Chronic Suppression of Angiogenesis following Radiation Exposure Is Independent of Hematopoietic Reconstitution

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Abstract
Radiation can potentially suppress neovascularization by inhibiting the incorporation of hematopoietic precursors as well as damaging mature endothelial cells. The purpose of these studies was to quantify the effect of radiation on angiogenesis and to examine the relationship between bone marrow reconstitution and neovascularization. Immune competent, severe combined immunodeficient, RAG1-deficient, and green fluorescence protein transgenic mice in the C57 genetic background, as well as the highly angiogenic 129S1/SvImJ strain of mice, underwent whole-body or localized exposure to radiation. The hematopoietic systems in the irradiated recipients were restored by bone marrow transfer. Hematopoietic reconstitution was assessed by doing complete blood counts. Angiogenesis was induced in the mouse cornea using 50 ng of purified basic fibroblast growth factor, and the neovascular response was quantified using a slit lamp biomicroscope. Following whole-body exposure and bone marrow transplantation, the hematopoietic system was successfully reconstituted over time, but the corneal angiogenic response was permanently and significantly blunted up to 66%. Localized exposure of the eyes to radiation suppressed corneal angiogenesis comparably to whole-body exposure. Whole-body irradiation with ocular shielding induced bone marrow suppression but did not inhibit corneal neovascularization. In mice exposed to radiation before tumor implantation, the reduced local angiogenic response correlated with significantly reduced growth of tumor cells in vivo. These results indicate that bone marrow suppression does not suppress neovascularization in the mouse cornea and that although hematopoietic stem cells can readily reconstitute peripheral blood, they do not restore a local radiation-induced deficit in neovascular response. [Cancer Res 2007;67(5):2040–5]

Introduction
At least eight distinct peripheral blood lineages are derived from a rare subpopulation of hematopoietic stem cells that reside in the bone marrow and circulate in the peripheral blood at low levels (1, 2). In addition, hematopoietic stem cells may have the capacity to transdifferentiate into diverse lineages, including muscle (3), liver (4), neural (5), and endothelial (6) cells. These findings were supported by studies indicating that transfer of wild-type (WT) bone marrow into angiogenic defective mice restored tumor angiogenesis and that incorporation of bone marrow–derived endothelial progenitor cells into neovessels was necessary for tumor angiogenesis (7, 8).

Radiation is well known to cause damage to endothelial cells (9, 10) and to inhibit neovascularization (11, 12). The finding that bone marrow–derived endothelial progenitors may participate in neovascularization suggests that radiation could potentially inhibit angiogenesis by suppressing the recruitment of bone marrow–derived endothelial progenitors. Several recent studies have used whole-body irradiation and hematopoietic reconstitution to examine the contribution of circulating bone marrow–derived cells to neovascularization (7, 8, 13–17). These studies did not, however, quantify the relative contribution of hematopoietic-derived and tissue resident endothelial cells to overall radiation-induced neovascular suppression. In the current study, we examined the relationship between radiation-induced bone marrow suppression and neovascularization and quantified the effect of radiation on angiogenesis using the mouse corneal micropocket assay.

The corneal micropocket assay uses purified angiogenesis factors implanted in the normally avascular cornea, where the neovascular response can be readily visualized and quantified (18). This model has been extensively used to study genetic variations in angiogenic potential (19–22) and to quantify the effects of pharmacologic agents on angiogenesis (23, 24). We found that mice exposed to a single dose of radiation used for bone marrow transplantation exhibited a long-term decrease in capacity to undergo neovascularization and tumor growth. Local exposure induced suppression of angiogenesis comparable with that induced by whole-body exposure. Moreover, bone marrow suppression induced by irradiation did not suppress neovascularization, provided that the cornea was shielded from radiation. These studies support the concept that although hematopoietic stem cells can readily reconstitute peripheral blood, they are not able to restore a radiation-induced deficit in neovascular response.

