

Molecular Mechanisms of Late Normal Tissue Injury

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Irradiation perturbs the homeostatic network linking parenchymal, mesenchymal, and vascular cells within tissues. Normal communication between cells through soluble, matrix, and cell-associated ligands and receptors is altered so as to set in motion a seemingly inexorable series of events aimed at tissue regeneration and healing. In late responding normal tissues where cell death is not compensated for by rapid regeneration, this process unfortunately often culminates in symptomatic complications of radiation exposure. Cytokines and their receptors are prominent in driving the cascade of molecular responses using the balance between seemingly mutually antagonistic molecules to control and direct the healing processes. There is strong evidence from preclinical models for the importance of cytokine-driven pathways in late radiation damage and growing evidence in humans for their relevance to radiation-induced disease. This review aims to show some general aspects of the molecular torments that drive responses in irradiated tissues before and during the development of late effects. It attempts to collate some of the findings from preclinical models of late lung, central nervous system, skin, and intestinal damage and from clinical studies in the belief that understanding how irradiation perturbs the cellular communication networks will allow rationale intervention for mitigating late radiation tissue damage and carcinogenesis.

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Cells acknowledge damage from radiation exposure through multiple sensor molecules and structures, including DNA, receptor tyrosine kinases, lipids, mitochondria, and proteasomes.¹ These sensors feed pathways that not only reprogram the cells to make appropriate internal responses but also produce extracellular factors that spread news about the “danger” to the tissue and the body and initiate molecular cascades that aim to orchestrate tissue repair processes. These tissue-healing responses contain many highly conserved features that might reflect their essential role in the evolution of multicellular and multitissue organisms. Characteristically, responses are highly integrated and show considerable redundancy in their use of effector molecules with overlapping functions, as befits such a critical process.

Not surprisingly, chemokines² and proinflammatory cytokines³ are highly prominent among the panoply of molecules expressed in tissues after irradiation. Perhaps less obvious is

the fact that anti-inflammatory cytokines can also be increased, some of which may participate in angiogenesis. This yin-yang cytokine balance is a common feature of inflammatory responses that is thought to reflect feedback control mechanisms regulated in time and space that allow phase transitions (eg, from proliferative to remodeling phases or for hypoxia-driven angiogenesis that is associated with wound healing). Cytokines, in turn, amplify further coordinated changes in additional cytokines, cell adhesion molecules, prostaglandins and leukotriene species, redox regulating enzymes and pro- and antioxidant species (manganese superoxide dismutase, inducible nitric oxide synthase, metallothionein, heme oxygenase, gamma-glutamylcysteine synthetase, and myeloperoxidase), matrix remodeling enzymes and inhibitors, plasminogen activators and inhibitors, and heat shock proteins. These components also often have mutually antagonistic aspects that allow tight control on the inflammatory and tissue-healing responses.

In many respects, the tissue responses to irradiation mimic the cytokine storms generated by many other tissue-damaging insults, although clinically relevant doses might be said to generate a cytokine breeze rather than storm. It is true that, unlike the low doses used in treating benign conditions,^{4,5} the accumulative doses that are used in cancer therapy are proinflammatory but each individual dose generates minimal

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inflammation. Indeed, one rationale for the use of low-dose fractionated protocols may have been the need to minimize inflammation resulting from high skin doses delivered by the early x-ray machines.⁶

Another aspect of the dualism inherent in inflammatory responses is the coexistence of tissue destructive components within a tissue-healing context. In other words, some cytokines may play a role in the pathogenesis of radiation damage as well as having a role in healing. Maintaining a balance between these mutually antagonistic forces within a changing microenvironment is critical. In this respect, there is an important and notable contextual difference between irradiation and other tissue-damaging insults. In irradiated tissues, proliferative and remodeling phases of healing proceed in sites in which functionally active cells harbor latent potentially lethal DNA damage and there are continuing episodic bouts of radiation-related cell death. The impact that cell death has on molecular and cellular responses in irradiated tissues has yet to be fully delineated. However, it is clear that molecular responses recur at periodic times in irradiated tissues, at least after damaging doses, as “waves.”³ Although it is possible that some radiation-induced cytokine-driven molecular responses become self-sustaining and chronic without radiation-induced cell death, it seems more likely that cell turnover and death are the reason molecular responses recur. If this is the case, then molecular responses in different tissues should have different periodicities in keeping with different turnover rates, although conclusive comparative studies are rare.

