Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice

John P. Chute¹, Garrett G. Muramoto¹, Alice B. Salter¹, Sarah K. Meadows¹, Dennis Rickman², Benny Chen¹, Heather A. Himburg¹, Nelson J. Chao¹

¹ Division of Cellular Therapy, Department of Medicine, Duke University Medical Center; ² Department of Neurobiology, Duke University Medical Center, Durham, NC

Key Words: Endothelial cells
Radiation
Hematopoiesis

Corresponding Author: John P. Chute M.D.
Associate Professor of Medicine
Division of Cellular Therapy
Duke University Medical Center
2400 Pratt Street
Durham, NC 27710
Ph 919-668-4706; Fax 919-668-1091
john.chute@duke.edu

Supported, in part, by grant AI067798-01 (J.P.C.) from the National Institute of Allergy and Infectious Diseases.

Authors’ contributions: J.C. designed the research, analyzed the data and wrote the paper; G.M., A.S., S.M., B.C., and H.H. performed research; D.R. performed research and analyzed data; N.C. helped design research and contributed to the paper.
Abstract

Flk-1$^+$ endothelial progenitors contribute critically to the definitive onset of hematopoiesis during embryogenesis. Recent studies have suggested that adult sources of endothelial cells also possess hematopoietic activity. In this study we sought to determine whether transplantation of primary vascular endothelial cells (ECs) could enhance the hematopoietic recovery and survival of irradiated mice. C57Bl6 mice were exposed to sublethal and lethal doses of irradiation and subsequently transplanted with either primary murine brain-derived ECs (MBECs) or fetal blood-derived ECs (FBECs). Mice transplanted with MBECs alone demonstrated accelerated BM cellular recovery, radioprotection of BM c-kit$^+$ sca-1$^-$$^-\text{lin}^-\text{progenitors and enhanced regeneration of c-kit}^+\text{sca-1}^+\text{lin}^-\text{(KSL) stem/progenitor cells following irradiation compared to controls. MBEC transplantation also facilitated the recovery of circulating white blood cells and platelet counts following radiation exposure. Remarkably, 57\% of mice transplanted with MBECs alone survived long-term following 1050 cGy exposure, which was 100\% lethal in control mice. FBEC transplantation was also associated with increased survival compared to controls, although these mice did not survive long-term. These data suggest that reestablishment of endothelial cell activity can improve the hematopoietic recovery and survival of irradiated mice.
Introduction

Recent studies have shown that hematopoietic stem cells (HSCs) reside in close association with osteoblasts within the BM niche and this association contributes to the maintenance of the HSC pool in vivo. HSCs also reside in intimate anatomic proximity to vascular endothelial cells (ECs) from the earliest stages of embryonic development, through their migration to the fetal liver, and ultimately throughout their adult residence within the BM compartment. During embryogenesis, mice lacking flk-1 endothelial precursor cells fail to initiate normal hematopoiesis and gene marking studies suggest that hematopoietic and vascular endothelial cells derive from a common precursor cell, the hemangioblast. Similarly, bone marrow-, umbilical vein- and yolk sac-derived endothelial cells elaborate growth factors that support the proliferation of myeloid, erythroid, and megakaryocytic progenitors in vitro. Our laboratory has shown that primary human brain-derived endothelial cells produce a soluble hematopoietic activity that supports a 1-2 log expansion of the most primitive assayable human hematopoietic cell, which is capable of long-term repopulation in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Li et al. have also confirmed that murine brain-and heart-derived endothelial cells can support the maintenance of colony forming unit-spleen day-8 colonies (CFU-S-8) in vitro. Taken together, these data implicate vascular endothelial cells as a source of proliferative and regenerative signals for hematopoietic stem and progenitor cells.

Although these studies have demonstrated the capacity of endothelial cells to support hematopoiesis in vitro, evidence of the in vivo contribution of endothelial cells to adult hematopoiesis has only recently begun to emerge. Blockade of N-cadherin on vascular endothelial cells in the BM has been associated with delayed megakaryocyte recovery in 5-FU treated mice and accelerated repair of this BM vascular niche has been associated with early megakaryocytopoiesis post-chemotherapy. More recently, adenoviral delivery of angiopoietin,
which can signal through the Tie-2 receptor on vascular endothelial cells, has been associated with improved hematological recovery following 5-FU treatment in mice.  Although the effects of Angiopoietin-1 delivery may have been accounted for by direct effects on BM HSCs, which also express Tie-2, these data suggest the possibility that vascular endothelial cells in the BM contribute to hematopoietic regeneration in vivo.  Based upon our own observations that primary ECs support the amplification of HSCs in vitro, we initiated studies to determine whether systemic transplantation of primary ECs alone, in the absence of hematopoietic stem cell transplantation, could ameliorate the myeloablative effects of high dose radiation therapy.  Our results indicate that systemic administration of primary ECs alone accelerates hematopoietic recovery and enhances the survival of lethally irradiated mice.  Moreover, this effect is mediated directly through the enhanced regeneration of BM stem and progenitor cells.

