Local Radiotherapy Induces Homing of Hematopoietic Stem Cells to the Irradiated Bone Marrow

Carlo Bastianutto,1,4 Asim Mian,1,4 Julie Symes,1,5 Joseph Mocanu,1,4 Nehad Alajez,1,4 Gillian Sleep,1,8 Wei Shi,1,3 Armand Keating,1,6 Michael Crump,1,6 Mary Gospodarowicz,1,7 Jeff Medin,1,5 Mark Minden,2,6 and Fei-Fei Liu1,2,4,7

Departments of 1Medical Biophysics, 2Medicine, and 3Radiation Oncology, University of Toronto; Divisions of 4Applied Molecular Oncology and 5Cell and Developmental Biology, Ontario Cancer Institute and 6Division of Medical Oncology and 7Department of Radiation Oncology, Princess Margaret Hospital, University Health Network; and 8Sunnybrook Health Sciences Centre, Toronto, Ontario, Canada

Abstract
Local breast radiotherapy (RT) is associated with a 3-fold increased risk of secondary acute myeloid leukemia. As a first step in determining the mechanism(s) underlying this observation, we investigated the role of RT in mediating the active recruitment of hematopoietic stem cells (HSC) to the site of RT. Our results show in a mouse model that local RT delivered to the left leg causes preferential accumulation of bone marrow mononuclear cells to the irradiated site, with maximum signal intensity observed at 7 days post-RT. This is associated with a 4-fold higher number of donor-derived HSC present in the left leg, demonstrating recruitment of HSC to the site of RT. SDF-1, matrix metalloproteinase 2 (MMP-2), and MMP-9 expression is significantly increased in the irradiated bone marrow, and their inhibition significantly reduced HSC recruitment to the irradiated bone marrow. Our data show that local RT has significant systemic effects by recruiting HSC to the irradiated bone marrow site, a process mediated by SDF-1, MMP-2, and MMP-9. These results raise the possibility that the exposure of increased numbers of HSC at a local site to fractionated irradiation may increase the risk of leukemogenesis. Our data also suggest some opportunities for leukemia prevention in breast cancer patients undergoing RT. [Cancer Res 2007;67(21):10112–6]

Introduction
Approximately half of all cancer patients are treated with radiation therapy (RT), which can give rise to secondary malignancies (1), such as secondary acute myeloid leukemia (sAML). In particular, 1% to 2% of breast cancer survivors after treatment with adjuvant chemotherapy and RT will develop sAML (2–7). Local breast RT alone (with or without lymph nodes) induces a 3-fold higher risk of sAML within 1 to 5 years (8), enhanced by 7-fold at 8 years if the RT dose delivered to the bone marrow exceeds 9 Gy (9).

Several lines of evidence indicate that AML is a stem cell malignancy (10, 11), suggesting that mutagenic events in the hematopoietic stem cell (HSC) could lead to AML development. Normal HSC migratory properties have been recently taken into account in a mathematical model designed to predict the cancer patient’s risk of developing sAML (12). However, previous studies have shown that HSC homing can be enhanced by total body radiation (13, 14). We hypothesized that local RT could increase the risk of sAML in breast cancer patients by enhancing homing of HSC to the RT field, in turn exposing a higher number of HSC to the mutagenic effects of RT, thereby increasing the probability of generating leukemic HSC. As a first step in this work, the role of local RT in mediating HSC homing to the site of RT was investigated.

Materials and Methods
Bone marrow mononuclear cell in vivo imaging. Bone marrow mononuclear cells (BMC; 8–12 × 10^6) were extracted from the femur and tibia of 4 to 24 weeks green fluorescence protein (GFP) transgenic BALB/c mice (15), pretreated i.p. with 150 mg/kg 5-Fluouracil for 3 days. BMCs were transduced with a luciferase lentivirus (Supplementary data) and injected 24 h later (1–1.5 × 10^6 cells per mouse) into the tail vein of BALB/c recipient mice. One day before BMC injection, the left leg of recipient BALB/c mice was irradiated with 5 Gy (100 kV; 10 Gy/min; Gemini Vertical X-ray Beam; Picker Industrial).

Bioluminescence imaging (BLI) was conducted at different time points to monitor the migration of BMC. Recipient BMC-injected BALB/c mice were anaesthetized (50 mg/kg ketamine, 10 mg/kg xylazine, and 1.5 mg/kg acepromazine) and then injected i.p. with 3 mg synthetic D-luciferin (Xenogen) in 100 μL PBS. Bioluminescence images were captured ~15 min later using the Xenogen IVIS 100 system (Xenogen), with an exposure time of 0.1 to 900 s (to maximize signal to noise). Elliptic regions of interest were drawn, and counts were integrated around the irradiated leg and adjacent regions (as background). The counts were background-subtracted and divided by the exposure time to yield relative light units per second (RLU/s) and then analyzed using the Living Image 2.50 software.

