Absence of Smad3 Induces Neutrophil Migration after Cutaneous Irradiation

Possible Contribution to Subsequent Radioprotection

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Our previous work showed that 6 weeks after cutaneous irradiation, mice null (knockout, KO) for Smad3, a cytoplasmic downstream mediator of transforming growth factor- β , demonstrate less epidermal acanthosis and dermal inflammation than wild-type (WT) Smad3 mice. Analysis of the kinetics of inflammation showed that 6 to 8 hours after skin irradiation, there was a transient sevenfold increase in neutrophil influx in Smad3 KO mice compared with WT. Herein we describe bone marrow transplantation and skin grafting between WT and KO mice to assess the contribution of the neutrophil genotype compared with that of irradiated skin to the induction of neutrophil migration after irradiation. Results from bone marrow transplantation showed that WT marrow transplanted into KO mice enhanced neutrophil migration 6 to 8 hours after irradiation by 3.2-fold compared with KO marrow in WT mice. KO skin grafted onto either WT or KO animals showed a sixfold elevation of neutrophils after irradiation compared with grafted WT skin. These results suggest that the genotype of the irradiated skin, rather than the inflammatory cell, controls neutrophil influx. Circulating neutrophils, increased in WT mice after injection of granulocyte colony-stimulating factor, resulted in increased neutrophil migration to the skin 6 to 8 hours after irradiation and less skin damage 6 weeks after irradiation compared with untreated WT mice. Thus, early responses, including enhanced neutrophil influx, appear to contribute to subsequent cutaneous

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Transforming growth factor- β (TGF- β) is a widely expressed, multifunctional cytokine that regulates a number of biological processes important in inflammation and fibrosis including cell proliferation, apoptosis, and chemotaxis, as well as promotion of extracellular matrix synthesis.¹ Several studies^{2–4} have demonstrated increased expression of TGF- β after exposure of tissues to ionizing irradiation and its sustained expression has been implicated in the subsequent chronic fibrotic response that often occurs after irradiation. Indeed, administration of neutralizing antibodies to TGF- β can decrease tissue damage and fibrosis caused by irradiation in several organ systems.^{5,6}

TGF- β signals through ubiquitously expressed serine-threonine kinase transmembrane receptors. Ligand binding to these receptors induces serine phosphorylation of cytoplasmic signal-transducing proteins known as Smads. After phosphorylation, pathway-specific Smad proteins (Smad2 and Smad3 for TGF- β signaling), heterodimerize with the common mediator Smad4 and the complex translocates to the nucleus to regulate the transcription of a number of target genes.^{7,8} Even though TGF- β can

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signal through multiple intercellular molecules, Smad3 is most predominantly involved in modulating radiation-induced tissue damage and fibrosis. We have previously shown⁹ that cutaneous irradiation of Smad3 wild-type (WT) mice results in marked epidermal acanthosis, as well as dermal inflammation and fibrosis, whereas all responses in Smad3 knockout (KO) mice are dramatically attenuated. The KO mice also show more rapid healing of incisional wounds made in previously irradiated skin,¹⁰ a response that can be compromised in cancer patients treated with a combination of radiation therapy and surgery. The central role of Smad3 in mediating a fibrotic response is seen in other organ systems, such as lung, liver, kidney, and eye where KO mice show less fibrosis in response to injury.¹¹

Within minutes ionizing irradiation activates latent TGF- β ,¹² which induces chemotaxis of neutrophils and macrophages into the irradiated area. Even though TGF- β is an extremely potent neutrophil chemoattractant at femtomolar concentrations, at nanomolar concentrations these effects are lost.^{13,14} Additionally, Smad3-null neutrophils¹⁵ and macrophages¹⁶ have impaired chemotactic responses to TGF- β and we have shown that 6 weeks after irradiation there are fewer inflammatory cells in KO skin as compared to WT.⁹ Because inflammatory cells are a potent source of TGF- β , fewer inflammatory cells leads to decreased levels of TGF- β present in the KO tissue,⁹ which contributes to the diminished fibrotic response in KO skin. In addition, *in vitro* studies have shown that Smad3 KO fibroblasts show decreased production of profibrotic genes after irradiation.¹⁷

Because cytokine production by inflammatory cells after irradiation is such a critical mediator of subsequent tissue damage and fibrosis, we have continued to examine the influx of inflammatory cells into WT and KO skin after irradiation and have found that surprisingly there is a dramatic transient enhancement of migrating neutrophils into KO skin at 6 to 8 hours after irradiation as compared to WT skin. This increased influx seems to result from signals emanating from KO, but not WT, irradiated skin rather than to result from differences in properties of WT and KO neutrophils. Additionally, the enhanced neutrophil migration seems to correlate with radioprotection at later time points.

