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Dickkopf-1 Treatment Stimulates Hematopoietic Regenerative Function in Infused Endothelial Progenitor Cells

Mindy M. Kim, Lauren Schluskel, Liman Zhao, Heather A. Himburg¹

Division of Hematology/Oncology, Department of Medicine, UCLA, Los Angeles, California

Abstract

Acute high-dose radiation injury damages the bone marrow hematopoietic stem and progenitor cell compartment. This damage compromises the functional ability of the bone marrow to produce mature blood cells and results in an increased risk of death due to hematopoietic complications. Past work has shown that the bone marrow endothelium provides critical cues, which promote hematopoietic stem cell regeneration after injury. Additionally, transfusion of endothelial cells after radiation injury has been shown to promote recovery of both the bone marrow vasculature and hematopoietic systems. In this work, we examined the regenerative capacity of intravenous infusion of umbilical cord-blood derived endothelial progenitor cells (EPCs) since this is a cell source which is easy to obtain, expand and cryopreserve. We show that pre-treatment with the Wnt-antagonist Dickkopf1 (Dkk1) augments EPC regenerative function in an allogeneic mouse transplant model. Here, hematopoietic recovery was assessed in Balb/c mice after 5 Gy total-body irradiation and transplantation with C57/BL6-derived EPCs either with or without Dkk1 pre-treatment. The Dkk1-treated EPC group had significantly faster recovery of peripheral white blood cells, total bone marrow cellularity, bone marrow progenitors and BM endothelial cells compared to EPC treatment alone or saline controls. Importantly, after an LD_{50/30} dose of 8 Gy in the Balb/c mouse, Dkk1-treated EPCs were able to rescue 100% of irradiated mice versus 80% in the EPC control group and only 33% in the saline-treated group. To understand how Dkk1 induces regenerative function in the EPCs, we screened for pro-regenerative factors secreted by the EPC in response to Dkk1. Dkk1-treated EPCs were observed to secrete high levels of the anti-fibrotic protein follistatin as well as several proteins known to promote regeneration including EGF, VEGF and G-CSF. This work demonstrates the potential for Dkk1-treated EPCs as a rescue therapeutic for victims of acute radiation injury.

INTRODUCTION

If a mass casualty nuclear disaster were to occur in a major metropolitan area, there are likely to be several hundred thousand victims of acute radiation syndrome (ARS) (6, 7). ARS victims will be exposed to varying levels of radiation depending on their distance and shielding from the event. Victims who receive 2–6 Gy total-body irradiation (TBI) will

¹Address for correspondence: UCLA Department of Medicine, Division of Hematology/Oncology, 414 Orthopedic Hospital Research Center Building, 615 Charles E. Young Drive South, UCLA, Los Angeles, CA 90095; hhimbura@mednet.ucla.edu.

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experience severe hematopoietic toxicity which, if untreated, may cause death within 2–8 weeks due to hematopoietic complications such as infection or anemia. While hematopoietic stem cell transplantation could potentially rescue individuals from lethal hematopoietic toxicity in these dose ranges, it would not be a feasible solution for most victims in a mass casualty event. Currently, the U.S. Strategic National Stockpile is amassing Neupogen® (G-CSF) for use in a nuclear or radiological emergency (8, 9). G-CSF stimulates myeloid precursors to produce mature neutrophils and is the current clinical standard of care for treatment of neutropenia in chemotherapy patients. In nonhuman primate animal models of lethal TBI, G-CSF has been demonstrated to increase survival by 38% when administered within 24 h (10). However, G-CSF showed no survival benefit in the nonhuman primate model when treatment was delayed 48 h postirradiation injury (11). These data and the limited data available on G-CSF administration to human victims of accidental radiation exposure suggest there is a substantial clinical need for more potent therapies that can induce rapid hematopoietic expansion even if treatment is delayed by several days.