Materials and Methods
Bone marrow transplantation. C57BL/6J immune competent donor mice were anesthetized by isoflurane inhalation and then sacrificed by cervical dislocation. Bone marrow cells were collected by irrigating the femur and tibia with saline using a syringe and a 26-gauge needle. The harvested cells were passed 10 times through a 26-gauge needle, and clumps were removed using a 40-μm nylon mesh filter. The cells were washed once and resuspended in saline at a density of 5 × 10⁷ cells/mL. WT and RAG1-deficient mice were exposed to 9.5 Gy, and severe combined immunodeficient (SCID) mice were exposed to 2.5 Gy. Mice were exposed to radiation from a Cs-137 source in a low-dose GammaCell 40 laboratory irradiator (Nordion International, Kamata, Ontario, Canada) at a rate of 88 rads/min. The irradiated mice were injected i.v. with approximately
2 × 10^6 donor bone marrow–derived cells in a 50 μL volume. The mice were housed in sterilized microisolator cages. Sulfatrim/bactrim was added to the autoclaved drinking water (30 mg/kg dose) for 2 weeks to prevent infections.

**Mouse strains.** Immune competent, SCID (B6.CB17-Prkdcsid/SjL), and RAG1 (B6.129S7- Rag1tm1Mom/J) mice in the C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME). Immune competent green fluorescence protein (GFP) transgenic mice (UBI-GFP/BL6) are C57BL/6J mice that express GFP under the control of the ubiquitin promoter (25). C57BL/6J-GFP-scid mice (16) were generated by crossing the transgene from UBI-GFP/BL6 mice into B6.CB17-Prkdcsid/SjL mice. The 129S1/SvJ mice were obtained from The Jackson Laboratory. Animals were housed in microisolator cages and fed autoclaved water and chow ad libitum.

**Corneal angiogenesis assay.** Angiogenesis was induced in the mouse cornea by implanting a polymer containing basic fibroblast growth factor (bFGF) in the mouse cornea as described previously (18). Briefly, mice were anesthetized with Avertin (400 mg/kg), and the eye was further anesthetized with topical proparacaine HCl (Alcon, Fort Worth, TX). Under an operating microscope (Zeiss, Jena, Germany), the eye was proptosed using forceps, and an intrastromal linear keratotomy was advanced to 1 mm from the limbus using a partially blunted von Graefe knife. Topical bacitracin-neomycin-polymyxin ointment (Pharmacia, Kalamazoo, MI) was applied once to the eye. Angiogenesis was quantified as anesthetized (Avertin 400 mg/kg) animals 5 days after implanting pellets using a slit lamp biomicroscope. Area of neovascularization (AON; in mm^2) was calculated as vessel length x vessel width x 0.0063 (18). Statistical significance for differences in angiogenesis was determined by a Kruskal-Wallis test. All statistical analyses were two sided, and a P value of <0.05 was considered statistically significant. Except when noted, blood was collected from the lightly anesthetized animals immediately after grading neovascularization. Samples were collected from the retro-orbital sinus using heparinized microhematocrit capillary tubes. Approximately 250 μL blood was collected into Microtainer (Fisher Scientific, Waltham, MA) tubes containing EDTA. Complete blood counts were determined in the Department of Laboratory Medicine at Children’s Hospital (Boston, MA).

**Fluorescence imaging.** To image neovessels and bone marrow–derived GFP^+ cells in the cornea, 100 μL of 5% lysine fixable, 2 million molecular weight TRITC-dextran (Molecular Probes, Carlsbad, CA) was dissolved in saline was injected i.v. via the retro-orbital sinus into mice anesthetized with Avertin. The mice were sacrificed after 5 min, and enucleated eyes were fixed in 2% paraformaldehyde for 30 min. The cornea was dissected from the eye and mounted onto microscope slides using Fluoromount-G (Southern Biotech, Birmingham, AL). TRITC-dextran and GFP^+ bone marrow–derived cells were imaged separately and then electronically merged using Corel Photo-Paint 12.0 (Ottawa, Ontario, Canada).