Similar molecules often seem to be involved in recurring responses, but this may in part be governed by what is under study. Later responses may be more dose dependent and more restricted in the molecules that are expressed,⁷ but the available evidence is minimal and allows no firm conclusion. Certainly, there is no a priori reason to believe that the molecular response recurs with an identical signature or has the same biological consequence, even if the same molecule is involved. Nor is there any strong evidence that the “waves” of response with time are causally linked. This is an important issue because it pertains to whether blocking an early molecular response can prevent the appearance of later complications. Currently available evidence is inconclusive on this issue. The very nature of “danger” signaling and its high level of redundancy suggest that it will not be easy to completely unravel all relationships until we understand the cellular processes that are associated with and impacted by each succeeding molecular phase. Our own studies tend to favor the view that the initial molecular responses to irradiation over the first few days bear little relationship to what happens weeks or months later, but there are dose-dependent molecular responses that precede overt damage that seem linked.^{8,9} Further research is, however, needed in this area.

The optimal outcome of the wound-healing process is regeneration of the original tissue with restoration of full function. Generally, complete regeneration requires balanced interplay between different cellular compartments, including a contribution from the postirradiation inflammatory infiltrate. Radiation-induced cell death or altered functionality in any

one compartment will affect this communication and the outcome. Acute responding tissues have a potentially large proliferative compartment, and, after clinically relevant doses of radiation, regeneration occurs early and will often be complete. This is not the case for late responding tissues that often have a small stem-cell component of variable significance and functional cells endowed with proliferative potential.

One consequence of failure of regeneration in many late normal tissues is fibrosis, a common complication of radiation therapy. Where tissue has been obliterated, radiation-induced fibrosis is enacted as the default pathway to fill the space, with a consequent loss of tissue function but maintenance of structure. Fibrogenesis itself may be a complex process and involve bidirectional interactions of epithelial cells with nonepithelial mesenchymal or interstitial cells, as in the lung, with expression of epithelial antigens reminiscent of fetal development.¹⁰ In fact, although fibrosis is often assumed to be an undesirable complication of radiation therapy, it may not always be a negative. The consequences of prevention of radiation-induced fibrosis could in some circumstances be more severe if it leads to structural collapse of the tissue. The intervention issue of whether prevention or reversal of fibrosis will allow for better normal tissue regeneration has not been fully answered.

The concept that molecular responses recur in tissues with time after exposure has many implications. One is for retreatment of cancer in which normal tissue tolerance is compromised by prior radiation therapy. Some molecular responses will be associated with attempted regeneration in response to cell loss that could lead to increased tissue tolerance. On the other hand, cells stimulated to proliferate might express latent DNA damage, and where these are structural or functional cells, as in the case of late responding tissues, an “avalanche” of lethality may be precipitated.¹¹ Having molecular markers that would indicate recovery or crisis in a particular irradiated tissue would be of considerable value for guiding retreatment. Conversely, understanding when the regenerative phase was underway would assist in elucidating the cytokines involved and identify targets for ameliorating late effects. If cytokines are being targeted, their role may vary with time after irradiation as phase transitions occur in the healing process. Picking the correct time for intervention could be critical to the outcome.

Inherent in the fact that the molecular tissue response to irradiation starts within minutes of exposure and recurs over and over again during the so-called latent period, when no clinical symptoms are obvious, up to the time when symptoms occur, is the concept that each dose of fractionated irradiation is given to a changing microenvironment and so the impact of each fraction may vary with time. For example, cytokines can alter intrinsic cellular radiosensitivity. In very general terms, signals that promote cell proliferation and survival tend to be radioprotective, whereas many antiproliferative and proapoptotic signals tend to radiosensitize.¹² Although there are probably exceptions to this rule, it is highly likely that the molecular responses to irradiation, including the development of hypoxia, will shift the position of the

cellular rheostat governing cell survival and death and the response to subsequent exposure.

The molecular characteristics of the tissue response to irradiation will be impacted by many factors, and it is often not clear how important the changes that are being observed are and what their spatial and temporal dependence is. Although there is information on some of the molecular changes in tissues with time, less is known about spatial dependency, which could be critical to the outcome. For example, although initial radiation injury is often diffuse, many late lesions, such as organizing alveolitis, brain necrosis, and fibrosis, often present focally and may progress. Little is known about the initiation of such lesions. They may result from local infiltration by inflammatory cells or local disruptions in blood flow because of endothelial cell damage or dysregulated interactions between distinct cell types. Information on the spatial relationships between initial damage, inflammatory infiltrates, vascular functionality, and these organizing foci would be of great value in understanding their origins. At a mechanistic level, such localized lesions may establish molecular gradients that spatially direct responses in the surrounding cells.