Recently published studies have suggested that cellular therapies, including those that use endothelial cells and mesenchymal stromal cells (MSCs), can promote regeneration of hematopoiesis after radiation injury (4, 12–18). Infusion of endothelial cells alone can accelerate hematopoietic recovery and were shown to rescue lethally irradiated mice from death due to hematopoietic syndrome (12). Furthermore, loss-of-function and gain-of-function animal models targeting the endothelium have demonstrated that a functional bone marrow (BM) endothelium is critical for hematopoietic regeneration after radiation injury (13, 19). Endothelial cells are known to secrete growth factors such as G-CSF that target hematopoietic progenitor cell populations (20). Additionally, endothelial cells also drive reconstitution of the entire blood and immune system by the secretion of growth factors that act on the hematopoietic stem cell (HSC). These HSC factors include epidermal growth factor (EGF), pleiotrophin and jagged-1 (4, 21–23). Endothelial cells have been demonstrated to promote better regeneration of the HSC compartment than G-CSF alone even when treatment was delayed to 48 h (24). Collectively, these studies emphasize the fundamental significance of the endothelium in regulating hematopoietic regeneration after radiation injury.

Here, we evaluate a novel cell therapeutic for mitigation of radiation-induced toxicity to bone marrow, which uses cord-blood-derived endothelial progenitor cells (EPCs). EPCs have been well characterized the Yoder group as well as others, as being both easy to isolate and highly proliferative in culture (1, 2). Ingram *et al.* demonstrated that a single EPC can undergo as many as 8,000 cell divisions in 14 days and can be passaged more than 100 times in culture without undergoing senescence (1). Previously, EPCs have been shown to augment both vascular and hematopoietic recovery after injury; however, this cell source was not potent enough to mitigate lethal injury without a stem cell transplant (12, 14). We hypothesized that EPC angiogenic activity could be stimulated by pre-treatment with the Wnt-antagonist Dkk1. Previously, Smadja *et al.* showed Dkk1 induces angiogenesis in EPCs by activation of VEGFR2, CXCR4 and SDF-1 (25). More recently, work from the Chute laboratory, showed Dkk1 infusion alone increased survival and hematopoietic regeneration after injury (26). Dkk1 treatment after radiation injury was observed to stimulate BM endothelial production of the regenerative growth factor EGF.

Based on these findings, we sought to determine whether Dkk1 could be used in combination with endothelial cell transplantation to augment hematopoietic reconstitution after radiation injury. The goal of this work was to develop an off-the-shelf cellular therapeutic for ARS victims. Here we evaluated Dkk1-treated EPCs in an allogeneic mouse transplant model.

MATERIALS AND METHODS

Statistical Analysis

Values are reported as mean $\pm$ SEM, unless stated otherwise. All comparisons were performed using GraphPad Prism version 7.0 (LaJolla, CA). All data were checked for normal distribution and similar variance between groups. Data are derived from multiple independent experiments from distinct mice or cell culture plates. Sample size for *in vitro* studies was chosen based on observed effect sizes and standard errors from prior studies. For all animal studies, a power test was used to determine the sample size needed to observe a twofold difference in mean between groups with 0.8 power using a two-tailed Student's *t* test. All animal studies were performed using sex- and age-matched animals. Animal studies were performed without blinding of the investigator and no animals were excluded from the analysis as outliers.

EPC Isolation and Characterization

EPCs were isolated from the fetal blood of C57/Bl6 mice and from human umbilical cord blood using the methodology first developed in the Yoder laboratory (1, 2) and detailed in video by Ravishankar *et al.* (3). EPC lines were verified by PCR to be positive for expression of the endothelial cell markers VE-cadherin (CD144), PECAM (CD31) and Endoglin (CD105). Additionally, cell lines were assessed using fluorescence-activated cell sorting (FACS) analysis for cell surface expression of VE-cadherin and PECAM. Endothelial cell cultures were maintained in EGM-2 growth medium (Lonza, Morristown, NJ). Mouse and human Dkk1 were purchased from R&D SystemseTM (Minneapolis, MN) and added at a concentration of 500 ng/ml. For transplantation studies, EPCs were grown to confluence (2–3 days) and switched to EGM-2 media containing 500 ng/ml Dkk1. At 24 h after Dkk1 treatment, the cells were rinsed, trypsinized and resuspended in phosphate buffered saline (PBS) for injection.