**Tumor experiments.** The Lewis lung carcinoma (LLC) line (26) was maintained in a humidified, 10% CO_2, 37°C incubator. The cells were cultured in DMEM (Invitrogen Life Technologies, Carlsbad, CA) containing 7% fetal bovine serum (Invitrogen Life Technologies), 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, and 100 units/mL penicillin/streptomycin. For in vivo studies, tumor cells were trypsinized, washed twice in sterile 1× PBS, and resuspended in saline at a density of 2 × 10^6 cells/mL. One million tumor cells in a 30 μL volume were injected in the s.c. space of the backs of shaved mice. Tumor dimensions were measured using calipers, and tumor volume was calculated (GEP) according to the following formula: volume = width^3 × length × 0.52.

All animal studies were done according to institutional regulations in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations of the U.S. Department of Agriculture, Department of Health and Human Services, and the NIH.

| Table 1. Angiogenesis in a corneal model of neovascularization following whole-body irradiation and bone marrow reconstitution in C57 mice |
|---------------------------------|-----------------|-----------------|-----------------|
| Gy                              | AON, mm^2 (n)^*| % Inhibition |
| C57BL/6j                       | 0.195 ± 0.29 (36) | NS |
| 2.5                            | 1.70 ± 0.36 (17) | 12.8 NS |
| 9.5                            | 0.77 ± 0.20 (15) | 60.5 <0.001 |
| C57BL/6-GFP                    | 0.194 ± 0.25 (10) | NS |
| 2.5                            | 0.78 ± 0.43 (25) | NS |
| 9.5                            | 0.86 ± 0.31 (29) | 55.9 <0.001 |
| C57BL/6-GFP-scid               | 0.187 ± 0.24 (7) | NS |
| 2.5                            | 0.88 ± 0.42 (9) | NS |
| 9.5                            | 0.69 ± 0.16 (10) | 64.6 <0.001 |

Abbreviation: NS, not significant.

*Corresponds to the number of eyes.

Percentage inhibition, relative to C57BL/6j control (0 Gy), was calculated as follows: [(1.95 – AON) / 1.95] × 100%.

Significant difference in AON compared with C57BL/6j control was determined by Kruskal-Wallis with Dunn’s multiple comparison posttest (GraphPad InStat version 3.02, GraphPad Software, San Diego, CA).

**Results**

**Long-term suppression of angiogenesis in a corneal model of neovascularization.** Previous studies have shown that the ability to respond to a defined quantity of an angiogenesis factor varies considerably between mouse strains (19), so the following experiments were done using mice having a C57BL/6j genetic background. We initially compared the neovascular response in several transgenic and immune deficient mice. As shown in Table 1, the neovascular responses of mice unexposed to radiation were comparable for all C57BL/6 substrains, including WT (C57BL/6j), immune competent GFP transgenic (C57BL/6j-GFP), SCID (C57BL/6j-scid), GFP transgenic SCID (C57BL/6j-GFP-scid), and RAG1-deficient (C57BL/6j-RAG1) mice. DNA-activated protein kinase, catalytic subunit deficiency (SCID mice), RAG1 deficiency, ectopic GFP expression, or a combination of the SCID mutation and GFP transgene expression therefore did not affect neovascularization.

We next determined the effect of radiation and bone marrow reconstitution on corneal neovascularization. C57BL/6j and C57BL/6j-RAG1 mice were exposed to a single dose of 9.5 Gy whole-body radiation. SCID mice, which are sensitive to ionizing radiation due to an inactivating mutation in the prkdc gene (27), were exposed to 2.5 Gy (28). A dose of 9.5 Gy was lethal for SCID mice even after bone marrow transplantation. The irradiated mice were injected i.v. with bone marrow cells from immune competent GFP transgenic mice. Eight weeks after irradiation and bone marrow transfer, complete blood counts were done to assess hematopoietic reconstitution. Flow cytometric analyses of peripheral blood confirmed that >90% of the CD45^+ leukocytes were donor-derived GFP^+ cells (data not shown). The neovascular responses, however, were diminished by approximately 56% to 65% compared with nonirradiated control mice (Table 1). At a dose of 2.5 Gy, the angiogenic response in...
C57BL/6J-scid mice was suppressed by 56% (P < 0.001). The neovascular response in WT C57BL/6J mice was unaffected at this dose.