Materials and methods

Isolation and passage of primary MBECs

To establish MBEC monolayers, 6-well culture plates (Corning Incorporated, Corning, NY) were coated with 0.2% gelatin (Sigma, St. Louis, MO) in DPBS, for 1 hour at room temperature.  Plates were then washed twice with DPBS, and 5ml/well of endothelial cell growth media, containing M199 (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 2mM L-glutamine (Invitrogen), 100 U/ml penicillin, 100 µg/ml streptomycin ([1% pcn/strp], Invitrogen), 4 U/ml heparin (Sigma), and 60 µg/ml endothelial cell growth supplement (ECGS, Sigma), was added.  MBECs were seeded at 1x10^5 /well and brought to >80% confluence by incubating for 48 hours at 37°C in 5% CO2.  GFP MBECs were generated from cortical brain vessel explants from C57BL/Ka-Thy1.1 GFP-transgenic
mice that were generated in the laboratory of Dr. Irving Weissman (Stanford University, Palo Alto, CA) and provided courtesy of Dr. Jos Domen (Duke University, Durham, NC).

**RT-PCR analysis of MBECs and Matrigel Assay**

Total RNA was extracted from $5 \times 10^6$ MBECs using a RNeasy Mini spin column (Qiagen, Valencia, CA), according to the manufacturer’s protocol. RNA was quantified using a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA), and total RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen), according the manufacturer’s instructions. PCR amplification of cDNA was conducted using Platinum Taq polymerase (Invitrogen) to detect the presence of mRNA for the following endothelial associated genes: vascular endothelial-cadherin (CD144), tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie 2), von Willebrand factor (VWF), Flk-1, and CD31 (PECAM) using sequence-specific primers as previously described.\(^6\)

Capillary-like tube formation assay was conducted using Matrigel basement membrane matrix (Becton Dickinson, San Jose, CA), according to the manufacturer’s recommended procedure. Briefly, 300µl/well of Matrigel was placed into a pre-cooled 24-well plate (Corning Incorporated) and incubated at 37°C for 30 minutes to solidify. $4 \times 10^4$ MBECs/well were placed onto the Matrigel matrix and incubated at 37°C in a 5% CO₂ humidified environment for 24-48 hours. Capillary tube formation was assessed via phase contrast microscopy as previously described.\(^6\)

**Transplantation of MBECs into irradiated C57Bl6 mice**

Eight to 10 week old C57Bl6 mice (Jackson Laboratory, Bar Harbor, ME) were irradiated with
700 cGy total body irradiation at a rate of 100 cGy/minute using the X-rad 320 irradiation system (AGFA NDT Inc., Lewistown, PA). Two hours following irradiation, mice were transplanted via either intravenous or intraperitoneal administration with MBECs (passage > 5) that had been washed twice and resuspended in PBS prior to tail vein injection. Mice were injected with MBECs daily from day 0 through day +4 (5 total doses) and then observed for survival. At days +5, +10, +15, +20, +25, and +30, subsets of transplanted mice and controls were euthanized and femurs were harvested. A subset of mice had fresh BM cells collected from the femurs, ficoll-hypaque collection of mononuclear cells (MNCs), and viable MNC counts were obtained. Another set of mice had femurs collected and fixed in 4% paraformaldehyde (Sigma) and dissolved in DPBS for 24 hours at room temperature. After fixation, one femur was processed for histology using hematoxylin and eosin (H&E) staining, and one femur was utilized for GFP visualization.

To detect native GFP, femurs were decalcified using daily changes of 14% EDTA (EMD Chemicals Inc., Gibbstown, NJ) (pH 7.1) for 7-10 days at 4°C. The femurs were washed thoroughly with D-PBS and soaked in 20% sucrose (Fisher Biotech, Fair Lawn, NJ) dissolved in D-PBS, overnight at 4°C. Decalcified bones were then placed into Tissue-Tek® cryomolds (Sakura Finetek, Torrance, CA), embedded in Tissue-Tek O.C.T. compound (Sakura Finetek), and frozen in a dry ice-ethanol bath. Cryosectioning was performed on a Leica CM1850 cryotome (Meyer Instruments Inc., Houston, TX), and 10µm serial sections were adhered to Poly-prep™ poly-L-lysine coated slides (Sigma). Slides were visualized on an Olympus FluoView FV500 confocal microscope (Olympus, Melville, NY). Wild-type and GFP+ BL6 mouse femur sections were used to distinguish and subtract background fluorescence.
Measurement of BM stem and progenitor cell content, hematological parameters and survival studies

Groups of mice were also irradiated with 700 cGy, transplanted with MBECs x 5 days and then sacrificed at day +10, +15 and +20 to measure BM stem and progenitor cell content. Untreated control mice, irradiated mice that were treated intravenously with MBECs and mice that received intraperitoneal MBEC injections were compared. Flow cytometric analysis was performed to measure the percentages of c-kit$^+$sca-1$^-$lin$^-$ progenitor cells and c-kit$^+$sca-1$^+$lin$^-$ (KSL) stem cell enriched populations within the BM in all mice. Briefly, bilateral femurs were harvested and BM cells were collected, following red blood cell lysis, as previously described. BM cells were incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-stem cell antigen-1 (sca-1), a phycoerythrin (PE)-conjugated anti-CD117 (c-kit), and an allophycocyanin (APC)-conjugated lineage antibody cocktail (Becton Dickinson), containing anti-CD3e, CD11b, B220, Ly-76, and Gr-1. The stained cells were washed with DPBS, resuspended in 100µl/sample DPBS+10% FBS+1% pcn/strp, and incubated with 5µl of 7AAD (Becton Dickinson) for 10 minutes on ice. Samples were analyzed using a FACSscalibur cytometer (Becton Dickinson).