Radiation Mitigation Studies

All animal procedures were performed in accordance with animal use protocols approved by the UCLA Animal Research Committee. All mice were purchased from Jackson Laboratory (Bar Harbor, ME). Female Balb/c mice, 8–10 weeks old (strain 001026) or congenic CD45.1 Balb/c mice (strain 006584) weighing 20 ± 2 g were used for all studies described. Mice received either a sublethal dose of 5 Gy or LD_{50/30} dose of 8 Gy TBI using a cesium-137 irradiator. At 24 h postirradiation, mice received a single 200- μ l intravenous (i.v.) injection of either saline, 1×10^6 EPCs, or 1×10^6 Dkk1-EPCs. On days 2–5 postirradiation, mice received a 100- μ l intraperitoneal injection of either saline, 2×10^6 EPCs or 2×10^6 Dkk1-EPCs.

FACS Analysis

Antibodies used for characterization of BM progenitor and HSC populations were purchased from BD Biosciences (San Jose, CA): Sca-1 (APC-CY7), c-Kit1 (PE) lineage cocktail (V450), CD34 (APC), Flt3 (FITC) or isotype controls. BM endothelial cells were isolated following a protocol by Poulos *et al.* (4). Briefly, mice were intravenously labeled with VE-cadherin antibody (25 µg/mouse; BioLegend® Inc., San Diego, CA) via i.v. injection. At 15 min after injection, mice were sacrificed, long bones were harvested and crushed with a mortar and pestle in PBS. The crushed bone product was then digested at 37°C for 10 min in a collagenase/dispase buffer (2.5 mg/ml Collagenase A; Sigma-Aldrich® LLC, St. Louis, MO), 1 unit/ml Dispase II (STEMCELL™ Technologies Inc., Vancouver, Canada). After digestion, bones were rinsed in ice-cold complete media [IMDM with 10% fetal bovine serum (FBS)], filtered, then column depleted of lineage committed hematopoietic cells (Miltenyi Biotec, Auburn, CA). Endothelial cells were identified as the CD45⁻7AAD⁻ VE-cadherin⁺ cell population. To discriminate apoptotic cells within this population, the CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Thermo Fisher Scientific™ Inc., Waltham, MA) was used per manufacturer's instructions. To identify donor EPCs engrafted in the bone marrow, we labeled transplanted EPCs or Dkk1-EPCs with the CellTrace Violet kit (Thermo Fisher Scientific). In this study, we performed bone marrow FACS analysis at day 7 after transplant to assess for cells with the CellTrace label within the CD45⁻7AAD⁻ VE-cadherin⁺ cell population.

Immunofluorescent Imaging

Whole femurs from VE-cadherin-labeled mice were fixed overnight in 4% paraformaldehyde, decalcified for 72 h in EDTA/PBS, incubated for 2 h in 20% sucrose and then embedded in OCT media. Femurs were sectioned at 8 µm. Sections were re-hydrated in PBS, permeabilized in 0.1% Tritone™ and blocked for 1 h in a solution of 1% bovine serum albumin, 0.1% Tween® in PBS. After blocking the primary beta-III-tubulin (βIII-tubulin) antibody (Neuron-specific beta-III Tubulin NorthernLight™ NL557-conjugated Antibody; R&D Systems). Slides were rinsed and mounted in ProLong Gold Antifade with DAPI.

CFC Assays

CFC assays were performed using STEMCELL Technologies MethoCult™ M3434 (mouse) or H4434 (human) following manufacturer's instructions.

in vitro Culture Assay

Human EPCs were grown to confluence in EGM-2 growth media in the lower portion of a 24-well transwell plate system. After reaching confluence, the media was replaced with HSC-supportive media IMDM containing 10% FBS, 1% pen-strep, 125 ng/ml stem cell factor, 50 ng/ml Flt3 ligand, and 20 ng/ml thrombopoietin with or without 500 ng/ml human Dkk1. Human bone marrow CD34⁺ cells (AllCellst®, Alameda, CA) were irradiated at 3 Gy and added in non-contact to the upper portion of the transwell at a dose of 10,000 cells/well. Cells were cultured for 7 days, viable cells were enumerated by trypan blue exclusion, and CD34⁺ phenotype determined by FACS analysis.

