such findings have prompted strong interest in the potential utility of agents that affect cell redox status. For example, a number of groups have investigated the efficacy of various salen-manganese compounds, which mimic the antioxidant enzymes, superoxide dismutase (SOD) and catalase. One of the best performing of these to date,

EUK-207, has exhibited activity as a radiation protectant (124), as well as a mitigator in a number of radiation-induced conditions, including radiation fibrosis, pneumonitis and dermatitis (125–127). A compound with a similar activity profile, AEOL 10150, an SOD mimetic without the catalase activity of the EUK series, also has been assessed for its efficacy as a potential countermeasure against a number of different terrorism-related delayed injuries, including radiation and chemical-biological attacks (128, 129). Although these and similar agents continue to show promise, as suggested earlier (60), their activity as free radical scavengers may not be sufficient to address the spectrum of affected pathways inherent in radiation-induced disease end points and likely will find greater utility used as part of a combination therapy.

Altering Cytokine/Chemokine/Growth Factor Signaling

The complex network of intra- and extracellular signals that many researchers have characterized during the progression from immediate radiation injury to both the early response and, especially, the late end points (130, 131) is an attractive, although relatively overwhelming, target for countermeasure development. The expression of major pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α), the interleukins (IL)-1 and IL-6, can be detected within minutes to hours of irradiation (132, 133), and multiple investigations have made attempts to modify this expression (134–137). Despite the strong likelihood that the immediate signal expression effects the initiation of wound repair, a recent SNP analysis has shown that genetic variation in many of these inflammatory genes correlates with radiation-induced toxicities, such as pneumonitis and esophagitis (138), supporting their potential role in injury progression. Growth factors associated with tissue remodeling, such as transforming growth factor- β (TGF- β), have been similarly targeted with the expectation that their suppression will mitigate the development of late conditions, in particular fibrosis (139, 140). Again, such strategies are considered despite the multifunctional roles of factors such as TGF- β , including acting as a counterbalance to pro-inflammatory signals and regulating wound repair (19, 141). Indeed, because of their pluripotent nature and intimate relationship with wound repair, rapid and intermittent increases in cytokine expression are seen in response to a wide range of toxic injuries (142–144), although in general, these resolve without leading to the spectrum of long-term detrimental outcomes observed after irradiation. So what currently remains unclear is the conditions under which, and the reason(s) behind why the molecular signaling response specific to radiation injury diverges from that associated with normal wound healing, leading to the development of radiation-induced normal tissue effects.

In a Failla Award lecture dedicated to normal tissue injury (19), McBride *et al.* drew parallels between radiation injury and the immunobiological responses associated with

FIG. 4. Radiation-induced cytokines and their homeostatic balance under wound conditions. Green circles connected by lines represent positive regulation by interactions along the line. Yellow circles joined to green circles represent negative regulation and mutually antagonistic effects. [Modified from McBride *et al.* (19)].

pathogens. They succinctly described the canonical and orchestrated responses that are seen after cellular and tissue injury, highlighting the changes in cytokine, growth factor and chemokine expression that form a highly regulated network of autocrine and paracrine communications. Furthermore, they noted that, through positive and negative feedback signals, most injured tissues maintain a homeostatic balance between inflammation and angiogenesis, culminating in a return to baseline conditions once a wound is fully repaired. The authors then went on to describe how, after some stress conditions, with particular reference to high-dose irradiation, the homeostatic balance is disrupted, becoming overtly tipped towards either chronic inflammation or excessive scarring (Fig. 4). Although this hypothesis likely gives undue weight to the role played by the immune system in the radiation response, and fails to identify the trigger for imbalance, work in other toxic injuries nevertheless indicates that it is entirely plausible that radiation-induced cytokine dysregulation leads to the creation of a pro-inflammatory, M1-mediated (destructive) microenvironment (145–147), a condition that would promote the development of the “itis-es” associated with early radiation reactions, e.g., dermatitis, pneumonitis, nephritis. Similarly, tipping the cytokine balance towards a more overtly aggressive M2 state would lay the groundwork for the dysfunctional tissue remodeling that typifies the late fibrotic response seen in many tissues and organs (118, 147–149).