Groups of mice were analyzed under the identical treatment conditions described above for measurement of recovery of WBCs and platelet counts in the peripheral blood. For these studies, peripheral blood samples (100 µL) were collected via the orbital vein beginning at day +5 and then every 5 days through day +30. Complete blood counts and differentials were measured within each sample using an Abbott CELL-DYN 3700 Hematology Analyzer (Abbott Laboratories, Abbott Park, IL). Equal numbers of mice transplanted with MBECs and non-transplanted control mice were evaluated to compare hematological recovery in each group. Statistical comparisons were performed using a t-test.

Additional groups of mice were irradiated with a lethal dose of irradiation and
subsequently transplanted systemically with either MBECs, murine fetal blood ECs (FBECs) or murine mesenchymal stem cells (MSCs, courtesy of Dr. Victor Dzau, Duke University) and their survival was compared to untreated control mice. Briefly, 8 to 10 week old C57Bl6 mice were irradiated with 1050 cGy irradiation (500 cGy and 550 cGy, split dose by 4 hours, dose rate 100 cGy/min) and transplanted beginning 2 hours post-irradiation with 1 x 10^6 MBECs intravenously x 1 and then 1 x10^6 MBECs daily x 4 days via intraperitoneal administration. Additional groups of mice were irradiated identically with 1050 cGy total body irradiation and subsequently transplanted with 1 x 10^6 FBECs or MSCs via identical route of administration and their survival was compared with control, irradiated mice. Survival analyses were conducted using the log rank test.

**Cytokine assays and cytokine administration in lethally irradiated mice**

The concentrations of known hematopoietic cytokines were measured in MBEC and FBEC-derived conditioned medium (CM) using enzyme-linked immunoabsorbent assay (ELISA). Conditioned media from MBECs and FBECs was produced as follows: MBECs and FBECs were seeded at 2x10^6 per flask in gelatin coated 175 cm^2 tissue culture flasks (Becton Dickinson) containing 25ml/flask of complete endothelial cell media as described above and allowed to come to >95% confluence over 10 days at 37°C in a 5% CO_2 environment. After 10 days, media was removed, the cells were washed twice with excess PBS to remove residual media, and the media was replaced with 35ml per flask of Iscove’s Modified Dulbecco’s Medium (IMDM) containing 1% pen/strp. The cultures were then incubated for another 10 days at 37°C in a 5% CO_2 and the media was collected, centrifuged to remove cell debris, and filter sterilized through a 0.2µm filter unit (Nalgene Nunc International, Rochester, NY). CM was concentrated to 50X in a stirred cell concentrator (Millipore Corporation, Bedford, MA) over a 3kD cellulose
ultrafiltration membrane (Millipore Corporation) using nitrogen gas, in a 4°C cold room. Following concentration, 50X CM was filter sterilized using a 0.2µm Acrodisc syringe filter (Pall Corporation, Ann Arbor, MI) and stored at -80°C in 0.5ml aliquots. For ELISA, CM was diluted to 1X concentration using sterile water.

ELISA analyses of CM were conducted using Quantikine ELISA kits (R&D Systems), according to the manufacturer’s suggested protocol. The following murine cytokines were measured: SCF, platelet-derived growth factor AA (PDGF-AA), interleukin 1-alpha (IL-1α), IL-1β, IL-6, TPO, granulocyte macrophage colony stimulating factor (GM-CSF), Flt-3 ligand, vascular endothelial growth factor (VEGF), IL-11, IL-3, tumor necrosis factor-alpha (TNF-α), stromal derived factor 1 (SDF-1), leukemia inhibitory factor (LIF), granulocyte colony stimulating factor (G-CSF), and angiopoietin 1 (Ang-1). ELISAs were also performed on serum collected via retro orbital bleed from isofluorane anesthetized C57BL6 mice at day +4 following 1050 cGy irradiation and 2 hours following the final dose of MBECs. Serum was also collected from 1050 cGy-irradiated mice that did not receive MBECs and from non-irradiated control mice. Blood was centrifuged in a tabletop microfuge at 5000 rpm for 10 minutes to pellet the cellular components and serum was collected and stored at -20°C prior to ELISA analyses. Analyses were conducted in triplicate on serum samples diluted 1:8 with PBS.