Materials and Methods

Generation of Mice

KO (*Smad3*^{ex8/ex8}) mice were generated by targeted disruption of the Smad3 gene by homologous recombination¹⁵ and are a mixed background of C57BL/6nCr, Tac: N:NIHS-BCFBR, and 129S6/SvEv-ATM<tm1awb>, which we have used previously.⁹ Mice heterozygous for the targeted disruption were intercrossed to produce homozygous offspring and genotyping was done by polymerase chain reaction of tail DNA.

Local Irradiation

Local irradiation (30 Gy) of flank skin of Smad3 WT and KO mice (~6 weeks of age) was performed as described.⁹ For most experiments both flanks were irradiated. At various times (1.5 hours to 5 months) after irradiation animals were euthanized and flank skin was removed and transferred to 10% neutral buffered formalin for 24 hours. After three washes in 70% ethanol, tissues were processed for paraffin embedding. All protocols involving animals were approved by the National Cancer Institute Animal Care and Use Committee.

Histological Analysis

Sections (5 μ m) of paraffin-embedded irradiated skin sections were cut and immunostained to identify neutrophils with rat anti-mouse neutrophil antibody (Serotec, Raleigh, NC) at 0.5 μ g/ml and macrophages with rat anti-mouse Mac-3 (BD PharMingen, San Diego, CA) at 2.5 μ g/ml as previously described.^{9,10} Briefly antigen-antibody complexes were detected using the Vectastain Elite rat ABC peroxidase kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions and were visualized with diaminobenzidine/H₂O₂. Total immunostained cells were counted in five $\times$ 400 high-power fields (HPFs) in the irradiated skin and expressed as mean $\pm$ SEM. Mast cells were identified by staining with low pH toluidine blue and counted in five $\times$ 400 HPFs. Significant intergroup differences were determined by applying the two-tailed assuming unequal variance *t*-test. Immunostaining for keratinocyte-derived cytokine (KC) and macrophage inflammatory protein-2 (MIP-2) were performed using a similar protocol with anti-mouse KC (Antigenix America, Inc., Huntington Station, NY) and anti-murine MIP-2 (Chemicon International, Temecula, CA) both at an IgG concentration of 1 μ g/ml.

Bone Marrow Transplantation (BMT)

Bone marrow was flushed from femurs and tibias of WT and KO mice. After T-cell depletion using CD4 and CD8 microbeads (Miltenyi Biotech, Auburn, CA), 7×10^6 cells were injected into the tail vein of WT or KO recipients that had received a lethal dose of 8 Gy total body irradiation 6 hours before injection. Donor marrow was matched to the sex of the recipient. The efficacy of the BMT was analyzed in the first experiment. Six weeks after BMT, mice were euthanized and bone marrow and splenocytes were isolated and analyzed by fluorescence-activated cell sorting to examine the extent of engraftment. Additionally, some of the bone marrow isolated after BMT was spun onto slides and Smad3 expression was analyzed by immunostaining. Briefly, the cytopspins were fixed in 2% paraformaldehyde and then boiled in 1 mmol/L ethylenediaminetetraacetic acid (pH 8) for 15 minutes. After cooling, endogenous peroxidase and nonspecific protein binding were blocked and cytopspins were incubated overnight at 4°C with 2 μ g/ml of anti-Smad3 IgG (Zymed, South San Francisco, CA). Antigen-antibody complexes

were detected as described above. In other experiments 6 weeks after BMT the flanks of mice were irradiated and histological samples were processed as described above.

Skin Grafting

Skin grafting was performed as described by Rosenberg.¹⁸ WT and KO donor mice were euthanized and skin was removed from the flank and back, cleaned of fat and subcutaneous tissue, and cut into 1 × 1.5-cm pieces. WT and KO recipient mice were anesthetized, the left flank shaved, and a 1 × 1.5-cm area of skin was removed from the left flank leaving the panniculus carnosus intact. A piece of donor skin was placed flat on the graft bed and covered with a square of Vaseline gauze (Johnson & Johnson, Arlington, TX). A Band-Aid strip was wrapped circumferentially around the mouse and then covered with Vetrap (3M, St. Paul, MN). Mice were housed individually for 10 days at which time they were anesthetized and the bandages and gauze were removed. The grafts were allowed to heal for ~2 additional weeks at which time both the left (grafted) and right (nongrafted) flanks of the mice were irradiated as described above.