The effect of radiation on angiogenesis following bone marrow reconstitution is independent of bone marrow status. To further investigate the relationship between hematopoietic reconstitution and angiogenesis, the corneal neovascular response was quantified over time following whole-body irradiation and bone marrow transplantation. In Fig. 1, four groups of WT C57BL/6J mice (five mice per group) were exposed to whole-body radiation (single dose of 9.5 Gy) and then injected with autologous donor bone marrow cells. On the same day following radiation exposure and bone marrow transfer (day 0), the mice were tested for their capacity to respond to bFGF. After 5 days, the neovascular responses were quantified, and a complete blood count was obtained for each mouse. This procedure was repeated with the remaining groups of mice 8, 27, and 69 days post-irradiation. As shown in Fig. 1, a 54% suppression of angiogenesis was observed on day 5 postirradiation, at which time the peripheral blood counts were severely reduced. The peripheral blood counts recovered over the 10-week (72 days) period, but the neovascular response was chronically suppressed between 50% to 60%. Thus, neovascular suppression in the corneal micropocket model following whole-body irradiation could not be rescued by hematopoietic reconstitution.

The effect of local irradiation on angiogenesis is comparable with the effect of whole-body exposure. To further address whether the defect in neovascular response caused by radiation was a local effect, we compared the effect of whole-body and local exposure on corneal neovascularization. As shown in Fig. 2 and Table 2, radiation applied locally to the head inhibited corneal neovascularization compared with whole-body exposure. This would support the notion that neovascular suppression was a
dependent angiogenic potential, defined by their neovascular marrow.

were derived from the local environment rather than from the bone newly formed vessels, which indicate that the endothelial cells after bone marrow transplantation by flow cytometric analysis of the C57BL/6J mice exposed to 9.5 Gy with ocular shielding were derived cells to neovascularization in the corneal micropocket assay. To visualize the presence of bone marrow–derived cells to neovascularization in the corneal micropocket assay, C57BL/6J mice exposed to 9.5 Gy with ocular shielding were reconstituted with bone marrow from GFP donor mice before stimulating neovascularization. Engraftment was confirmed 8 weeks after bone marrow transplantation by flow cytometric analysis of the peripheral blood. Before stimulating neovascularization, few GFP cells were observed in the cornea. Neovascularization was then induced with 80 ng bFGF, which produced a normal response in the shielded cornea after 5 days (similar to Fig. 2A, bottom right and Table 2). Epifluorescence examination readily revealed an influx of GFP bone marrow–derived cells into the cornea. The GFP cells were loosely or perivascularly associated with but not incorporated into the newly formed vessels, which indicate that the endothelial cells were derived from the local environment rather than from the bone marrow.

Previous studies by Shaked et al. (22) showed that strain–dependent angiogenic potential, defined by their neovascular response in the corneal micropocket assay, correlated with levels of circulating endothelial or putative endothelial progenitor cells. To address whether the lack of bone marrow contribution to cornal neovascular response was a C57BL/6 strain–dependent phenomena, the highly angiogenic 129S1/SvImJ strain of mice (19, 22) was also tested in the ocular shielding experiments. The AON in control nonirradiated 129S1/SvImJ mice (AON, 2.95 ± 0.58 mm²; n = 8) induced with 80 ng bFGF was considerably higher than in C57BL/6 strain of mice (Table 2), which is consistent with previous studies (19, 22). In mice exposed to 9.5 Gy with ocular shielding, the neovascular response (AON, 3.26 ± 0.78 mm²; n = 8) was slightly higher than in control mice, although the difference was not statistically significant (P > 0.05, based on a Kruskal-Wallis ANOVA with a Dunn's multiple comparison posttest). In some of the animals, hyphema was noted, which was attributable to a severe reduction in platelet counts coupled with a robust neovascular response and fragile neovessels. As expected, mice, in which only the eyes were exposed to 9.5 Gy, exhibited a significant reduction (46%; AON, 1.60 ± 0.17 mm²; n = 9; P < 0.01) in corneal neovascular response compared with control mice. Taken together, these results show that bone marrow suppression does not inhibit corneal neovascularization and that bone marrow reconstitution cannot rescue the inhibitory effect of local irradiation on corneal neovascularization in both the C57BL/6 strain and the highly angiogenic 129S1/SvImJ strain.