Survival studies were also performed on additional sets of mice that were lethally irradiated with 1050 cGy total body irradiation and subsequently received 5 days of VEGF, PDGF, SDF-1, IL-6 or the combination of these 4 cytokines, beginning at 2 hours post-irradiation. 1050cGy-irradiated C57BL6 mice were given either 100 ng murine VEGF, 100 ng rat PDGF-AA, 2 µg IL-6, 1.5 µg SDF-1 (R&D Systems, Minneapolis, MN) daily x 5 days via intraperitoneal administration, or the combination of these cytokines at the same doses. Doses of
each growth factor were determined based upon prior studies demonstrating the systemic activity of each cytokine following administration in rodents. Log rank analyses were performed to compare the survival of the cytokine-treated animals versus control, irradiated mice.

Results

Characterization of primary murine brain endothelial cells (MBECs) and systemic administration in irradiated mice

Since our previous studies demonstrated the hematopoietic capacity of primary human brain-derived vascular endothelial cells as compared to ECs from other tissues, we generated primary murine brain-derived endothelial cell cultures (Murine Brain Endothelial Cells, MBECs) from mouse brain cortical vessel explants (Anterior Cerebral Artery, Middle Cerebral Artery) harvested from 6-8 week old C57Bl6 mice, as previously described. In order to facilitate in vivo tracking of transplanted ECs, primary MBEC cultures were also generated from brain vessels obtained from C57Bl6 mice that were transgenic for expression of green fluorescent protein (GFP). Transcriptional analysis demonstrated that these primary cells expressed VE-cadherin, Von Willebrand Factor, Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) and PECAM (CD31) and readily formed vascular tubes when plated in Matrigel, verifying their endothelial origin (Figure 1A, B).

We next sought to determine whether transplanted MBECs could engraft in the BM of recipient mice. As a preliminary experiment, we irradiated C57Bl6 mice (n=5 per condition) with 700 cGy and transplanted the mice with $5 \times 10^5$ GFP+ MBECs via tail vein injections daily x 5 days, beginning 2 hours after radiation exposure on day 0. Beginning at day +5 and continuing every 5 days through day +30, mice were sacrificed, bilateral femurs were harvested and BM was analyzed for evidence of MBEC engraftment. Spleens and lung tissue were also
harvested from mice to assess engraftment in other tissues. GFP\(^+\) cells were not detected in the BM in any mice analyzed, whereas GFP\(^+\) cells were identified consistently at low levels (range 0.5 -1.0% of cells per high power field) in the lung tissue in all treated animals. For comparison, additional mice also received GFP\(^+\) MBECs via intraperitoneal injection and these animals also demonstrated no detectable GFP\(^+\) cells in the BM at any time point, indicating a lack of engraftment of MBECs in the BM in these mice (data not shown).

Since 700 cGy total body irradiation can be expected to acutely induce hypoplasia within the BM, we next examined the recovery of BM cellularity in mice transplanted with MBECs versus irradiated, control mice. For these measurements, bilateral femurs were collected from groups of mice in each condition (n=5 per condition) and total viable cell counts were performed every 5 days from day 0 (pre-irradiation) through day +30. Both control mice and MBEC-transplanted mice displayed a marked reduction in BM cell counts through day +10, but MBEC-transplanted mice demonstrated significantly increased BM cell counts at day +15, +25 and +30 compared to untreated controls (P=0.004, P=0.001, P=0.019; Figure 2). These quantitative results corresponded with microscopic analysis of BM cellularity in tissue sections as well (data not shown). In addition, the MBEC-treated mice showed earlier recovery of BM vascular architecture by day +20 compared to the irradiated, control mice which demonstrated persistent disruption of the vascular architecture through day +30.

**MBEC transplantation radioprotects BM progenitors and enhances the regeneration of BM stem/progenitor cells**

In order to determine whether systemic administration of MBECs could mediate a radioprotective or regenerative effect on BM stem and progenitor cells following radiation injury, additional mice (n=2-4 per condition) were irradiated with 700 cGy and subsequently
transplanted with $5 \times 10^5$ MBECs per day for 5 days via either intravenous or intraperitoneal injection. At day +10 following 700 cGy irradiation, untreated control mice demonstrated a 5.1-fold decline in c-kit $^+$ sca-1 $^-$ lin progenitor cells in the marrow compared to normal mice (mean 14.0% ± 2.0 vs. 60.7% ± 4.4 of the lin-population, $P=0.002$; Figure 3A, B). Conversely, mice treated intravenously or intraperitoneally with MBECs maintained significantly higher numbers of BM c-kit $^+$ sca-1 $^-$ lin- progenitor cells at day +10 compared to untreated control mice (mean 31.5% ± 7.1 and 34.1% ± 9.0 of lin- population vs. 14.0% ± 2.0, respectively; $P=0.04$ and $P=0.04$), suggesting that administration of MBECs protected BM progenitor cells from early radiation-induced toxicity. We next examined the recovery of BM c-kit $^+$ sca-1 $^+$ lin- (KSL) cells which are enriched for hematopoietic stem cell content. At day +10 following 700 cGy exposure, untreated control mice demonstrated a 90-fold reduction in the percentage of KSL cells compared to normal mice (mean 0.001% ± 0.001 vs. 0.09% ± 0.01 of the total BM population, $P=0.002$; Figure 3A,B), reflecting radiation-induced loss of BM stem/progenitor cells at this early time point. Mice treated with MBECs via either intravenous or intraperitoneal administration also demonstrated significant reduction in KSL populations at day +10 compared to normal mice (mean 0.007% ± 0.002 and 0.005% ± 0.002 vs. 0.09% of the total BM population, respectively; $P=0.002$ and $P=0.002$, respectively; Figure 3B). However, BM analysis between day +15 and day +20 following irradiation revealed accelerated recovery of the KSL population within the MBEC-treated animals as compared to the irradiated control animals (Figure 3C, D). At day +20, mice that were treated with MBECs via either intravenous or intraperitoneal administration demonstrated significantly increased percentages of KSL cells in the BM compared to untreated control mice (mean 0.04% ± 0.01 and 0.07% ± 0.02 vs. 0.0003% ± 0.0001 of the total BM population; $P=0.01$ and $P=0.03$, respectively). Taken together, these data
suggested that systemic administration of MBECs mediated the radioprotection of BM progenitor cells and promoted early regeneration of hematopoietic stem/progenitor cells following radiation injury. Of note, to exclude the possibility that transplanted MBECs contained contaminating cells with hematopoietic capacity that could account for the observed results, we examined the peripheral blood and BM of MBEC-transplanted mice between day +10 and +30 post-irradiation and found no donor-derived GFP CD45$^+$ cells (data not shown). These results indicated a very low probability that transplanted MBEC grafts contained contaminating hematopoietic progenitor cells or underwent dedifferentiation in vivo to account for the hematopoietic recovery we observed.