Given the high degree of redundancy in healing responses, it seems likely that multiple molecules will drive responses. Genetic factors will tip the balance toward different outcomes, but the nature of the participating cells and the tissue involved is probably what adds most to any unique time and dose dependency in the pattern of response. For this reason, we will compare and contrast what is known about the molecular responses in preclinical models of late radiation damage in the lung, central nervous system (CNS), skin, and gastrointestinal tract. Clinical correlates of these responses will be covered elsewhere in this volume.

Lung

Pneumonitis and fibrosis are distinct features of the lung's response to radiation damage. The former may occur after 6 to 16 weeks and is typified by inflammation and interstitial pneumonia. The latter is a more chronic response lasting months to years with progressive obliterative fibrosis. Mouse strains, particularly those with C3H and C57BL/6 backgrounds,^{9,13,14} vary with respect to their tendency to develop these 2 endpoints and have been exploited in genetic studies¹⁵ that will not be elaborated on here because they are dealt with in detail elsewhere in this volume (Travis). Type II pneumocytes have traditionally been considered "target" cells for radiation whose loss leads to inflammation, desquamation of epithelial cells from the alveolar surface, edema, and discharge of proteinaceous material into the alveoli. More recently, the cellular response has come to be viewed more in its entirety as involving multiple cell types with the outcome being dependent on the genetically determined molecular profile that drives the wound-healing process.

Similar to other tissues, the lung's response to radiation involves an inflammatory response, although in this case the infiltrates tend to be extensive and have been characterized in some detail.^{8,9,16} This is in part because of the ease with which

bronchoalveolar lavage (BAL) can be performed; however, it should be noted that this is only one compartment of the lung and is a poor reflection of what occurs in the lung as a whole.⁹ After initial decreases in total cell number, neutrophils then lymphocytes infiltrate and are found elevated in BAL for months.^{9,16,17} The initial loss is predominantly because of the loss of macrophages, although these recover with a turnover time of about 1 week and mitotic macrophages as well as foamy macrophages are found.^{8,17} Despite the evidence for dynamic changes within the BAL population, they contribute less to radiation-induced cytokine alterations than interstitial cells.⁸ Hong and coworkers⁹ suggested that a molecular and cellular change in the pattern of response in the irradiated mouse lung at 1 to 2 months might be a critical step in the development of radiation damage. The role of infiltrating, as opposed to resident, cells in this switch is not clear. Of interest is the possible role of lymphocytes. Westermann and coworkers¹⁸ isolated CD4-positive cells from irradiated rat lung that expressed IL-4 messenger RNA (mRNA), suggesting a Th2 phenotype that could enhance collagen production by fibroblasts. We have shown that thymectomy can decrease radiation-induced pneumonitis in the mouse, suggesting that T cells were detrimental.¹⁹ Hallahan and coworkers²⁰ have shown that antibodies to radiation-induced cell adhesion molecules, specifically intercellular adhesion molecule-1 (ICAM-1), that might be expected to be involved in promoting cellular infiltration, could block radiation pneumonitis.

Again, cell infiltration may be a double-edged sword that could both promote healing and cause tissue damage depending on the phenotype of the infiltrate and its interaction with parenchymal and endothelial cells, but modifying the infiltrate seems a possible means of therapeutic intervention.

Rubin and coworkers²¹ first used the cytokine "cascade" in the lung after irradiation to argue that there was no real "latent period" before the genesis of fibrosis and suggested that this was a self-sustaining continuous process. It is perhaps natural that much of the focus of cytokine studies in the lung has been on transforming growth factor β (TGF- β) because this cytokine has been implicated as a tissue sensor of oxidative stress²² and is implicated as "par excellence" in fibrosis. TGF- β 1 drives procollagen 1 production by fibroblasts, myofibroblasts, and other reparative cells through the Smad transcription factor pathway in addition to controlling many other aspects of extracellular matrix homeostasis.²³ TGF- β has, however, other numerous biological functions. These include inhibiting proliferation of many cell types including lymphocytes and type II pneumocytes²⁴ and being involved in carcinogenesis,²⁵ cell migration, and apoptosis.²⁶ In some models, TGF- β 1-induced fibrosis and remodeling requires Egr-1-mediated caspase-dependent activation of apoptosis,²⁶ providing a potential target for intervention.