Effect of prior host exposure to radiation on the growth of transplanted tumor cells in vivo. To determine if the reduced neovascular response in irradiated animals affected tumor growth, we implanted Lewis lung tumor cells into the s.c. space of mice that previously underwent radiation exposure and bone marrow transplantation. Indeed, the growth of transplanted tumor cells, which were never exposed to radiation, was significantly suppressed even when implanted 8 weeks after the mice had been exposed to radiation. Figure 3A shows the tumor volumes in nonirradiated WT and GFP transgenic control mice and in irradiated (9.5 Gy) mice that had previously undergone reciprocal bone marrow transfers. No statistical difference in tumor volume was observed between the transgenic and WT mice within the nonirradiated control mice. However, the average tumor volume in

<table>
<thead>
<tr>
<th>Exposed area*</th>
<th>Measured dose, Gy (n = 3)</th>
<th>C57BL/6J</th>
<th>129S1/SvImJ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AON, mm²</td>
<td>P</td>
<td>% Suppression</td>
</tr>
<tr>
<td>Control (no exposure)</td>
<td>—</td>
<td>2.11 ± 0.37</td>
<td>—</td>
</tr>
<tr>
<td>Whole body</td>
<td>—</td>
<td>—</td>
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<tr>
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<td>2.10 ± 0.46</td>
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<tr>
<td>Body</td>
<td>7.8 ± 0.4</td>
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* Mice were positioned inside a 1-inch-thick lead cylindrical shield to selectively expose either the head or body to an applied dose of 9.5 Gy. Mice undergoing whole-body or body only exposure received bone marrow engraftment to prevent death from infection.
† The radiation dose was measured using thermoluminescent dosimeters placed at strategic locations on the mice. The dosimeters were processed by a National Voluntary Laboratory Accreditation Program vendor to determine radiation dose.
‡ P values were determined by Kruskal-Wallis with a Dunn's multiple comparison posttest.
† Percentage suppression was defined as [(vessel area of control) − (vessel area of irradiated)] / (vessel area of control) × 100%.

Table 2. Effect of local and whole-body exposure on neovascularization

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the irradiated mice (volume, $373 \pm 51 \text{ mm}^3$) was 60% smaller than in the nonirradiated mice (volume, $932 \pm 332 \text{ mm}^3$), and the difference was highly significant ($P = 0.0011$, significance was determined by an unpaired $t$ test with Welch correction; $n = 10$ animals per group). We also compared the growth rate of tumors in control animals and in animals that had undergone irradiation with bone marrow transfer 3 days prior and 56 days before tumor cell implantation. As shown in Fig. 3A, comparable inhibition of tumor growth was observed for tumor cells implanted 3 days postirradiation, when the peripheral blood counts were drastically suppressed (Fig. 1), and 56 days postirradiation, when the peripheral blood was reconstituted. Taken together, the results indicate that local irradiation inhibits a subsequent neovascular response both in the corneal pocket model as well as in the tumors.

Discussion

Whole-body irradiation followed by bone marrow reconstitution has been widely used to study hematopoietic development and function in various model systems (1, 7, 8, 13–17, 25, 29). The effect of whole-body exposure on the neovascular response, however, has not been quantified previously. We therefore examined the effect of radiation exposure and hematopoietic reconstitution on angiogenesis in a corneal micropocket assay. We found that mice that had undergone bone marrow transplantation exhibited long-term latent neovascular suppression, which was evident even 8 weeks after the mice had undergone bone marrow transplantation.

Previous studies have shown that hematopoietic-derived cells significantly contribute to the architecture of newly forming vessels (7, 8), which would predict that bone marrow status should affect the neovascular response. However, mice exposed to whole-body irradiation exhibited a permanent neovascular defect that was unaffected by hematopoietic reconstitution. Radiation applied locally to the eye was comparable with whole-body exposure with respect to inhibition of neovascularization. Moreover, bone marrow suppression induced by radiation did not inhibit neovascularization in nonirradiated cornea. These results indicate that neovascular suppression was due to the direct effect of radiation on the eyes and unrelated to either bone marrow suppression or defective recruitment of circulating bone marrow–derived cells that may be necessary for a normal neovascular response. Infusing bone marrow–derived cells, which was necessary following radiation-induced bone marrow suppression, itself possibly contributed to suppressing neovascularization. However, bone marrow suppression with ocular shielding did not stimulate neovascularization, so infusing bone marrow cells would not be expected to attenuate neovascularization.