Neutrophil Enhancement

Smad3 WT mice were injected subcutaneously with 250 µg/kg of recombinant murine granulocyte colony-stimulating factor (G-CSF) (Biosource, Camarillo, CA) in phosphate-buffered saline (PBS)/0.1% low endotoxin bovine serum albumin (Sigma Chemical Co., St. Louis, MO) (vehicle) or with vehicle alone. At times from 6 to 72 hours after injection, peripheral blood smears were obtained and stained with Protocol Hema 3 (Fisher Diagnostics, Middletown, VA) according to the manufacturer's instructions. The number of neutrophils per 100 white blood cells was determined for each smear. For radiation experiments, mice (five per group) were injected with G-CSF or vehicle and 40 hours later skin on both flanks was irradiated as described above. Six hours after irradiation peripheral blood smears were obtained and circulating neutrophil counts were determined by Hema 3 staining. In one experiment mice were euthanized at this time point and flank skin was processed for determination of numbers of infiltrating inflammatory cells as described above. In other experiments mice were maintained for 6 weeks after irradiation during which time the skin reaction was scored as described below. At the end of this time, mice were euthanized, and flank skin was analyzed for quantitation of neutrophils and macrophages as described above.

Phenotypic Scoring of Irradiated Skin

In some experiments from ~2 to 6 weeks after irradiation the skin reaction was evaluated every few days according to the following phenotypic scale¹⁹: 1, normal; 2, hair loss; 3, erythema; 4, dry desquamation; 5, <30% moist

desquamation; 6, >30% moist desquamation. Values were averaged from five mice per group in which both flanks of the mice were irradiated.

Results

Smad3 KO Mice Have Enhanced Cutaneous Neutrophil Influx at Short Times After Irradiation

We previously demonstrated⁹ that 6 weeks after irradiation of the skin, Smad3 KO mice showed significantly less dermal inflammation than did Smad3 WT mice. The same trend was seen from the time period of 14 to 100 days after irradiation when neutrophil influx was examined (Figure 1A). After this time neutrophil infiltration into the dermis of the WT mice decreased significantly. Enhanced macrophage infiltration in WT compared to KO skin was observed for ~6 weeks and after that there were similar increases in the number of macrophages in the skin of both genotypes greater than levels in nonirradiated skin (Figure 1B). Surprisingly, when we examined the influx of inflammatory cells at short times (up to 48 hours) after irradiation, there was a sevenfold increase of neutrophils present in the KO skin compared to the WT skin at 6 to 8 hours after irradiation (Figure 1C). The peak neutrophil influx in WT skin occurred at 4 hours and then decreased to near control levels. The neutrophil peak in KO skin continued to increase until 8 hours after irradiation, when it was 20-fold greater than levels in nonirradiated KO skin, before returning to control levels at 24 hours. Figure 1E shows typical differences between the neutrophil influx in WT and KO skin at 8 hours after irradiation. Changes in macrophage influx after irradiation were similar in WT and KO skin at the time points examined (Figure 1D).

Skin Genotype, Rather than Neutrophil Genotype, Determines Extent of Neutrophil Influx

We used two approaches to determine whether the difference in neutrophil influx between WT and KO mice resulted from the genotype of the migrating neutrophils or from the genotype of the irradiated skin. In the first approach we transplanted bone marrow from WT mice into KO mice and vice versa. Recipient mice received a dose of 8 Gy total body irradiation before transplantation. We have previously shown that this dose is lethal for all genotypes of Smad3 mice⁹ because of bone marrow toxicity, so mice that survive the BMT procedure do so because they have been reconstituted with donor marrow. Fluorescence-activated cell sorting analysis of bone marrow and spleen from transplanted animals confirmed that 6 weeks after total body irradiation and transplantation the bone marrow of the mice had reconstituted similarly, regardless of genotype (data not shown). Six weeks after transplantation, bone marrow was isolated and stained for Smad3 to confirm its genotype (Figure 2, A–C). Using rabbit IgG as the primary antibody gave no

staining (Figure 2C). When WT bone marrow was transplanted into KO mice $\sim 40\%$ of the cells expressed Smad3 similar to the percentage of positive cells when WT bone marrow was transplanted into WT mice (Figure 2D). A small number of cells were stained with anti-Smad3 when KO marrow was transplanted into either WT or KO mice, probably attributable to slight cross-reactivity of the Smad3 antibody with Smad2 as suggested by the product literature.