**MBEC transplantation accelerates WBC and platelet recovery and improves survival of irradiated mice**

In order to determine whether the accelerated recovery of the BM stem and progenitor cell pool in the MBEC-transplanted animals impacted hematological recovery, we collected peripheral blood (PB) from mice irradiated with 700 cGy beginning at day +7 and every 3 to 4 days through day +20 (n=10 mice per condition). Total white blood cells (WBCs) and platelet counts (Plts) were measured in each animal over time. From day +7 through day +14 following irradiation, all MBEC-treated and untreated control mice demonstrated severe leukopenia and thrombocytopenia (Figure 4A, B). However, at day +20, mice treated with MBECs via either intraperitoneal or intravenous administration demonstrated increased WBCs in the PB compared to untreated controls (mean WBC 0.7 and 0.9 vs. 0.1, respectively; $P=0.01$ and $P=0.05$; Figure 4A). Similarly, mice treated intravenously with MBECs demonstrated significantly increased platelet counts at day +17 versus untreated controls (mean Plts 176 vs. 68, $P=0.04$; Figure 4B) and both groups of MBEC-treated mice had higher platelet counts at
day +20 compared to untreated controls (mean Plts 377 and 232 vs. 78, respectively; \( P = 0.03 \) and \( P = 0.06 \); Figure 4B). These data suggested that systemic administration of MBECs accelerated hematologic recovery in irradiated animals.

Since systemic administration of MBECs was strongly associated with enhancement of hematopoietic recovery in irradiated mice, we sought to determine whether such therapy could impact the survival of animals following lethal dose irradiation. We irradiated a group of C57Bl6 mice with 1050 cGy (split dose), which we have previously shown to be 100% lethal in this strain of mice by day +30 (LD100/30). We then examined whether systemic administration of MBECs alone, in the absence of transplanted HSCs, beginning 2 hours post-exposure, could improve the survival of these animals compared to irradiated controls. One hundred percent of irradiated control mice died prior to day +30 (Figure 5A). In contrast, 57% of mice that were irradiated with 1050 cGy and subsequently transplanted with MBECs survived through day +60 with no signs of morbidity (Figure 5A). These data demonstrated that systemic administration of MBECs alone, in the absence of HSCs, significantly increased the survival of animals after exposure to lethal dose irradiation (\( P = 0.04 \), log rank test).

We next sought to determine whether the effect of MBEC transplantation on the survival of lethally irradiated mice was specifically due to an effect of MBECs or could be mediated by other sources of ECs or non-endothelial cells. For this purpose, we compared the survival of groups of mice irradiated with 1050 cGy without treatment (controls) versus 1050 cGy-irradiated mice that were transplanted with equal doses of FBECs or MSCs. One hundred percent of the control mice (\( n = 9 \)) that received 1050 cGy without treatment died by day +21 as did 100% of irradiated mice transplanted with MSCs alone (\( n = 7 \), Figure 5B). Interestingly, mice that were irradiated and transplanted with FBECs (\( n = 9 \)) demonstrated a significant increase in survival
compared to both control mice and MSC-transplanted mice, although these mice failed to survive long-term (P=0.002 and P=0.002, respectively; Figure 5B).