BM Endothelial Cell Permeability

For analysis of BM endothelial cell permeability, we used a methodology developed by Zhou *et al.* (5), measuring BM endothelial cell permeability with Evans Blue Dye (EBD) extravasation in the BM space. Briefly, at 72 h postirradiation and treatment, mice were i.v. injected with 200 μ l of a 0.5% solution of EBD. Then 15 min later, the mice were euthanized and the BM supernatant was collected. EBD concentration was determined by measuring absorbance on a spectrophotometer at 610 nm.

Luminex and ELISA Analyses

Luminex and ELISA were performed on twofold concentrated cell culture supernatants from four independent wells of a six-well plate at day 5 after treatment with Dkk1. The Millipore mouse bone, angiogenesis and 32-plex cytokine panels were performed at the UCLA Immune Assessment Core. Individual ELISAs for IGFII (R&D Systems), and for BDNF, neuregulin, GDNF and CNTF (all from Biomatik USA, Wilmington, DE), were performed according to manufacturer's directions.

RESULTS

Mitigation of Hematopoietic Injury in Allogeneic Transplant Model

To evaluate whether Dkk1 pre-treatment increases the benefit of transplanted EPCs, recombinant mouse Dkk1 was added to established cultures of EPCs derived from C57/Bl6 mouse fetal blood. Dkk1-treated EPCs (Dkk1-EPCs) or untreated control EPCs were harvested 24 h after treatment and transplanted into irradiated Balb/c mice at 24 h post-TBI (Fig. 1A). We evaluated vascular recovery in the EPC groups relative to saline-treated mice. After 5 Gy TBI, mature white blood cells (WBCs), neutrophils and lymphocytes reach a nadir around day 10 (Fig. 1B). Infusion of Dkk1-EPCs accelerated the recovery of WBCs, neutrophils and lymphocytes compared to EPC or saline treatment (Fig. 1B). Total BM cellularity was similarly reduced in all treatment groups at day +2 after 5 Gy irradiation. However, Dkk1-EPC treatment increased total BM cellularity faster and was significantly better than EPC and saline treatment at days 7 and 10 (Fig. 1C).

Within the BM, we assessed recovery of stem and progenitor cell populations by FACS analysis. At day 10 after 5 Gy TBI, saline-treated mice exhibited typical loss of phenotypic stem and progenitor cell markers compared to nonirradiated controls (Fig. 2A). However, mice treated with Dkk1-EPCs had significantly greater recovery of the stem and progenitor enriched population of lineage⁻ c-Kit⁺Sca-1⁺ (KSL) cells and the long-term HSC enriched population of CD34⁻Flt3⁻ KSL cells (Fig. 2A). Over time, Dkk1-EPC treatment was observed to significantly increase total numbers of BM KSL cells compared to saline control (Fig. 2B). Consistent with the increase in phenotypic stem and progenitor cells, Dkk1-EPC treatment improved BM colony forming capacity after injury (Fig. 2C). Most importantly, Dkk1-EPC treatment was observed to rescue Balb/c mice from hematopoietic mortality after a LD_{50/30} of 8 Gy TBI (Fig. 2D). Here, 100% (15/15) Dkk1-EPC-treated mice survived compared to 80% (12/15) of EPC-treated mice and 33% (5/15) of saline-treated mice.

Since mesenchymal stromal cells (MSCs) have also been shown to promote hematopoietic recovery after radiation injury (17, 18, 27), we evaluated the effect of the same cell dose of the bone marrow-derived stromal cell line M210B4. At day 10 after 5 Gy TBI, M210B4 transplantation did not significantly improve recovery of complete blood counts (CBCs), BM cellularity or BM KSL cells (Supplementary Fig. S1A–C; <http://dx.doi.org/10.1667/RR15361.1.S1>).