Because the BMT was successful we then irradiated the flank skin of the transplanted mice and quantitated the neutrophils and macrophages in the dermis at 6 to 8 hours after irradiation (Figure 3). In our control studies when KO mice served as both donor and recipient, there was a fourfold greater increase in neutrophils in the skin than when WT mice were used as both donor and recipient. In three separate experiments when WT bone marrow was transplanted into KO mice there were 2.9-, 2.6-, and 4.1-fold more neutrophils migrating into the dermis than when KO bone marrow was transplanted into WT mice (Figure 3A). Thus it appears that the enhanced neutrophil influx is a property of the irradiated KO skin rather than a property of the KO neutrophils themselves. Macrophage influx was generally similar in all of the transplant groups (Figure 3B). The differences in the y axis scale in Figure 3, A and B, show that the main infiltrating cell population is neutrophils (peak cell count of 262 cells per five HPFs) as compared to macrophages (peak cell count of 79 cells per five HPFs).

This observation was confirmed in skin-grafting experiments (Figure 4). In each animal the graft was placed on the left flank. When the graft had healed completely (~ 4 weeks after surgery), both the left and right flanks were irradiated and the neutrophil influx was quantitated 6 hours later. Each animal served as its own control because the influx into the grafted skin on the left flank was compared to the nongrafted right flank. Because of the high degree of interanimal variation the results comparing the left and right flanks of individual animals are plotted in Figure 4. When the genotype of the graft and recipient were the same, there was good agreement in the numbers of neutrophils that invaded the grafted and nongrafted flanks with KO flanks showing approximately sixfold more neutrophils than WT flanks (Figure 4, A and B; far right). Even though there was a significant amount of variability between animals, possibly attributable to the additional surgical step, when KO skin was grafted onto WT mice there were more neutrophils present in the graft skin in 80% of the mice with an average increase of 5.9 ± 1.5 -fold (Figure 4A). Similarly when WT skin was grafted onto KO animals 87% of the animals showed greater numbers of neutrophils in the nongrafted KO skin (control) compared to the grafted WT skin with an average increase of 6.7 ± 1.6 -fold (Figure 4B). These results again suggest that the genotype of the irradiated skin is controlling the extent of neutrophil influx. In contrast, macrophage quantitation showed that there was an average difference of $74 \pm 4\%$ between grafted and nongrafted

sides of each animal suggesting again that the enhanced influx was specific to neutrophils and not a general property of inflammatory cells.

Expression of MIP-2 and KC Are Similar in WT and KO Skin after Irradiation

Neutrophils are recruited to sites of injury in response to local production of chemotactic cytokines. In humans interleukin-8 (IL-8), a member of the CXC chemokine family, is the primary neutrophil chemoattractant, but there is no murine IL-8 homologue. In mouse the two most well-defined neutrophil chemoattractants are KC and MIP-2, which are members of the IL-8 family.²⁰ Mast cell activation has been reported to induce neutrophil migration via secretion of KC²¹ and MIP-2.²² There are significantly more mast cells present in irradiated KO skin compared to WT skin at 4 to 8 hours after irradiation [42 ± 3 mast cells per five HPFs in WT versus 84 ± 5 mast cells per five HPFs in KO ($P < 0.005$) at 6 hours], suggesting that there may be increased levels of MIP-2 and KC in KO skin compared to WT skin after irradiation that could account for the enhanced neutrophil influx. Figure 5 shows immunostaining for MIP-2 (Figure 5, A–D) and KC (Figure 5, E–H) in WT (Figure 5, left) and KO (Figure 5, right) skin. There is very little MIP-2 expression in nonirradiated skin (Figure 5, A and B), but by 6 hours after irradiation similar levels of extracellular MIP-2 are seen in WT and KO skin (Figure 5, C and D). MIP-2 staining is