**MBEC-derived cytokines fail to rescue lethally irradiated mice**

Since transplantation of MBECs supported the survival of mice following lethal dose irradiation, we sought to determine whether MBECs produced soluble hematopoietic growth factors that could account for the results we had observed. ELISA analysis of a broad panel of candidate hematopoietic cytokines revealed that MBEC-CM (1X) contained significantly increased levels of VEGF, PDGF-AA, SDF-1 and IL-6 compared to all other cytokines tested (P<0.03 for all comparisons, Tukey-Kramer multiple comparison test; Figure 6A). MBECs notably produced no significant levels of TPO, SCF, GCSF, GMCSF, or IL-3. For comparison, ELISA analysis of FBEC-CM was also performed, which revealed increased levels of SCF and IL-11 but not VEGF, PDGF, SDF-1 or IL-6 (data not shown). These results indicated that MBECs and FBECs were distinct in their production of known hematopoietic cytokines.

We next tested whether the in vivo administration of the most overexpressed MBEC-derived cytokines (VEGF, PDGF, SDF-1, IL-6) or their combination could reproduce the effect of MBEC transplantation toward promoting the survival of lethally irradiated mice. For these studies, the survival of control C57Bl6 mice that were irradiated with 1050 cGy was compared with 1050 cGy-irradiated mice that were treated, beginning 2 hours post-irradiation, with either 100 ng VEGF, 100 ng PDGF-AA, 2 μg IL-6 or 1.5 μg SDF-1 or the combination of all 4 cytokines given intraperitoneally x 5 days. All control mice (n=10) died by day +23 and no mice treated with VEGF alone (n=10), PDGF-AA (n=10), IL-6 (n=10), SDF-1 (n=10) or the combination of all 4 factors (n=10) survived past day +28 (Figure 6B). Taken together, these
data suggested that these growth factors alone did not account for the in vivo survival benefit that was observed in mice transplanted with MBECs.

As a final analysis to determine whether MBEC transplantation might have indirectly promoted the survival of irradiated animals via the systemic induction of anti-apoptotic or hematopoietic growth factors in vivo, we compared the levels of cytokines in the serum of mice that were irradiated and transplanted with MBECs versus irradiated controls and non-irradiated controls. Interestingly, 1050 cGy irradiation was associated with a significant increase in the serum concentrations of GCSF and Flt-3 ligand compared to non-irradiated control mice (P=0.002 and P<0.001, respectively) and mice that were irradiated and transplanted with MBECs displayed a further increase in GCSF levels and a significant decrease in PDGF-AA levels in the serum compared to irradiated, untreated mice (P=0.02 and P=0.03, respectively).

Discussion

Administration of high dose chemotherapy or radiotherapy in the treatment of cancer is limited by the potentially prolonged BM aplasia and pancytopenia that can occur due to toxicity to proliferating stem and progenitor cells. The only established treatment for the resulting marrow aplasia is transplantation of autologous or allogeneic hematopoietic stem cells coupled with intensive supportive care. Experimental studies have demonstrated that supportive cells within the BM niche (stromal cells, endothelial cells) are also severely damaged by exposure to therapeutic doses of ionizing radiation. We postulated that cellular therapy directed at replenishing normal endothelial cell activity could potentially accelerate hematopoietic recovery following radiation-induced BM aplasia. Our results suggest that systemic administration of vascular endothelial cells provides radioprotection to BM progenitor cells, induces the regeneration of primitive stem/progenitor cell populations, accelerates the recovery of mature
peripheral blood counts and facilitates the improved survival of mice following radiation injury. These results indicate that the targeted restoration of endothelial cell activity is a potentially viable strategy to accelerate hematological recovery following myelosuppressive chemo- or radio-therapy.

Although the contribution of vascular endothelial precursors to hematopoiesis has been well demonstrated during embryogenesis, the contribution of ECs to adult hematopoiesis in vivo has been less well characterized. Two recent investigations have suggested that signals provided by the bone marrow vascular niche may be important in hematopoietic regeneration following chemotherapy-induced myelosuppression. Montfort et al. demonstrated that transplantation of segments of thoracic aorta or inferior vena cava under the kidney capsule was associated with radioprotection in lethally irradiated mice, and Li et al. have preliminarily reported that transplantation of PECAM/CD31\(^+\) cells was radioprotective in mice as well (Li B et al, Blood 2002, abstract #1120). Our results significantly extend these observations by demonstrating that transplanted ECs exert a pro-survival effect on susceptible BM progenitor cells in vivo during the early period post-irradiation (day 0 - 10) and a regenerative effect on the more primitive BM stem cell population which occurs later (day 10 - 20). The combination of these effects coincided temporally with the earlier recovery of mature WBCs and platelets at day +20 post-irradiation. Furthermore, 100% of lethally irradiated, EC-transplanted mice that were alive at day +20 ultimately survived long-term, suggesting a causal relationship between the recovery of hematopoiesis and survival. These data indicate that restoration of the hematopoietic activity normally provided by vascular endothelial cells can enhance survival following myelosuppressive radiotherapy.

Additional studies performed here suggest the possibility that transplantation of other
sources of ECs, particularly fetal blood ECs, has the potential to ameliorate radiation-induced toxicity. In a preliminary study, fetal blood EC transplantation was significantly less potent than transplantation of brain-derived ECs, perhaps reflecting the distinct pattern of cytokine production between the 2 sources of ECs. Nonetheless, we plan to further study the utility of fetal blood ECs to augment hematopoiesis and survival following myelosuppressive therapy in mice since this could be a model for the use of umbilical cord blood ECs to augment human cord blood transplantation. Interestingly, transplantation of MSCs had no impact on the survival of irradiated mice, suggesting that the beneficial effects of MBEC transplantation reflect an endothelial cell-specific activity, rather than a non-specific effect inducible by different cell types.