Dkk1-EPCs Promote Vascular Recovery in Allogeneic Transplant Model

We hypothesized that Dkk1-EPC infusion mediated accelerated hematopoietic recovery by restoring BM vascular integrity and function. To assess BM vascular integrity after injury, we performed immunofluorescent imaging of VE-cadherin⁺ BM vascular endothelial cells after 5 Gy TBI and treatment with saline, EPCs or Dkk1-EPCs (Fig. 3A). At day 7 post-TBI, irradiated control mice exhibited dilated and highly disorganized vascular structures in the BM. However, treatment with either EPCs or Dkk1-EPCs improved vascular architecture. We quantified endothelial cell numbers using FACS analysis of the CD45[−] VE-cadherin⁺ fraction (Fig. 3B). Since the bone marrow hematopoietic compartment is largely depleted by radiation injury, the relative percentage of endothelial cells within the marrow space was actually observed to increase after radiation injury (Fig 3D, left side). However, total BM endothelial cells declined after radiation injury (Fig 3D, right side). We observed a significant increase in both BM endothelial cell percentage and total endothelial cell number in the DKK-EPC-treated group (Fig. 3B and D). Additionally, Dkk1-EPC treatment significantly lowered apoptotic cell death within BM endothelial cells (Fig. 3C and E). Finally, we assessed barrier function of the BM endothelium by measuring Evans Blue Dye-labeled albumin (EBD-albumin) transit from the circulation into the BM space. Consistent with prior reports, radiation-induced damage to the BM vasculature increased BM vascular permeability to EBD-albumin. Dkk1-EPC treatment significantly reduced permeability compared to saline or EPC treatment alone.

Regenerative Factors Produced by EPCs and Dkk1-EPCs

Allogeneic transplantation of EPCs does not result in long-term engraftment in the bone marrow or other hematopoietic tissue (14) (Supplementary Fig. S2; <http://dx.doi.org/10.1667/RR15361.1.S1>). It is more likely that transfused EPCs promote regeneration of the vascular and hematopoietic tissues via secretion of regenerative factors. In a previously published study, Dkk1 was shown to induce BM endothelial secretion of EGF (26). Here, we screened for factors secreted by EPCs in response to Dkk1-EPC treatment using a combination of ELISA and Luminex® (Austin, TX) panels; we screened for a wide range of hematopoietic, angiogenic, bone and neural factors. Of the 66 unique factors represented on this panel, 13 were detectable above a threshold of 20 pg/ml in the cell culture supernatant of either EPCs or Dkk1-EPCs (Table 1). Consistent with prior analysis of EPC lines, we observed that EPCs secrete VEGF, SDF-1 and HGF (28). In this study, the most highly secreted protein was follistatin (FST), which was further increased (2.8-fold) by Dkk1 treatment. Dkk1 treatment induced a 2-fold increase in eight proteins, including a >20-fold induction in SDF-1 and OPG. Additionally, a fivefold increase in EGF and sevenfold increase in VEGF were observed with Dkk1 treatment. Surprisingly, we observed

that both EPCs and Dkk1-EPCs secreted high levels of brain-derived neurotrophic factor (BDNF). Dkk1-treatment induced a modest but significant increase in BDNF secretion.

Endothelial Transfusion Hastens Neural Regeneration

We anticipated that EPCs would secrete hematopoietic and angiogenic cytokines; however, we did not expect expression of the neural growth factor BDNF. To determine if there was a functional consequence to EPC secretion of BDNF, we looked for anatomical differences in β III-tubulin-labeled nerve fiber organization between our treatment groups. At day 10, saline-treated mice had marked disruption of nerve fiber organization compared to nonirradiated controls. Treatment with either EPCs or Dkk1-EPCs improved nerve fiber organization. To assess for functional recovery of BM sympathetic nerves, we measured sympathetic nerve-dependent circadian oscillations in hematopoietic progenitor cell mobilization (36). Consistent with previously reported studies, we observed increased mobilization of hematopoietic progenitors to the peripheral blood at midday (5 h after lights on; ZT + 5) compared to early evening (1 h after lights off; ZT + 13) in nonirradiated mice (Fig. 4A). After 5 Gy TBI, peripheral blood HSC mobilization was significantly impaired and no effect of light cycle was observed (Fig. 4B). However, mice transplanted with either EPCs or Dkk1-EPCs showed evidence of recovery of circadian oscillation in HSC mobilization to the peripheral blood, as evidenced by a significant difference in mobilized progenitors at ZT 5 vs. ZT + 13 (Fig. 4C).