Flow cytometry. For the quantification of murine HSC, flow cytometry was done by dividing BMC into groups and incubating with 0.1 μg purified antimouse CD16/CD32 Fcγ III/II receptor (BD Bioscience), followed by simultaneous staining with 0.2 μg each of PE-Cy5.5-conjugated antimouse CD117 (eBioscience), PE-conjugated antimouse Thy1.1 (Abcam), and APC-conjugated antimouse Sca-1 (BioLegend) for 1 h at 4°C. Control cells were stained with matched isotype controls PE-Cy5.5-conjugated rat IgG2b (BioLegend), PE-conjugated rat IgG2a
(BioLegend), and APC-conjugated rat IgG2a (BioLegend). Stained samples were acquired using BD FACSCalibur (BD Bioscience) and analyzed using Cell Quest software version 3.3. To avoid bias, the acquisition and analysis were done in a blinded fashion (the identity of the samples was not revealed to the flow cytometry operator).

SDF-1, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression and inhibition. The expression of SDF-1, matrix metalloproteinase 2 (MMP-2), and MMP-9 were assessed using quantitative real-time PCR (qRT-PCR) as described in the Supplementary data. To confirm the functional role of SDF-1, MMP-2, and MMP-9 in the homing process,

**Figure 1.** Recruitment of BMC to the site of local RT. A, in vivo imaging of luciferase expressing BMC in four representative mice 7 d after RT. Images were obtained with a CCD camera, and the BLI images were overlapped with a photographic image. R, right leg; L, left leg. B, relative luminescence signal intensity between the left (irradiated, red), and right (unirradiated, blue) legs of injected mice as a function of days post-RT. Mice were RT-treated on day 0 and injected with BMC on day 1. Points, average RLU from nine mice; bars, SE. *, P < 0.01, calculated by comparing the corresponding data time points between the left and right legs.

**Figure 2.** Recruitment of HSC to the site of local RT. The numbers in each panel indicate the percentage of HSC defined as Sca1+, c-Kit+, and Thy1.1low cells, which are GFP+. A, a representative analysis done with BMC extracted from GFP+ BALB/c mice. The subpopulation of BMC positive for Sca1 and with low expression of Thy1.1 was further analyzed for the expression of c-Kit and GFP. B, percentage of GFP+ HSC cells in GFP+ BALB/c mice, irradiated (left), and unirradiated (right) legs (0.52%, 3.77%, and 0.35%, respectively).
before injection, half of the BMC were incubated for 1 h in PBS with a rabbit polyclonal isotype control antibody (Abcam) and the other half with an antibody against CXCR4 (Torrey Pines Biolabs), with subsequent injection into the CXCR4 or control groups of mice. One hour before the injection of the BMC, a second group of recipient mice were injected i.p. with a common inhibitor of MMP-2 and MMP-9 (EMD Biosciences) or with BMC pretreated with vehicle. The combination group of mice was pretreated with the MMP-2–MMP-9 inhibitor, along with the combination of BMC pretreated with the anti-CXCR4 antibody (MMP-2–MMP-9/CXCR4 group), or isotype control (MMP-2–MMP-9 group). At day 6, the percentage of GFP+, Sca1+, Thy1.1low, c-Kit+ cells between the irradiated (left) and unirradiated (right) legs was compared in each treatment group using flow cytometry, as described above.

Results

To show that local RT could recruit HSC to the irradiated bone marrow (Figs. 1 and 2), we used BMCs isolated from transgenic GFP-expressing mice. The BMC were infected with a lentivirus expressing luciferase and injected into the tail vein of syngeneic GFP−/− recipient mice. The left legs of the recipient mice were irradiated (5 Gy) 24 h before injection of BMC, and at different time points, migration of the injected BMC was monitored using BLI. A differential BLI signal between the irradiated and the unirradiated leg becomes detectable after 3 days (133 versus 18 RLU, respectively; \( P < 0.01 \)), peaking at day 7 post-RT (1,033 versus 78 RLU; \( P < 0.01 \)) and becoming undetectable after 14 days.

The recruitment of murine HSC in the irradiated leg was confirmed by flow cytometry. The BMC cells were extracted from the right and left legs at the time of maximum BLI signal (day 7 post-RT) and analyzed for expression of GFP and HSC markers c-Kit, Sca-1, and Thy1.1 (Fig. 2A). No significant difference in the total number of HSC was observed between the two legs (data not shown), suggesting that complete bone marrow repopulation might have been achieved by day 7 post-RT. However, a 6-fold to 10-fold increase of 3.77% versus 0.35% in GFP+ HSC was observed in the irradiated leg compared with the right leg, respectively (Fig. 2B). These data, combined with the BLI results, confirmed that local RT induced homing of HSC to the site of RT.