We also sought to determine whether the beneficial effects of MBEC transplantation could be ascribed to the production of known hematopoietic cytokines. Not surprisingly, MBEC-CM contained increased levels of VEGF, PDGF-AA and SDF-1, along with IL-6. However, when we administered pharmacologic doses of these 4 growth factors alone or in combination to lethally irradiated mice, we did not observe a significant improvement in the survival of any mice compared to controls. While additional dose and scheduling studies will be necessary to confirm these results, the current data suggest that additional, perhaps novel, MBEC-derived soluble factors account for the effects we have observed. It is also possible that MBEC transplantation indirectly mitigates radiation damage in vivo via the induction of other systemic growth factors. However, preliminary studies we have performed indicate that non-contact culture with MBECs directly reduces apoptosis and increases the recovery of viable BM progenitor cells harvested from irradiated mice (Chute J, manuscript in preparation).

In order to advance the observations presented here for therapeutic purposes, it will be
important to delineate the precise mechanism(s) through which ECs mediate hematopoietic regeneration following radiation damage. Our results suggest that transplanted ECs promote hematopoietic repair and regeneration via a soluble or endocrine effect. First, we found no evidence that intravenously administered MBECs engrafted in the recipient BM within the first 30 days post-transplant, yet these recipient animals had earlier recovery of BM cellularity, stem and progenitor cell content and circulating WBCs and platelets. Second, mice treated with intraperitoneal administration of MBECs also did not demonstrate detectable GFP+ MBECs in their BM and these animals demonstrated nearly identical protection of the BM progenitors, regeneration of more primitive c-kit$^+$ sca-1$^+$ lin$^-$ (KSL) cells and recovery of mature blood counts compared to mice that received intravenous MBECs. Taken together, these results suggest that transplanted MBECs mediate their in vivo activity via soluble effects on the BM stem and progenitor cell compartment. We plan to confirm the mechanism of action in two ways: first, we will examine the efficacy of systemically administered MBEC-derived Conditioned Media alone in the treatment of irradiated animals and second, we will test whether direct intrafemoral injection of MBECs, which will eliminate homing inefficiencies, can further augment the hematopoietic recovery and survival in mice following high dose irradiation.

The most surprising result from this study was the demonstration that transplantation of primary MBECs alone, in the absence of HSCs, promoted the survival of mice following lethal dose total body irradiation. It has been established in experimental models and clinical practice that survival following lethal dose irradiation depends upon either the transplantation of sufficient numbers of HSCs to repopulate the ablated host marrow or transplantation of myeloid-erythroid progenitor cells which can serve as a “bridge” providing short-term (2-4 weeks) hematopoietic production until endogenous hematopoietic stem/progenitor cell activity
occurs. Our study suggests that a third strategy can be applied to facilitate survival following exposure to lethal dose irradiation: replenishment of endothelial cell-derived hematopoietic activity. Since the BM vascular niche is significantly damaged following high dose radiation exposure, it is plausible that diminished vascular niche activity contributes to the delayed recovery of hematopoiesis that invariably ensues. This study provides proof of principle that provision of vascular endothelial cell activity can accelerate hematopoietic recovery following radiation injury. Pharmacologic or cellular therapies aimed at restoring endothelial cell-mediated activity represent a novel approach to enhance hematopoietic recovery in vivo following radiation-induced myelosuppression.
References


Figure Legends

Figure 1. MBECs express endothelial-specific genes and display endothelial functional characteristics. (A) Primarily passaged MBECs were collected and total RNA was isolated and prepared for reverse-transcriptase PCR analysis for expression of multiple endothelial cell genes. VE-Cadherin, Von Willebrand Factor (VWF), VEGFR-2 and PECAM (CD31) were all expressed by MBECs, confirming their endothelial cell lineage. (B) Primary MBECs ($5 \times 10^4$) were plated in 24 well tissue culture plates pre-coated with Matrigel to analyze for evidence of capillary tube forming capability. As shown, MBECs readily formed tube-like structures, consistent with an endothelial cell lineage.

Figure 2. Mice transplanted with MBECs demonstrate accelerated BM cellular recovery following total body irradiation. C57Bl6 mice were irradiated with 700 cGy total body irradiation and then observed without treatment or transplanted with $5 \times 10^5$ MBECs daily x 5 days. BM cells were collected from bilateral femurs from mice in each condition at day 0 and every 5 days through day +30 and mean viable mononuclear cell (MNC) counts were performed. MBEC-transplanted mice demonstrated significantly increased numbers of viable MNCs at day +15, +25 and +30 compared to irradiated control mice. * indicates a significant difference at each time point; P=0.004, P=0.001, P=0.019, respectively.