Co-culture with Dkk1-EPCs Increases Irradiated Human CD34⁺ Colony Capacity

To assess the potential for Dkk1-EPCs to accelerate human hematopoietic recovery after radiation injury, human BM CD34⁺ cells were 3 Gy irradiated and co-cultured with human cord blood-derived EPCs in a non-contact culture system. Total cell expansion and maintenance of CD34⁺ BM progenitor cell population were measured at day 7. Irradiated BM CD34⁺ cells in cytokine-supplemented media alone did not expand in culture. Consistent with prior findings by the Chute laboratory, addition of Dkk1 alone improved hematopoietic recovery as evidenced by increased total CD34⁺ cells (Fig. 5A and B). However, the total *ex vivo* expansion of cells was more robust when the CD34⁺ BM progenitors were co-cultured with EPCs. Here we observed that co-culture with either EPC treatment group significantly increased the total number of cells. The addition of Dkk1 to the co-culture system significantly expanded total cells compared to EPC co-culture alone (Fig. 5A). Additionally, total CD34⁺ cells were more than doubled in the Dkk1-EPC treatment group compared to EPC culture alone (Fig. 5B). Finally, irradiated BM CD34⁺ cells co-cultured with Dkk1-EPCs had significantly greater colony forming capacity than EPCs or media alone (Fig. 5C). Together, these data demonstrate that Dkk1-EPCs mediate the recovery of human hematopoietic cells after radiation injury.

DISCUSSION

There is conflicting evidence on the effect of Dkk1 on endothelial function. Smadja *et al.* showed Dkk1 treatment increases the angiogenic potential of EPCs and promotes *in vivo* vascularization of Matrigel® plugs (25). More recently, Choi *et al.* demonstrated that Dkk1 promotes angiogenesis of human umbilical vein endothelial cells (HUVECs) by activation of

VEGFR2 signaling. In this study, Choi *et al.* did not observe inhibition of Wnt signaling and posited that Dkk1 activated VEGFR2 via a Wnt-independent pathway (37). Conversely, Glaw *et al.* demonstrated that Dkk1 inhibits canonical Wnt signaling to induce microvascular remodeling (38). In a tumor angiogenesis model, Dkk1 blocked tumor angiogenesis while its family member Dkk2 promoted angiogenesis (39). These published findings suggest that Dkk1 effects on the endothelium appear to be context- and endothelial cell-type specific.

In the current study, we evaluated the hematopoietic regenerative function of EPCs after Dkk1 treatment. Transplantation of Dkk1-EPCs improved recovery of hematopoietic function as evidenced by increased BM cellularity, hematopoietic progenitor content, mature circulating blood cells and a marked benefit in survival after 8 Gy TBI. Importantly, infusion of Dkk1-EPCs also promoted recovery of the bone marrow niche as evidenced by increased functional recovery of BM vessels and nerve fibers. We also observed that Dkk1-treated human EPCs promoted expansion of irradiated BM CD34⁺ cells in a non-contact system. Although Dkk1 alone is known to promote hematopoietic regeneration (26), Dkk1-EPC treatment robustly expanded total numbers of human BM CD34⁺ cells *ex vivo*. The ability of Dkk1-EPCs to promote hematopoiesis in a non-contact system suggests that Dkk1-EPC activity is mediated by secretion of soluble growth factors. It is unknown whether the Dkk1 promotes secretion of soluble factors via inhibition of Wnt signaling or through a Wnt-independent mechanism. The cellular mechanism of Dkk1 action on the EPC will be the focus of future work.

In our cytokine screen, we observed Dkk1 induced production of the hematopoietic growth factors EGF and G-CSF. EGF treatment alone has been shown to potently induce HSC regeneration after ablative injury and promote survival of lethally irradiated mice (23). G-CSF is well known for its ability to mitigate radiation injury by accelerating recovery of mature myeloid cells (10, 11). Additionally, we observed that EPC infusion visibly improved recovery of the supportive BM vascular and neural microenvironment. EPC infusion may play a direct role in regeneration of the BM niche by the secretion of proangiogenic factors such as VEGF and the neural growth factor BDNF. Future work will be necessary to determine whether the EPC-mediated recovery of BM sympathetic nervous function is directly related to EPC-secreted factors or simply a consequence of structural recovery of the bone marrow.