After lung irradiation of fibrosis-prone C57BL/6 mice, Johnston and coworkers²⁷ found TGF- β 1 mRNA levels elevated 8 weeks after 5 Gy and 12.5 Gy and again at 26 weeks for 12.5 Gy, but not after 16 weeks, and not in fibrosis-resistant C3H mice. Although active protein was not evaluated, these data tend to support the idea that TGF- β 1 is involved in the onset of pulmonary fibrosis that was coinci-

dent with increased collagen I and III levels at 26 weeks after 12.5 Gy. In addition, collagen IV levels, indicative of basement membrane events, were increased after 8 weeks as well as at 26 weeks. Franko and coworkers²⁸ showed that immunoreactive TGF- β was present in fibrotic lesions of irradiated lungs of C57L/J mice that develop small, tightly packed areas of inflammation and exhibit progressive fibrosis but was absent in C3HeB/FeJ mice that develop classical pneumonitis.

Other cytokines have, however, been implicated in radiation-induced pulmonary injury. Johnston and coworkers² have shown increases in chemokines and chemokine receptors at 26 weeks in fibrosis-sensitive (C57Bl/6) but not fibrosis-resistant (C3H/HeJ) mice irradiated with a single dose of 12.5 Gy to the thorax. Buttner et al²⁹ reported elevated levels of the type 2 cytokine interleukin (IL)-4 in the rat lung after irradiation. Rubin and coworkers³⁰ reported that TGF- β mRNA was elevated at 2 and 8 weeks after lung irradiation in C57Bl/6 mice, but this was not sustained. However, IL-1 β mRNA, which was briefly expressed at 2 weeks, was highly expressed at 8 weeks through to 26 weeks during lung fibrosis, as was IL-1 α and tumor necrosis factor α (TNF- α).³¹ We showed that these proinflammatory cytokines and interferon-gamma (IFN- γ) were produced in both fibrosis-prone and fibrosis-resistant mice within a day of irradiation, before subsiding and reappearing^{8,32} over the next 1 to 2 months, with little difference between the two strains. Still, at the time C3H/HeN mice began to develop pneumonitis, their lungs had very high levels of TNF- α and IL-1 α/β mRNA, which was not the case for C57Bl/6 mice. IL-1 α/β and IFN- γ were highly expressed in C57Bl/6 mice when they later developed fibrosis, but the TNF- α response remained muted. The hypothesis was advanced that C57Bl/6 mice were better able to control their proinflammatory cytokines, especially TNF- α expression, possibly with TGF- β production, allowing them to survive longer, albeit with fibrosis.

Although these studies provide little more than vignettes of the molecular events in the lung after irradiation, they can provide some useful hypotheses as to the role of pro- and anti-inflammatory cytokines in radiation damage. For example, the links between TGF- β and TNF- α and IL-1 α and - β have been studied in some detail. TNF- α ³³ and IL-1³⁴ are known to upregulate TGF- β expression. However, simple induction says little about the interaction between these cytokines given the time-dependent complex expression patterns and multiple roles they play in the evolution of responses. TNF- α , IL-1 α , and β are often categorized as type 1 (T1) cytokines that are produced during active inflammation, whereas TGF- β is classified as a type 2 (T2) cytokine because of its antiproliferative, immunosuppressive, and profibrotic actions,³ as are IL-4, IL-10, and IL-13. In most situations, T1 and T2 cytokines are mutually antagonistic. The association of a T1 cytokine profile with pneumonitis and a T2 profile with fibrosis is therefore reasonable, as is the concept that cytokines like TGF- β might inhibit the development of pneumonitis and prolonging tissue function and life while allowing fibrosis to progress. This hypothesis has yet to be proven, but it suggests that targeting radiation-induced inflammation or fibrosis may not provide a complete answer to mitigating

radiation-induced lung damage and expanding the epithelial stem/precursor cell pool may be needed. Having said that, multiple approaches have been suggested that do target pneumonitis and fibrosis, including the use of T1 cytokines such as IFN,³⁵ restoration of the antioxidant balance,³⁶ inhibition of pneumonitis by transfer of soluble TGF- β type II receptor,³⁷ neutralizing antibody to TGF- β ,³⁸ and various other strategies, some of which have shown some success at least preclinically.