Targeting Microvascular Injury and/or Hypoxia

A final mechanism for consideration is the role played by the injured microcirculation in radiation-induced normal

tissue effects, the primary critical defect identified by Rubin and Casarett (12, 13). After irradiation, there is a dose-dependent, acute loss of endothelial cells (up to ~15%) followed by progressive loss over the ensuing weeks to months with doses >2 Gy (150). This can result in vascular pruning and loss of vascular density (151, 152), accompanied by tissue hypoxia. At higher doses, vasogenic edema, a consequence of wall rupture, can also lead to hypoxia. Thus, some investigators have proposed that hypoxia is a significant factor in the progression towards late effects (153). Interestingly, because free radical generation has been shown to be elevated under conditions of low oxygen concentration (154), the presence of hypoxia may provide additional support for the role of oxidative stress in radiation injury. Furthermore, it has been suggested that the presence of both ROS and hypoxia regulates macrophage activation and polarization (155, 156), conditions shown to be associated with late radiation injury (148, 157).

With respect to direct radiation injury of the vasculature *per se*, recent elegant studies from the Milwaukee group have demonstrated the delayed pruning of small vessels from such tissues as the heart and lung (152, 158). This has led researchers to suggest that the significant mitigation of cardiopulmonary and renal damage seen after postirradiation administration of a number of ACE inhibitors (159, 160) was due, at least in part, to the agents’ effects on the vasculature, since there was an observed reduction in radiation-induced vessel loss and an improvement in vessel distensibility (152). Although the vasculature may indeed play a role in late effect pathology, the broad physiological contributions of the renin-angiotensin system to inflammation, redox signaling and cellular proliferation (161–163)

FIG. 5. Interrelationship between homeostatic spheres of imbalance after irradiation.

temperers this conclusion since the ACE inhibitors are likely affecting a wide range of systemic and local physiological systems beyond their more commonly associated pharmacologic role in correcting hypertension.

DISCUSSION

There are few who would argue that radiation therapy has not contributed significantly to current cancer treatment, with approximately 50% of all cancer patients receiving radiotherapy at some point during their treatment (23). Although some branches of oncology may argue that the observed recent improvements in outcome rates of many cancers have little to do with radiation delivery, the technological advancements seen in the field, such as image-guided delivery, respiration gating and altered fractionation protocols, cannot be discounted. However, despite the radiation oncologist's ability to tightly conform beams to a tumor, thereby minimizing normal tissue involvement, the majority of cancer survivors are still plagued by side effects of their treatment (164–167). Although some of the outcomes are relatively benign, many are serious and even life threatening, drastically affecting patients' quality of life. Blame for these issues cannot be laid solely at the feet of radiation oncologists, nonetheless eliminating, or simply reducing the risk of radiation-induced disease in normal tissues would provide a profound benefit to the growing population of cancer survivors. This rationale, together with the current geopolitical risk of a terrorism-related radiological or nuclear event that may involve mass casualties, provides a strong justification for a concerted effort to develop agent(s) that will counter the acute and chronic changes

seen in normal tissues after irradiation, a goal that has, unfortunately, remained elusive for over 70 years.

To date, the vast majority of investigations in this field have evolved and revolved around the conditions and symptoms seen during the immediate and early stages of progression of normal tissue effects. As described in this review, such efforts have therefore focused on the development and assessment of agents that target acute pathophysiologic outcomes, such as cell loss, oxidative stress, molecular signaling, as well as the delayed, more overt, symptoms, such as chronic inflammation and fibrosis. It is therefore not surprising that, in the recent upsurge in countermeasure efforts prompted by the events of 9/11, focus has mostly been on developing agents that counter the acute symptoms and syndromes associated with radiation injury. But as suggested by McBride *et al.* (19) and others (168), the acute symptoms themselves may not be end points per se, but a reflection of dysregulated homeostatic mechanisms that are consequent to, or the initiators of a disrupted microenvironment (Fig. 5). Thus, no single approach, targeting only one of the pathways or conditions, is likely to provide long-term normalization of an irradiated tissue or organ, explaining the limited efficacy seen with respect to late outcomes after successful treatments of acute symptoms in accident victims and bone marrow transplant patients (79, 169).

If, as it appears from the evidence, irradiation creates a multiplicity of disrupted conditions, then a more rational approach to long-term mitigation would be the use of combination therapies, targeting two or more different aspects of the disrupted environment. The likelihood of success would be improved further if agent administration is temporally informed, possibly through the use of biomarkers that indicate pertinent levels of dysregulation.