Figure 3. MBEC transplantation provides radioprotection of BM progenitors and augments the regeneration stem cell-enriched populations. BM mononuclear cells were collected from mice on days +10, +15 and +20 following 700 cGy of total body irradiation. The percentages of c-Kit $^+$ sca-1 $^-$ lin progenitors and the more primitive c-kit $^+$ sca-1 $^+$ lin $^-$ (KSL) cells were measured by flow cytometry in the BM at each timepoint in normal mice (A), in 700 cGy...
irradiated control mice, and in mice treated with MBECs intravenously or via intraperitoneal administration x 5 days. As shown in (B), control mice demonstrated marked reduction in viable c-kit$^+$ sca-1$^-$ lin$^-$ progenitors at day +10, whereas all animals treated with MBECs maintained significantly higher numbers of this population following radiation exposure. At days +15 (C) and day +20 (D), both groups of MBEC-treated animals demonstrated recovery of c-kit$^+$ sca-1$^-$ lin$^-$ (KSL) cells compared to untreated controls. Percentages shown in the upper quadrants of each figure represent the percentage of that population within the lin$^-$ subset.

Figure 4. MBEC transplantation is associated with accelerated recovery of mature blood counts. Peripheral blood was collected every 3-4 days following the administration of 700 cGy total body irradiation to C57Bl6 mice. Groups of mice were transplanted with MBECs intravenously (n=10, blue line) or via intraperitoneal injection (n=10, gray line) and hematologic recovery was compared with that of irradiated control mice (n=10, black line). The mean total white blood cells (WBCs) and platelet counts (Plts) were markedly reduced in all mice through day +14. By day +20, mice that received either intravenous or intraperitoneal MBECs demonstrated increased total WBCs compared to control mice (A). (B) Mice that received intravenously administered MBECs displayed significantly increased platelet counts at day +17 compared to untreated controls and mice that received either intravenous or intraperitoneal MBECs had higher platelet counts at day +20. * indicates a significant difference in the levels between the group that received intraperitoneally administered MBECs and control mice and ^ indicates a significant difference between the mice that received intravenously administered MBECs versus untreated control animals.
Figure 5. Transplantation of MBECs is associated with improved survival in mice following 1050 cGy total body irradiation. (A) Control C57Bl6 mice were irradiated with 1050 cGy total body irradiation and their survival was compared with mice that were irradiated with 1050 cGy and then transplanted with MBECs x 5 days as described in the Materials and Methods. Whereas 100% of control, irradiated mice (n=11) died prior to day +30 (gray line), 57% of mice transplanted with MBECs (n=7) survived through day +60 without evidence of morbidity (black line, P=0.04). (B) The survival of control C57Bl6 mice that were irradiated with 1050 cGy (n=9, gray line) was compared with the survival of C57Bl6 mice that were irradiated with 1050 cGy and transplanted with FBECs (n=9, solid black line) or MSCs (n=7, dashed black line). FBEC-transplantation was associated with a significant increase in percent survival compared to controls or MSC-transplanted mice (P=0.002 and P=0.002, respectively), although these mice failed to survive long-term.

Figure 6. Cytokine production does not account for the in vivo effects of MBECs. (A) ELISAs performed on MBEC-CM (1X) revealed significantly increased concentrations of VEGF, PDGF-AA, SDF-1 and IL-6 compared to all other cytokines that were measured (* indicates a P<0.03 for multiple comparison test). (B) C57Bl6 mice were irradiated with 1050 cGy and subsequently treated with 5 days of intraperitoneal administration of either VEGF, PDGF-AA, SDF-1, IL-6 or VEGF/PDGF-AA/SDF-1/IL-6 combined, beginning at 2 hours post-irradiation. Percent survival in each treatment group is shown as compared to control, irradiated mice. No animals in any group survived beyond day +28.
Figure 1
Figure 3
Figure 3

B

Untreated

IV ECs

IP ECs

Ig G

C-kit

sca-1

Lin -

0.05%

0.20%

0.25%

15.6%

36.5%

40.4%
Figure 3

C

Untreated

IV ECs

IP ECs

Ig G

Lin -

19.9%  0.06%

36.7%  0.31%

40.0%  0.88%

Ig G  c-kit  sca-1
Figure 3

D

Untreated

IV ECs

IP ECs

Lin -

1.0% 0.001%

37.9% 0.65%

43.4% 2.57%
Figure 4

A

![Graph showing changes in WBC x 10^9/L over days (7 to 20). The graph indicates a significant change at 20 days marked with an asterisk.](image-url)
Figure 4

B
Figure 5 A
Figure 5 B
Figure 6 A

The graph shows the concentration (pg/ml) of various cytokines and growth factors. The concentration of each factor is represented by a bar, with error bars indicating the variability. The factors include VEGF, FLT3, G-CSF, GM-CSF, IL-6, IL-1beta, SCF, SDF, TPO, TNFalpha, IL-11, IL-1alpha, IL3, LIF, PDGF-A, and ANG-1. The concentration levels vary significantly across these factors, with some showing a marked increase or decrease compared to others.
Figure 6 B
Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice

John P Chute, Garrett G Muramoto, Alice B Salter, Sarah K Meadows, Dennis Rickman, Benny Chen, Heather A Himburg and Nelson J Chao