CNS

The cardinal features of radiation exposure of the CNS are demyelination, gliosis, and vascular damage. Pathologically, multiple alterations are seen, with the most serious being hemorrhagic necrosis and white matter necrosis that may occur with or without obvious vascular injury. The late effects that irradiation elicits in the CNS may be preceded by transient episodes of nausea, headaches, and somnolence within a week of exposure that may recur between 1 month and 6 months. Late damage occurs as permanent and irreversible paralysis in the case of spinal irradiation and in the brain as persistent dementia after large field irradiation or neurological complications from radionecrosis after more localized exposure. These patterns of responses are, again, consistent with dynamic molecular changes in the CNS after radiation exposure.³⁹

It is of interest that inflammatory cytokines appear to have unique roles in the CNS as neuromodulators of normal function, such as sleep and the neuroendocrine axis.⁴⁰ After irradiation, their production is increased, similar to what is seen in lung. RNase protection assays showed increases in IL-1 and TNF- α , ICAM-1, and other inflammation-related molecules within 4 to 6 hours of irradiation delivered to the whole brain of mice, generally with doses above 7 Gy.^{41,42} Gaber and coworkers⁴³ reported a similar marked increase in TNF- α and ICAM-1 expression by real-time polymerase chain reaction after whole-brain irradiation with 20Gy. Within 2 hours, TNF- α mRNA had risen 10-fold. Most responses returned to normal levels within 48 hours. The increase in TNF- α and ICAM-1 was more modest and significantly delayed if fractionated doses were given rather than single large exposures.

The possible significance of early cytokine production after brain irradiation has been most investigated with respect to TNF- α . One of the early cellular events after brain irradiation is apoptosis, which preclinical studies have shown to occur in 3 different cell types. One is oligodendrocyte apoptosis that is p53-⁴⁴ and TNFR1-dependent, at least in vitro (McBride and Olsen, unpublished data, 2003) with the clonogenic capacity of oligodendrocyte type 2 astrocyte (O-2A) progenitors also affected.⁴⁵

A second is endothelial cell apoptosis⁴⁶ that is acid sphingomyelinase but not p53 dependent and may be responsible for early failure of the blood-brain barrier and edema.⁴⁴ Platelets can bind to exposed matrix resulting in basal membrane thickening and increased leukocyte infiltration. These leukocytes may be a primary source of TNF- α ,⁴⁷ although it can

also be produced by astrocytes and microglia.⁴⁸ TNF- α itself may also disrupt the blood-brain barrier.⁴⁰ The third cell type to apoptose in a p53-⁴⁹ and TNFR1-dependent⁵⁰ manner is the neural precursor cell that resides primarily in the subventricular zone and hippocampal dentate gyrus.^{50,51} These cells allow neurogenesis to persist in adult brain and can differentiate into the 3 major neural cell types. The role of these cells in recovery from radiation-induced brain damage and cognitive injury is unknown but long-term loss of proliferative potential has been found after x-ray exposure,⁵¹ and the suggestion has been made that this might be why hippocampus-related task behavior is impaired at later time points.⁵² Neurons, being fully differentiated, are themselves not radiosensitive, at least in the adult,⁵³ although neurons in neonatal mice are susceptible to radiation-induced apoptosis, a potential that declines rapidly after birth and is insubstantial at 2 weeks.⁵⁴

The potential roles that radiation-induced proinflammatory cytokines might play in the CNS are numerous. TNF- α may mediate some of the cell apoptosis mentioned earlier. It is cytotoxic to oligodendrocytes,⁵⁵ is either neurotrophic or neurotoxic to neurons,⁵⁶ upregulates GFAP expression and gliosis,⁵⁷ and it may be directly responsible for dopaminergic cell degeneration,⁵⁸ which is another late consequence of brain irradiation,⁵⁹ something that TGF- β may protect against.⁶⁰

Chiang⁶¹ presented clear evidence of further increases in TNF- α expression 2 weeks, 2 to 3 months, and 5 to 6 months after mouse brain irradiation. These cyclical increases in expression of TNF- α and other proinflammatory molecules correlated with demyelination during the subacute and late phases.⁶¹ In normal mice, oligodendrocyte markers decreased at 2 months and again at 4 to 6 months, followed by decreased levels of myelin basic protein and myelin staining that was obvious at 3 months and very marked at 6 months, which was the time of death in mice receiving the highest doses. Gliosis and proliferation were also observed.⁶² This pattern of molecular and cellular fluctuations was thought to reflect abortive attempts at remyelination and healing. The role played by TNF- α in the irradiated brain may be best exemplified by the use of TNFR knockout mice. Mice lacking TNFR2 developed severe brain injury earlier than wild-type controls that was associated with extensive demyelination, indicating that TNF acting through this receptor can be neuroprotective,⁵⁰ whereas most of its harmful effects are through TNFR1, which contains death domains. TNFR2 knockout mice also showed increased early stem-cell apoptosis, decreased proliferative gliosis at 1 month, increased demyelination at 2 to 3 months, and succumb early to the effects of brain irradiation.⁵⁰ These responses indicate how one pleiotropic cytokine might act on several cell types to affect multiple aspects of the response of a tissue to irradiation.