Based on the literature, the mediators for this process could include SDF-1, MMP-2, and MMP-9 (16, 17). To address this possibility, BALB/c mice were treated with RT (5 Gy) to the leg and, at different time points, total RNA was extracted from the BMC of both legs independently, and the expression of SDF-1, MMP-2, and MMP-9 was measured using qRT-PCR. The data show up-regulation of all three transcripts by 16 h post-RT, lasting for at least 48 h (Fig. 3). In particular, expression of MMP-2 is most significantly up-regulated after RT (16.7-fold at 24 h, \( P < 0.01 \)), indicating that RT-induced up-regulation of these chemokines and MMPs is not a strain-specific process.

The involvement of SDF-1, MMP-2, and MMP-9 was confirmed using specific inhibitors or blocking antibody (Fig. 4), as described in Materials and Methods. After 7 days post-RT, significant inhibition of HSC homing to the site of RT was observed in the mice treated with the MMP-2 and MMP-9 inhibitor, or with the MMP inhibitor plus the CXCR4 antibody (down to 0.33 or 0.4, respectively; \( P < 0.05 \); Fig. 4B). Administration of the CXCR4 antibody reduced recruitment to 0.52 (\( P < 0.01 \)), indicating that both MMPs and SDF-1 are involved in RT homing of HSC (Fig. 4B).

Discussion

RT remains one of the most efficient cancer therapeutic modalities for at least half of the patients with solid malignancies. However, significant long-term side effects remain, ranging from local fibrosis to secondary malignancies (1). Until recently, the complications induced by RT were believed to be an exclusively local phenomenon. Previous studies on the effect of total body RT in HSC homeostasis (13, 14) have shown that whole body RT could alter HSC dynamics. In this work, we show for the first time that...
Local RT also influences HSC homeostasis, amplifying the effects of local RT to a systemic level. The importance of HSC dynamics in the risk of sAML was recently addressed elegantly by Shuryak et al. (12), using a mathematical model. This model calculates the risk of sAML by including a variable that accounts for the normal migratory properties of HSC, as well as the capacity of preleukemic HSC to leave the radiation field. In that model, the combination of both variables resulted in an overall reduction of leukemic risk (12). Our data, however, show that RT actually increases the homing of HSC to the RT site, suggesting a dynamic process of HSC homing above that of normal recruitment. Hence, future models for RT-associated leukemia should consider including a new variable that reflects the increased recruitment by RT.

This dynamic change seems to peak by day 7 post-RT, after which, the luciferase signal progressively attenuates, suggesting that by 2 weeks, normal homeostasis has been reestablished in the irradiated bone marrow. The almost null number of migrant HSC to the unirradiated right leg suggests that the recruited HSC do not relocate to nonirradiated bone marrow sites. This dynamic perturbation of HSC homing could be potentially further amplified by the repetition of this process with each fraction of RT delivered over a course of curative or adjuvant RT.

SDF-1 and its receptor CXCR4 are the key effectors of HSC homing and engraftment to the bone marrow both in human and mice (18, 19). The expression of SDF-1 can be up-regulated by 24 to 48 h after chemotherapy or total body RT, and this...

![Figure 4](https://www.aacrjournals.org/doi/figure/10.1158/0008-5472.CAN-07-0713)

**Figure 4.** Inhibition (inh.) of MMP-2 and MMP-9 blocks HSC recruitment to the irradiated bone marrow. A, representative flow cytometry results, comparing the percentage of HSC-GFP+ cells in the left and right leg, achieved with the different treatments. B, columns, ratio of HSC-GFP+ cells between the left and right leg normalized to the ratio of the control group; bars, SE. *, P < 0.05, calculated by comparing the different treatments to the control group. Representative of the average of three independent experiments, each consisting of two recipient mice per group.
up-regulation correlates with efficiency of engraftment of injected BMCs (13, 14). Our data suggest that local RT also causes a similar kinetics in SDF-1 expression in the bone marrow, with progressive increase in gene expression peaking at 48 h (Fig. 3). However, the functional experiments show that inhibition of CXCR4 only partially blocks the recruitment of HSC to the site of RT (Fig. 4), suggesting that either incomplete inhibition was achieved or additional factors are necessary for HSC homing. Our data show up-regulation of MMP-9 and particularly MMP-2 in the bone marrow after exposure to local RT (Fig. 3) and significant reduction in HSC homing with a common inhibitor of MMP-2 and MMP-9 (Fig. 4), indicating that either or both MMPs also participate in homing of HSC to the irradiated bone marrow.

In conclusion, this work shows for the first time that local RT alters the dynamics of HSC homeostasis by increasing their homing to the site of RT. These results are important to understand the mechanism(s), which might be responsible for the sAML observed in cancer patients treated with RT. This observation has additional implications, with the increasing use of intensity modulated RT, which exposes a much larger integral volume of normal tissues (and bone marrow) to ionizing radiation, thereby potentially, even further, increasing the risk of secondary malignancies (20). Hence, a clearer understanding of the biology underpinning this process could provide opportunities for interventions, which might ultimately prevent this fatal iatrogenic complication.

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References

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