Notably, the most highly secreted factor was the secreted glycoprotein protein follistatin. In cancer radiotherapy patients, follistatin expression negatively correlates with fibrosis development (40). Follistatin dosing *in vivo* has been shown to reduce the chronic fibrotic response in irradiated mice via binding and inactivation of the TGF- β family member activin (29). Although follistatin has not been shown to directly promote BM regeneration after injury, it has been shown to promote tissue regeneration in skeletal muscle injury (41) and in planarian loss of tissue regeneration models (42). In our acute radiation injury model, EPC-derived follistatin may reduce induction of the fibrotic response and prevent long-term fibrotic damage.

In summary, this study demonstrates the potential for Dkk1-treated EPCs as a rescue therapeutic for victims of acute radiation injury to the hematopoietic system. Dkk1-EPC treatment was superior to MSC cell line M210B4 in promoting recovery of bone marrow cellularity and function at day 10 after 5 Gy radiation injury. Additionally, our preliminary data show that administration of Dkk1-EPCs is still effective when administered at 24 h after radiation injury. Therefore, this potential therapy addresses the unmet clinical need for mitigators that can be delivered when treatment is delayed beyond 24 h. Finally, since we observed Dkk1-EPCs produce angiogenic and anti-fibrotic factors, it is possible that this therapy may also promote multi-organ recovery after radiation injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Dkk1-EPC transplantation accelerates hematopoietic reconstitution. Panel A: Schematic of allogeneic transplantation model. At 24 h after 5 Gy TBI, Balb/c mice received an i.v. dose of either saline, 1×10^6 EPCs or 1×10^6 Dkk1-EPCs. On days 2–5 postirradiation, mice received intraperitoneal injection of either saline, 2×10^6 EPCs or 2×10^6 Dkk1-EPCs. Panel B: Recovery of total WBCs, neutrophils and lymphocytes over time in the treatment groups. Data represent mean $\pm$ SEM. $N = 5$ –10 mice per group and time point. Panel C: Left side, representative H&E-stained longitudinal femur sections from day 10 mice. Right side, quantification of total bone marrow cellularity over time. Data represent mean $\pm$ SEM. $N = 5$ –10 mice per group and time point. * $P < 0.05$ and ** $P < 0.01$ based on two-way ANOVA with multiple comparison test.

FIG. 2.

Dkk1-EPC transplantation increases hematopoietic stem cell content. Panel A: Top left side, representative FACS plots showing expression of c-Kit and Sca-1 within the BM lineage⁻ (lin⁻) cell fraction of nonirradiated and 5 Gy irradiated mice at day 10. Top right side, quantification of the percentage c-Kit⁺Sca-1⁺lin⁻ (KSL) cell population within the treatment groups. Lower left side, representative FACS plots showing expression of CD34 and Flt3 expression within the KSL gate. Lower right side, quantification of percentage CD34⁺Flt3⁻ KSL HSCs (N = 10 mice/group; **P* < 0.05 one-way ANOVA with multiple comparison test). Panel B: Mean KSL cells per femur over time. (N = 5–10 mice per group and time point; **P* < 0.05 for significantly different treatment groups based on two-way ANOVA.) Panel C: CFC assay from day 10 treatment groups (N5 mice/group; *P*, 0.05 one-way ANOVA with multiple comparison test). Panel D: Survival after 8 Gy TBI: 15/15 Dkk1-EPC, 12/15 EPC and 5/15 saline. (N = 15 mice/group; *P* < 0.001 log-rank test).

FIG. 3.