Kim and coworkers⁴⁷ irradiated the right side only of rat brains with a single 10 Gy dose and described increased expression of TNF- α and TGF- β weeks and months after irradiation. Levels of both proteins were 20% to 30% higher on the irradiated than the nonirradiated side. Indeed, the

anti-inflammatory cytokines TGF- β and IL-10 are upregulated in many central and peripheral CNS diseases/disorders.⁶³ TGF- β obviously does not have a fibrogenic role in the brain. It is produced by astrocytes and microglial cells and is generally thought to be neuroprotective.⁶⁴ It increases neurogenesis⁶⁵ and is involved in the complex interplay between glial cells and neurons,⁶⁶ controlling astrocyte proliferation and differentiation in response to injury. Again, TGF- β has been found to neutralize many of the effects of TNF- α and the proinflammatory cytokines, including vascular leakage,⁶⁷ to maintain homeostasis.

Vascular damage as evidenced by imaging and leakage of compounds across the blood-brain barrier also has been found to recur up to 24 weeks after irradiation⁶⁸ and a 22-Gy single dose to the rat spinal cord increased vessel permeability after 20 weeks.⁶⁹ The increase in permeability was exclusively in the white matter and correlated with significant upregulation of vascular endothelial growth factor (VEGF) secreted by glial cells. Interestingly, this excess VEGF failed to promote the growth of new endothelial tissue and microvessel density even slightly decreased. Nordal and coworkers⁷⁰ also showed an increase in VEGF expression 16 to 20 weeks after irradiation of rat spinal cord with doses in excess of 18 Gy. This response was mediated by astrocytes, was strongly dose dependent, and mirrored HIF1 α kinetics. The white matter showed increased permeability and was hypoxic. These mice eventually developed white-matter necrosis and hind-limb weakness and paralysis. Interestingly, mice engineered to express lower levels of VEGF suffered milder late effects after radiation damage.

The brain therefore shows similar cytokine cascades as the lung, although the final outcome is very different. The same cytokines have different roles in this tissue and act on different cells, the response being dictated by the receptor profiles that are expressed. In this respect, it is interesting to note the mutually antagonistic roles of TNFR1 and TNFR2 and the potential there is for therapeutic exploitation of these dueling pathways.

Skin

The acute effects of skin damage are erythema and desquamation. Transient erythema appears within the first hours of irradiation and subsides after 24 to 48 hours and is due to inflammation. The main erythematous reaction is caused by loss of epidermal basal cells that produce the 10 to 20 layers of keratinizing epithelial cells in humans and that have a transit time between 12 to 48 days. Dry or moist desquamation is therefore seen after 3 to 6 weeks.⁷¹

The late radiation effects in the skin derive largely from damage to the vascular network and fibroblasts in the dermis and can include dermal atrophy, telangiectasia, necrosis, and fibrosis that occur many months after irradiation. There can also be a late erythematous reaction associated with dermal ischemia and necrosis after high doses, appearing around 8 to 16 weeks.^{71,72} Dermal atrophy manifests as a thinning of the dermal tissue and shrinking of the skin in the irradiated area and can reoccur over a year after exposure. This has been well

documented in pig skin and has been associated with loss of endothelial cells and a reduction in capillary density.⁷¹ Telangiectasias also may be considered as failure in the regrowth of damaged vasculature. Necrosis is typically precipitated by trauma in atrophic skin where the reaction of the vasculature to injury may be impaired. The wound-healing capacity of irradiated skin may be severely impaired, which is of considerable clinical importance where surgical resection in preirradiated sites is required.

Several groups have investigated the roles of cytokines in the skin response to irradiation. Members of the IL1 and TGF- β families have been implicated as mediators of fibrosis both in vivo and in vitro,^{73,74} and both assist wound healing in irradiated skin although they seem to promote different phases of healing.⁷⁵ IL-1 enhances proliferation of keratinocytes and fibroblasts and helps to regulate matrix deposition and remodeling by stimulating metalloproteinases.⁷⁶ It also can bring about phenotypic changes in dermal microvasculature that are not dependent on cell proliferation,⁷⁷ although other proangiogenesis cytokines will also be present in the postirradiation milieu.