GFP$^+$ bone marrow–derived cells in mice that had previously undergone hematopoietic reconstitution were loosely or perivascularly associated with neovessels in nonirradiated corneas. The lack of bone marrow–derived cell incorporation into vessels is consistent with a corneal neovascular response that is unaffected by bone marrow suppression and hematopoietic reconstitution. Hematopoietic-derived cells have been shown to potentially promote angiogenesis in a paracrine manner by secreting or mobilizing growth factors (17, 30). Bone marrow–derived cells may have a negligible effect on the magnitude of the neovascular response in the corneal micropocket assay; a relatively high dose of an exogenously supplied angiogenesis factor (80 ng bFGF) may mask or minimize potential contributions from hematopoietic-derived paracrine factors. The corneal micropocket assay may thereby reflect the inherent angiogenic capacity of resident endothelial cells. The lack of bone marrow contribution to neovascularization in the mouse cornea was observed in both the C57BL/6j and the 129S1/SvImJ strains; strain-dependent differences in neovascular responses are therefore most likely due to inherent differences in the endothelial cells rather than in bone marrow–derived cells (such as an inflammatory cell) that may modulate endothelial cell responses.

Ionizing radiation causes cellular damage by inducing DNA double-strand breaks, which can be repaired by a nonhomologous DNA end-joining pathway (31). An enzyme that is critical to this repair pathway is the catalytic subunit of the DNA-dependent protein kinase DNA-PKcs, which is also required for immunoglobulin and T-cell receptor gene rearrangements. SCID mice lack DNA-PKcs and are radiation sensitive as well as immune compromised due to lack of T and B cells. Lymphocyte deficiency had no effect on angiogenesis because SCID, RAG1-deficient mice, and immune competent WT mice responded equally to bFGF. However, angiogenesis in SCID mice were inhibited at a dose of 2.5 Gy, which had no effect on WT mice. This indicates that a DNA-PKcs-dependent pathway is capable of restoring a normal neovascular...

Figure 3. Inhibition of LLC implanted in C57BL/6J mice following whole-body irradiation and bone marrow transplantation. A, tumor volumes of LLC measured 12 d postimplantation in nonirradiated control WT and GFP transgenic mice as well as irradiated mice (9.5 Gy) that underwent reciprocal bone marrow transfer between WT and GFP transgenic mice [direction of donor to recipient transfer (arrow)]. B, nonirradiated LLC tumor cells implanted in control mice and mice that underwent autologous WT bone marrow transplantation either 56 or 3 d before implanting tumor cells.
response following exposure to a lower dose of 2.5 Gy but not to a higher dose of 9.5 Gy. These results are consistent with previous studies in a dorsal skin window model showing that the SCID mutation and pharmacologic DNA-PKc antagonists did not affect tumor neovascularization but significantly sensitized vessels to the effect of ionizing radiation (11). The DNA-PKc–dependent repair pathway is not necessarily specific to endothelial cells, however, because the SCID mutation causes a general defect in DNA repair (32), and exposing mice to a dose sufficient to suppress angiogenesis in WT and SCID mutant mice also resulted in loss of hair pigmentation (data not shown).

The therapeutic benefit of radiation is generally attributed to a direct killing of tumor cells. In our studies, irradiating the host reduced its capacity to respond to an angiogenic stimulus and also impaired the growth of transplanted tumor cells that were never exposed to radiation. The current work suggests that a persistent anti–endothelial cell effect significantly contributes to overall tumor response to radiotherapy. In cases where radiation has been used to prevent tumor recurrence as well as seeding and metastasis (33, 34), radiation may prevent relapse in part by inducing a longer-term defect in capacity of the host to undergo a neovascular response to tumor cells.

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