strongest just under the epidermis. In both genotypes the expression of MIP-2 decreases until it is close to control levels by 24 hours after irradiation (data not shown). Light staining for KC is seen in keratinocytes and hair follicles with stronger staining in scattered cells throughout the dermis (Figure 5, E–H; arrows) in WT (Figure 5E) and KO (Figure 5F) nonirradiated skin. There is a small increase in the numbers of dermal cells expressing KC at 6 hours after irradiation, which is similar in WT (Figure 5G) and KO (Figure 5H) skin with no significant change in epidermal staining. Again by 24 hours after irradiation KC staining returns to levels similar to control. No staining was seen when primary antibody was replaced by rabbit IgG (data not shown). Thus, the enhanced influx of neutrophils into the KO skin after irradiation is not explained by differences in immunoreactive MIP-2 and KC in irradiated WT and KO skin.

Enhanced Neutrophil Influx at Early Time Points Correlates with Later Radioprotection

The compromised health of KO mice complicated our attempts to determine whether the enhanced neutrophil influx at 6 to 8 hours after irradiation of the flank contributed to the subsequent radioprotection.⁹ Smad3 KO mice generally die by 6 months of age because of defects in mucosal immunity and skeletal abnormalities¹⁵ making long-term experiments difficult. In BMT studies mice are transplanted at 6 to 8 weeks of age and allowed to

Figure 3. Quantitation of neutrophils (A) and macrophages (B) after irradiation in mice receiving BMT. The results of three BMT experiments are shown using the indicated donor and recipient genotypes. Each donor-to-recipient group contained four to six mice. Six weeks after BMT both flanks of each mouse were irradiated. Six hours after irradiation, mice were euthanized and irradiated skin was processed and neutrophils and macrophages were identified by immunostaining and quantitated as described in Materials and Methods. Mean $\pm$ SEM. * $P < 0.05$ between pairs in each experiment.

reconstitute for ~6 weeks before flank irradiation. We then wait an additional 6 weeks before scoring the skin response to irradiation. In a long-term BMT experiments, all KO mice that had received WT marrow died at 3 to 4 weeks after irradiation whereas all WT mice that had received KO marrow were healthy. Although the experiment was not taken to completion the average phenotypic score of the WT mice that had received KO marrow was 3.4, whereas that of the KO mice receiving WT marrow was 1.5. Since our previous results (Figure 3) showed that KO mice had greater neutrophil influx than WT mice independent of the genotype of the bone marrow, these results support the idea that an early response that includes enhanced neutrophil influx correlates with radioprotection.

Long-term skin graft experiments with KO recipients were also difficult because of mortality of the KO recipient mice. In experiments in which WT or KO skin was grafted onto WT recipients and irradiated, at 6 weeks after irra-

Figure 4. Quantitation of neutrophils in mice receiving skin grafts. Results from WT recipients (A) and KO recipients (B) are shown. For each mouse the indicated genotype skin was grafted onto the left flank whereas the right nongrafted flank served as the control. After recovery from surgery, both flanks of each mouse were irradiated. Six hours after irradiation, mice were euthanized and irradiated skin was processed and neutrophils were immunostained and quantitated as described in Materials and Methods. In both A and B the results on the far right of the graph were from mice in which the donor skin matched the genotype of the recipient.

diation the grafts tended to have a lower phenotypic score than the nongrafted site regardless of the genotype of the graft. There also were fewer neutrophils that migrated into the grafts (KO or WT) compared to the irradiated nongrafted WT skin. Because surgery before the irradiation seemed to alter the subsequent long-term wound healing and inflammatory response in the WT mice, another approach was used to investigate the relationship between early neutrophil influx and subsequent radioprotection.

Circulating neutrophils were increased in WT mice by injections of G-CSF. A preliminary time course experiment showed that circulating neutrophils increased threefold to fourfold at 24 to 48 hours after G-CSF injection. The flanks of WT mice were irradiated 40 hours after injection with G-CSF or vehicle and 6 hours later blood samples and irradiated skin were collected and processed for analysis. Wild-type mice injected with G-CSF showed 33.6 ± 1.5 circulating neutrophils per 100 white blood cells compared to 10 ± 0.5 for WT mice injected with PBS. Untreated KO mice showed 11 ± 0.9 neutrophils per 100 white blood cells. The increase in circulating neutrophils resulted in enhanced migration of neutrophils into WT irradiated skin at 6 hours after irradiation with 165 ± 22 neutrophils per five HPFs in WT mice receiving G-CSF compared to 48 ± 7 neutrophils per five HPFs in WT mice injected with vehicle ($P < 0.02$).