As in other tissues, TGF- β in the skin promotes collagen production by dermal fibroblasts. In pig skin, TGF- β production was elevated after irradiation during both erythema and fibrosis.⁷⁸ During the later phases of fibrosis, from 6 to 12 months after irradiation, the TGF- β 1 gene was highly expressed in the repaired skin and the underlying muscular fibrotic tissue, with 10- and 8-fold maximal increases, respectively. TGF- β 1 was present in capillaries, in myofibroblasts, and in the collagenous matrix of the fibrotic tissue. TGF- β has also been shown to mediate radiation-induced fibroblast senescence, enhancing collagen production, which may be an important mechanism in radiation skin fibrosis.⁷⁹ This may explain why irradiation given postoperatively can sometimes actually enhance wound tensile strength, at least transiently.⁸⁰

Fetal healing responses to injury differ markedly from those of the adult. The acute inflammatory response is absent, few fibroblasts participate, and no collagen is deposited. TGF- β seems to be a major effector of the scarring reaction because it is diminished in fetal wounds. Injecting TGF- β into fetal wounds in rabbits induced fibrosis and scarring while injecting neutralizing antibodies to TGF- β healed adult wounds with less scarring.⁸¹ IL-6 and IL-8 are also diminished during fetal wound repair and knockout of IL-6 promotes scarless healing,⁸² whereas knockout of IL-10 has the opposite effect.⁸³

In normal dermal wound repair, inflammatory cells and in particular activated macrophages are thought to coordinate connective tissue responses and capillary growth and to be the major sources of cytokines. The role of bone marrow-derived inflammatory cells in the process can be seen in the finding that wound healing is compromised more by whole body than local irradiation.⁸⁴ Experiments with IL-6 knockout mice suggest that this cytokine can play a major role in regulating infiltration and wound healing,⁸⁵ but other cytokines are likely involved especially initially. Keratinocytes are important sources of IL-1 β in the skin.⁸⁶ After irradiation,

production is increased and it may diffuse to the adjacent dermis providing a major initial paracrine signal of skin damage.⁷⁸ Later, production of IL1 α and IL1 β is likely to be primarily from activated macrophages.⁸⁶

The role of IL-1 and TGF- β in skin radiation responses has been elucidated using knockout mice. IL-1R1 knockout mice have milder histopathological responses to skin irradiation than wild type with little desquamation at early time points and later hyperkeratosis and fibrosis is reduced.⁸⁶ By using an excisional wound healing model, others have found oral wound healing to be compromised by lack of IL1R, but dermal healing was not affected.⁸⁷ Mice that have no IL-1R antagonist (IL1ra), which can bind to IL-1R but fails to associate with the IL-1R associated protein to activate IL-1 function, also have impaired wound healing as shown by diminished collagen deposition and delayed neovascularization.⁸⁸ In skin wound sites, levels of Smad 2 and 3, key downstream mediators of TGF- β , were decreased but Smad 7 was increased, suggesting that IL-1ra may drive TGF- β -mediated wound healing.⁸⁸ The response of mice lacking Smad 3 to skin irradiation has been studied in some detail. They had reduced epidermal acanthosis, cellular infiltrate, and fibrosis as well as an accelerated healing response in previously irradiated skin, suggesting that Smad 3 could be a target for reducing fibrosis and enhancing wound healing.⁸⁹ These experiments with knockout mice clearly show the role of IL-1 and TGF- β in radiation-induced skin late effects, although as we have argued previously,⁹⁰ these cytokines are probably working by different mechanisms or at least at different phases in the healing continuum.

Gut

The severity and frequency of intestinal radiation-induced toxicity depends on many factors including radiation dose, treatment volume, fractionation regimen, and concurrent administration of chemotherapeutic agents and other drugs. However, it is important to note that chronic symptoms of intestinal dysfunction present in 60% to 90% of patients⁹¹ and severely impacts the quality of life for cancer survivors. Late effects develop, gradually or suddenly, months to years after radiotherapy with symptoms ranging from slight to severe.

Acute toxicity manifests clinically as enteritis and ulceration in the intestine and loss of epithelium integrity and increased secretion of mucus in the rectum. Patients experience chronic diarrhea with the development of tissue edema and hyperemia.⁹² Radiation-induced intestinal dysfunction and dysmotility continue to worsen into the late stage effects, with an average onset of roughly 8 to 12 months. Clinical manifestation and symptoms include intestinal obstruction, bleeding, increased stool frequency, incontinence, fistula formation, and persistent diarrhea. Delayed radiation enteropathy can be described by ischemia related to vascular insufficiency with the development of fibrotic lesions.⁹³ Additionally, it is also reported that bacterial overgrowth may accompany intestinal irregularity and play a role in digestive malfunction after radiation.⁹² The nature of the bacterial flora

in the gut, as well as diet, may well be critical to the development of late effects. The finding that injection of lipopolysaccharide or IL-1 before abdominal irradiation of mice greatly enhanced peritoneal adhesion formation 2 to 4 months later,⁹⁴ supports this view.