Support for such an approach has been seen in preclinical models, with combined therapies of, for example, antioxidants administered together with an anti-inflammatory and/or an ACE inhibitor, showing greater than additive effects when compared to the efficacy of the single agents alone (107, 127, 170). Importantly, for treatment purposes after either localized or whole-body injuries, the contributions that the variously disrupted homeostatic conditions will make towards normal tissue outcomes will, in all probability, vary from organ to organ, given the inherent differential pathophysiology and functional diversity that make up the tissues of the human body. For example, in addition to compensating for hippocampal stem cell loss through the use of expansion or replacement therapies, mitigation strategies for the brain may require additional measures that will normalize the vasculature due to the unique and critical properties of the blood-brain barrier. In contrast to this cell loss/vascular approach, the successful use of pentoxifylline and alpha-tocopherol together with clodronate in the treatment of osteoradionecrosis takes advantage of their anti-inflammatory and antioxidant properties, respectively (171).

Other, more general characteristics may also affect combination treatment considerations, such as age, sex, smoking status, etc. Our group has demonstrated, in both adult and neonate models of lung irradiation, that low-dose irradiation leads to late susceptibility to pulmonary infections (54–56). However, further investigations have indicated these altered immune responses are subsequent to effects on different cell populations dependent on the age at the time of irradiation, i.e., loss of epithelial (club) cells (54) in the adult animals versus alterations in secondary lymph organs in the neonates (172), indicating that age and/or development status may also inform mitigation strategies. It is therefore likely that combination approaches will need to be tailored to the most critically affected organs or those most at risk, depending on the affected volume.

Under mass casualty conditions, prioritization of treatment strategies will be a daunting task (173), with clinicians possibly having to use unreliable indicators of exposure, such as areas of skin erythema, to indicate the organs with the highest exposure levels. The validation of circulating biomarkers that could indicate damage in specific organs, such as club cell secretory protein (54) and citrulline (69, 174) for the lung and gut, respectively, may provide invaluable assistance to emergency medical personnel when determining therapy under uncertain conditions. In contrast, determining tissues and organs at risk will be a relatively easier task in the radiation oncology clinic, given the highly defined and circumscribed nature of the modern radiation treatment volume. However, the presence of a tumor in the radiation field adds additional layers of complexity to our understanding of normal tissue injury. Indeed, it is currently unknown as to how the presence of a tumor within a tissue

or organ affects the response of the surrounding normal tissue to radiation and vice versa.

With respect to countermeasure development for the clinic, significant care will need to be taken to ensure that measures to mitigate and/or treat radiation-induced damage in normal tissue do not adversely affect the overall goal of eliminating the cancer. But it is intriguing that many of the identified normal tissue changes described in this review as consequences of irradiation mimic the effects that a tumor imposes on its immediate environment. For example, the induction of capillary leakiness seen in the brain (175, 176), lung (177), skin (178), etc., which has been associated with upregulation of VEGF (176, 179) and hypoxia (180), is reminiscent of the increased vascular permeability (181, 182), aberrant pro-angiogenic signaling (183, 184) and areas of hypoxia (185, 186) characteristic of many tumors. Similarly, there is a growing understanding of the transition in macrophage state between M1 and M2 associated with the progression of radiation-associated late effects (118); alterations in the M1:M2 ratio are a common feature of the tumor microenvironment (187, 188) and are associated with immune tolerance (189, 190). Furthermore, chronic inflammation is now recognized as major factor in malignant disease (191).

In conclusion, these parallel states suggest that countermeasure strategies that normalize the irradiated microenvironment in order to mitigate normal tissue injury might have the added advantage of adversely affecting the pro-survival conditions generated by a tumor. This is not a new idea; for over a decade, Jain *et al.* have promulgated the suggestion that normalizing the tumor vasculature will improve therapeutic outcome (192, 193). However, we believe that “normalization” needs to move beyond the vasculature alone and instead, address multiple areas of radiation-induced pathologic disruption. By taking this multipronged approach, we believe that rational combinations of countermeasures will augment agents used in the immediate aftermath of a mass radiological or nuclear event, mitigating the delayed effects that, until now, have seemed almost inevitable. Furthermore, by targeting those areas of dysregulation that are common to both the tumor and the irradiated normal tissue microenvironment, we hypothesize that such countermeasures will also find utility in the clinic by improving the therapeutic ratio, not only by limiting the initiation and progression of normal tissue injuries that affect the quality of life of cancer survivors, but also by enhancing tumor therapy. By normalizing dysregulated homeostatic conditions within the entire treatment volume, there will not only be a reduction in normal tissue detriment, but by reducing the pro-survival advantages generated in the immediate tumor microenvironment, there will be the potential for limiting tumor recurrence and the likelihood of secondary malignant tumors by lowering conditions of genotoxic stress, a win-win for all.

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