Figure 6, A and B, and Table 1 show the phenotypic score and numbers of inflammatory cells present in flank skin 6 weeks after irradiation of two separate neutrophil enhancement experiments. In each experiment 1.6-fold more neutrophils migrated into the skin in WT animals that had received G-CSF than in WT animals injected with vehicle (Table 1). In experiment 2, the number of migrating neutrophils in G-CSF-treated WT mice was similar to

Figure 5. Immunostaining of WT and KO skin for MIP-2 and KC. Smad3 WT (A, C, E, G) and KO (B, D, F, H) skin sections were stained for MIP-2 (A–D) and KC (E–H) as described in Materials and Methods. Sections shown were from nonirradiated skin (A, B, E, F) or skin collected 6 hours after irradiation (C, D, G, H). Extracellular staining for MIP-2 is similar in WT and KO skin at 6 hours after irradiation (C, D). KC staining is seen in scattered cells in the dermis (E–H, arrows). There are similar increases in stained cells in WT and KO skin after irradiation. DAB with hematoxylin. Original magnifications: $\times 100$ (A–D); $\times 200$ (E–H).

that in KO mice. The numbers of migrating macrophages were similar in all treatment groups. In each experiment the phenotypic score of the irradiated skin was significantly lower in WT mice treated with G-CSF than in those treated with vehicle from days 23 to 45, although they were not as low as scores in KO mice (Figure 6). These data suggest that enhanced neutrophil migration at early times after irradiation at least partially contributes to subsequent radioprotection.

Discussion

Significant neutrophil influx has been reported in the spleen²³ and thymus²⁴ in mice at 6 to 9 hours after exposure to ionizing irradiation. Cutaneous irradiation of Smad3 WT and KO mice induces a neutrophil influx to the dermis that is sevenfold greater in irradiated KO skin at 6 to 8 hours after irradiation compared to irradiated WT skin. Within minutes ionizing irradiation activates TGF- β ,¹² a potent neutrophil chemoattractant that may contribute to the initial influx of neutrophils in WT skin; however, Smad3-

null neutrophils do not respond to the migratory effects of TGF- β . This suggests that Smad3-null neutrophils respond differently to other chemotactic signals secreted by irradiated skin or that irradiated KO skin secretes different signals than irradiated WT skin.

To distinguish between these possibilities we used BMT and skin grafting to examine the response of different combinations of neutrophil and skin genotype. In three separate experiments in which bone marrow was swapped between WT and KO mice there was threefold to fourfold greater neutrophil migration in KO mice with WT marrow than in WT mice with KO marrow in good agreement with control experiments using syngeneic recipients. Thus, the enhanced neutrophil migration appears to be more dependent on the genotype of the irradiated skin than the genotype of the neutrophils. Results of skin grafting experiments between WT and KO mice supported this conclusion. Syngeneic grafts showed good agreement after irradiation in neutrophil influx in grafted versus nongrafted skin suggesting that the surgery did not alter the early neutrophil influx. KO skin showed elevated neutrophil levels after irradiation whether it was grafted onto WT or KO skin as compared to WT grafts. Additionally, when a large enough area around the graft was irradiated to permit analysis, significant differences in neutrophil numbers were seen between the irradiated grafted skin compared to the irradiated skin adjacent to the graft. There was good agreement in numbers of neutrophils between the irradiated nongrafted flank and the irradiated area outside of the graft (data not shown). This again suggests that the irradiated KO skin is generating different signals than the irradiated WT skin shortly after irradiation and these signals are enhancing neutrophil migration into the local area.

We attempted to identify the early responses that were different between WT and KO skin after irradiation that might contribute to enhanced neutrophil accumulation in KO skin. Increased expression of KC and MIP-2, two potent murine neutrophil chemoattractants, have been reported in a variety of cell types in skin after thermal or incisional wounding at times from 4 hours to several days after injury,^{25,26} but there are no reports concerning the response of KC and MIP-2 to localized ionizing irradiation. Using immunohistochemistry we saw significant increases in extracellular MIP-2 and modest increases in KC-positive dermal cells after irradiation, but did not find any consistent difference in expression of KC and MIP-2 in WT and KO skin at various times. TGF- β suppresses lipopolysaccharide-induced expression of KC and MIP-2 in macrophages via inhibition of p38 MAPK.²⁷ Our data suggest that this suppression occurs via a Smad3-independent mechanism because we saw no differences in KC and MIP-2 expression between Smad3 WT and KO mice.