Initial responses to radiation in the gut are characterized by radiation-induced cytokine expression, as in other tissues.⁹⁵ Within hours of radiation exposure the rat ileal muscularis layer expresses IL-1 β , TNF- α , and IL-6.⁹⁶ TGF- β 1 is activated at 24 hours after irradiation and remains high throughout later responses, whereas IL-10 decreased. At this time, TGF- β 1 is found in the inflammatory cells and surrounding extracellular matrix, with PDGF-AA induction in inflammatory cells and fibroblasts.⁹⁷ Additional studies showed TGF- β 1 association with mucosal ulceration, membrane thickening, and epithelial atypia 2 weeks after irradiation.⁹⁸ A significant increase in the number of inflammatory cells in the mouse intestine can be observed at 24 hours after irradiation,⁹⁷ and IL-8 is upregulated at about 3 days after irradiation.⁹⁶

Long-term changes in cytokine expression in the bowel of mice after irradiation implicate TNF- α , IL-1, IL-6, and TGF- β 1 in late radiation-induced bowel fibrovascular toxicity.⁹⁹ The TGF- β family members TGF- β 1, TGF- β 2, and TGF- β 3 were all increased in rat ileum 2 weeks after radiation doses of 12 to 21 Gy,¹⁰⁰ but only TGF- β 1 remained high into the late response, in keeping with its major role in fibrosis. Connective tissue damage and increased collagen deposition is accompanied by high expression of smooth muscle actin and increased levels of the fibrogenic growth factor connective tissue growth factor (CTGF).¹⁰¹ CTGF may be involved in radiation-induced fibrogenic differentiation in intestinal smooth muscle cells. The Rho/ROCK pathway has been shown to regulate CTGF expression and may serve as a target for intervention.¹⁰²

Microvascular injury in both acute and chronic radiation injury to the gut has been investigated in some depth and ascribed to dysfunction of the thrombomodulin (TM)-protein C (PC) system.¹⁰³ The TM-PC system is a critical physiological anticoagulation system in which TM forms a complex with thrombin to promote anticoagulation. This complex activates PC, an anticoagulant and anti-inflammatory protein, rather than protease-activated receptor-1 (PAR-1).¹⁰⁴ TM and activated PC have important anti-inflammatory properties.^{104,105} Studies have shown that radiation therapy causes a significant reduction in endothelial TM levels in intestinal vasculature.^{106,107} Deficient TM levels are likely a result of initial direct oxidative damage to TM, decreased transcription of TM by inflammatory cytokines, and release of TM into the circulation.¹⁰⁸ Cytokines such as IL-1, TNF- α , and TGF- β 1, all induced after radiation, reduce the transcription of TM.¹⁰⁹ TM deficiency is found early postirradiation and continues to persist into chronic radiation injury, paralleling cytokine expression. This mechanism may allow persistence of the late cytokine cascade and damage.

Conclusions

The molecular mechanisms activated in an irradiated tissue incorporate canonical, highly conserved pathways that communicate not only between cell types within the tissue but also between the vasculature, its coagulation system, and the bone marrow-derived inflammatory and immune systems. Late normal tissue damage reflects a failure to regenerate functional tissue, whether through a lack of stem cells or radiation-induced dysregulation of the normal healing process. A common alternative pathway of tissue restoration is by fibrogenesis, which is often invoked to maintain structural integrity. After irradiation, there are recurring patterns of expression of pro- and antiinflammatory cytokines, molecules that promote and diminish cell adhesion, proteases and antiproteases, oxidants, and antioxidants. This interactive network of factors is critical to tissue regeneration and healing after irradiation, and it changes with time and space to move the healing processes forward in a controlled way. The severity of injury, extent of cell death, nature and extent of disease, and adjunctive therapies will all impact the balance of forces within the network. The degree of prominence that can be ascribed to any individual molecule within the network depends on the tissue and on the endpoint. Clearly, there are multiple possible targets for intervention aimed at improving the outcome, although minimizing the effect of any one molecule may not have the same effect in all tissues, at all intervention times, and at all levels of damage. Intervention aimed at physiological processes underlying the symptoms of late damage may be just as effective as those aimed at altering regeneration, restoration, remodeling, or the recurring cytokine breezes that may drive the phase transitions. There is much we need to know before these efforts can bear fruit, but there are many indications that it is possible to modify late radiation damage and further studies are greatly warranted.

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