Analysis of BM vascular recovery at day 10 after 5 Gy TBI. Panel A: Top row, 103 immunofluorescent images of nonirradiated and irradiated femurs showing BM vasculature (VE-cadherin, red) and total cells DAPI (nuclear stain, blue). Scale bar = 100 μ M. Middle row, same field showing the VE-cadherin (white) signal alone to highlight the differences in vasculature between treatment groups. Bottom row, inset box showing magnified view of VE-cadherin (white) vasculature. Red arrows indicate recovering sinusoidal blood vessels. Panel B: Representative FACS plots of the CD45⁻ VE-cadherin⁺ endothelial cells within the BM lin⁻ cell fraction of nonirradiated and 5 Gy irradiated mice at day 10. Panel C: Representative FACS plots of apoptosis within the CD45⁻ VE-cadherin⁺ cell population as measured by active caspase 3/7⁺ staining. Panel D: Left side, quantification of the percentage CD45⁻ VE-cadherin⁺ cells within the BM lin⁻ population. Right side, calculation of total VE-cadherin⁺ endothelial cell based on the total BM cellularity and percentage VE-

cad⁺ (N 6 mice/group; $P < 0.05$ one-way ANOVA with multiple comparison test). Panel E: Quantification of the percentage caspase 3/7⁺ cells within the CD45⁻ VE-cadherin⁺ cell population (N = 6 mice/group; $P < 0.05$ one-way ANOVA with multiple comparison test). Panel F: EBD extravasation to the BM space from the vasculature (N = 4–6 mice/group; $P < 0.05$ one-way ANOVA with multiple comparison test).

FIG. 4.

Analysis of BM nerve regeneration at day 10 after 5 Gy TBI. Panel A: 20 $\times$ immunofluorescent images of nonirradiated and irradiated femurs showing BM nerve fibers (μ III-tubulin, white) adjacent to BM vessels (VE-cadherin, red) and counterstained with DAPI (nuclear stain, blue). Scale bar 100 μ M. Panel B: Mean KSL cells per ml of peripheral blood in nonirradiated mice at ZT + 5 (5 h after lights on) and ZT 13 (1 h after lights off) demonstrating typical sympathetic nervous system regulation of HSC mobilization. (N = 5 mice/group; * P < 0.05, two-tailed Student's t test). Panel C: Mean KSL cells per ml of peripheral blood in treatment groups at day 10 after 5 Gy TBI (N = 5 mice/group; * P < 0.05, two-tailed Student's t test).

FIG. 5.

Expansion of 3 Gy irradiated human BM CD34⁺ cells. Panel A: Total cell expansion after one week in either liquid culture $\pm$ 500 ng/ml Dkk1 or in non-contact co-culture with human EPCs $\pm$ 500 ng/ml Dkk1. Input cells = 10,000 BM CD34⁺ cells. Panel B: CD34⁺ cell expansion. Panel C: Hematopoietic colony forming assay after one week of co-culture. (N = 5 wells per liquid culture group and N = 6 wells per EPC treatment group; * P < 0.05, Student's t test).

TABLE 1

Soluble Factors Produced by EPCs in Response to Dkk1 Treatment

Cytokine	Concentration (pg/ml)				Sig	Potential role in BM regeneration	Ref(s).
	EPC	Dkk1-EPC	Difference	Fold			
Follistatin	13,429	38,037	246,08	2.8	*	Reduces radiation-induced fibrosis	(29)
EGF	1,122	5,759	4,638	5.1	*	Promotes HSC regeneration	(19, 23)
Insulin	6,403	8,760	2,357	1.4	*	Unknown	
SDF-1	80	1,735	1,655	21.7	*	BM HSC retention, Angiogenesis	(30, 31)
VEGF	228	1,643	1,416	7.2	*	Angiogenic	(32)
IP-10	553	884	331	1.6	*	Unknown	
HGF	90	320	230	3.6	*	Radioprotective endothelial cells	(33)
OPG	5	121	116	26.5	*	Angiogenic	(34)
BDNF	724	897	173	1.2	*	Neural Regeneration	(35)
G-CSF	27	74	47	2.7	*	Stimulates BM myeloid recovery	(9)
LIF	123	159	37	1.3	NS	Unknown	
MCP-1	1,243	1,201	-42	1.0	NS	Unknown	
Amphiregulin	172	102	-70	0.6	*	Unknown	

Notes. Factors are listed in order of the greatest difference in factor secretion between Dkk1-stimulated and control. Where applicable, fold change indicates induction after Dkk1 treatment relative to EPC control.

* $P < 0.001$ based on multiple t test with Bonferroni-Dunn correction.