Activation of TGF- β immediately after irradiation may initially induce migration of neutrophils, but as TGF- β concentrations increase throughout time, possibly from production by invading inflammatory cells, the WT neutrophils no longer are induced to migrate.^{13,14} In fact TGF- β 1 has been identified as the factor present in supernatants from stored red blood cells that inhibits neutrophil migration in transfusion patients.²⁸ Because

Figure 6. Phenotypic score of WT mice injected with G-CSF or vehicle compared to KO mice. Mice were treated as indicated and both flanks were irradiated with 30 Gy. At the indicated times after irradiation, mice were evaluated for a skin reaction according to the phenotypic scale given in Materials and Methods. **A** and **B** represent two independent experiments. Values are the mean $\pm$ SEM from five mice per group scoring two flanks per mouse. * $P < 0.05$ for WT (V) versus WT (GCSF); + $P < 0.05$ for KO versus WT (V).

Smad3-null neutrophils have lost their ability to respond chemotactically to TGF- β ,¹⁵ they are not affected by alterations in TGF- β levels and continue to respond to other chemotactic signals. Alternatively, other inhibitors of neutrophil infiltration, such as neutrophil inhibitory factor²⁹, S100A9,³⁰ and angiostatin³¹ may be less effectively induced in KO skin. We have performed microarray analysis of irradiated WT and KO skin, but are yet to identify any likely candidates that might account for the differences in neutrophil infiltration into the skin after irradiation.

Additionally, we have shown that enhanced neutrophil influx at 6 hours after irradiation correlates with cutaneous radioprotection 6 weeks later because increasing neutrophil influx into WT skin by systemic injection of G-CSF to increase circulating neutrophils, improved the cutaneous phenotypic score at 6 weeks toward that of KO skin. An augmented neutrophil presence might be expected to enhance tissue damage because an increased respiratory burst may lead to increased oxidative stress and damage. However, it has also been shown that tissues subjected to injury undergo adaptive responses making them better able to withstand subsequent injury. This

phenomenon, known as thermotolerance, is believed to be mediated by the accumulation and subsequent degradation of heat shock proteins^{32,33}. Pre-exposure to reactive oxygen species can confer resistance to ionizing irradiation in cultured cells³⁴ and *Caenorhabditis elegans*.³⁵ The preconditioning of an oxidative burst by neutrophils in KO or G-CSF-treated WT mice may help to lessen the subsequent complex injury initiated by ionizing irradiation in the skin. The increased DNA damage sensing and repair mechanisms that we have shown to be operative in Smad3 KO dermal fibroblasts¹⁷ suggest that the stromal compartment may play a role in this protection.

Radiotherapeutic injury of tissues is a multidimensional, complex process that involves the interaction between a number of different cells types, as well as multiple biologically active extracellular signaling molecules.^{36–38} The relationship between the early acute effects of irradiation such as skin erythema and dry and moist desquamation, and the late effects, such as fibrosis, which can occur months after, are not well understood. Because Smad3 KO mice demonstrate decreased injury from ionizing radiation than do WT mice, they provide a model to begin to analyze their differences in the early response to irradiation. Although inflammatory responses have been demonstrated to contribute directly to radiation-induced damage and fibrosis, this model suggests that perhaps a transient, acute aggravated response might be beneficial with potential therapeutic value. A better understanding of the early effects of ionizing radiation on all cell types involved in the process may suggest novel interventions that might limit the damaging late effects.

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Table 1. Inflammatory Cells in Dermis 6 Weeks after Irradiation

Genotype	Treatment	Number per high-powered field $\pm$ SEM	
		Neutrophils	Macrophages
WT-1	Vehicle	63 $\pm$ 8	100 $\pm$ 5
WT-1	G-CSF	39 $\pm$ 4*	101 $\pm$ 5
WT-2	Vehicle	156 $\pm$ 26	85 $\pm$ 8
WT-2	G-CSF	96 $\pm$ 19*	109 $\pm$ 9
KO-2	None	82 $\pm$ 10†	69 $\pm$ 5

* $P < 0.02$ versus WT (vehicle) for each experiment.
† $P < 0.05$ versus WT-2 vehicle.

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