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Prostaglandin E2 increases hematopoietic stem cell survival and accelerates hematopoietic recovery after radiation injury

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Abstract

Hematopoietic stem and progenitor cells (HSPCs), which continuously maintain all mature blood cells, are regulated within the marrow microenvironment. We previously reported that pharmacologic treatment of naïve mice with prostaglandin E2 (PGE2) expands HSPCs. However, the cellular mechanisms mediating this expansion remain unknown. Here we demonstrate that PGE2 treatment in naïve mice inhibits apoptosis of HSPCs without changing their proliferation rate. In a murine model of sub-lethal total body irradiation (TBI), in which HSPCs are rapidly lost, treatment with a long-acting PGE2 analogue (dmPGE2) reversed the apoptotic program initiated by TBI. dmPGE2 treatment in vivo decreased the loss of functional HSPCs following radiation injury, as demonstrated both phenotypically and by their increased reconstitution capacity. The antiapoptotic effect of dmPGE2 on HSPCs did not impair their ability to differentiate in vivo, resulting instead in improved hematopoietic recovery after TBI. dmPGE2 also increased microenvironmental cyclooxygenase-2 expression and expanded the α -SMA+ subset of marrow macrophages, thus enhancing the bone marrow microenvironmental response to TBI. Therefore, in vivo treatment with PGE2 analogues may be particularly beneficial to HSPCs in the setting of injury by targeting them both directly and also through their niche. The current data provide rationale for in vivo manipulation of the HSPC pool as a strategy to improve recovery after myelosuppression.

Introduction

Success of stem cell transplantation (SCT) is in part determined by delivery of adequate numbers of hematopoietic stem and progenitor cells (HSPCs) to efficiently reconstitute the hematopoietic system in the recipient. A potential strategy to expand HSPCs and improve their engraftment is through modulation of marrow microenvironmental components that normally regulate HSPCs¹. This strategy is feasible in the case, for example, of parathyroid hormone-mediated stimulation of marrow osteolineage cells^{2–5}. One recently discovered

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microenvironmental factor that regulates HSPCs is prostaglandin E2 (PGE2). Prostaglandins are synthesized by many cell types in the marrow microenvironment, including osteoblastic cells^{6, 7}. PGE2 and other metabolites in the prostaglandin pathway expand the HSPC pool through activation of the EP2 and EP4 receptors and thereby improve their repopulating ability^{8–10}. While we have demonstrated that systemic administration of PGE2 in mice expands a subset of HSPCs with limited self-renewal¹¹, the mechanisms by which this occurs have not been defined. Moreover, while the effects of ex vivo PGE2 treatment have been explored both in murine models and non-human primates^{8, 10, 12}, it is unclear whether administration of PGE2 in vivo has beneficial effects on hematopoietic recovery. This issue has pragmatic implications since the long-acting PGE2 analogue 16,16-dimethyl-PGE2 (dmPGE2) is well tolerated by patients^{13, 14}.

Therefore, in this manuscript we tested the hypothesis that in vivo PGE2 treatment may decrease apoptotic rates of HSPCs in both naïve mice and during hematopoietic stress.

Materials and Methods

Animals

All studies were performed in 6-12-week-old male C57BL/6 (CD45.2) and B6.SJL-*Ptprca*^d*Pep3^{fl}*/BoyJ (CD45.1) mice (Jackson Laboratory). All experiments were approved by the Institutional Animal Care and Use Committee at the University of Rochester School of Medicine.

Total body irradiation, PGE2 and dmPGE2 treatment

PGE2 treatment in naïve mice was performed as described¹¹. For total body irradiation (TBI), mice were treated once with a single exposure to gamma radiation from a ¹³⁷Cs irradiator. Different groups of mice were given single doses of TBI ranging from 0.5 to 6.5 Gy, as labeled in each experiment. Immediately after radiation exposure, mice were injected intraperitoneally with either 2.0 mg/kg body weight dmPGE2 (Cayman Chemicals) or vehicle (4% ethanol in molecular grade water). Subsequent treatments were given at 24, 48, or for 72 hr post-radiation.

Flow Cytometric Analysis

Bone marrow mononuclear cells (BMMCs) obtained by flushing the long bones (femur and tibia) of experimental mice (10^6 to 10^7 cells/mouse) were stained to identify lineage-Sca-1+ c-Kit+ (LSK) cells and subsets as previously described^{5, 11}. To identify apoptotic HSPCs, cells were washed and separated into two aliquots. 1×10^7 cells were resuspended in Annexin-binding buffer (BD Pharmingen, FITC Annexin Flow Cytometry Kit) with 5 μ l anti-Annexin V antibody and DAPI and incubated at room temperature for 30 minutes. For active-Caspase 3 staining, 4×10^6 cells were fixed and permeabilized using the protocol for Active Caspase-3 Apoptosis Kit (BD Pharmingen). After washing, cells were then stained for active Caspase-3 with PE-conjugated active Caspase-3 antibody for 30 minutes at room temperature. Analytical data were collected on a LSR-II flow cytometer. For sorting, BMMCs were incubated with biotinylated-Lineage cocktail on ice for 15 minutes, washed and incubated with streptavidin-coated magnetic beads (Invitrogen) for 30 minutes to remove Lin⁺ cells (4 rounds of 4 minutes each on an iMagnet, BD). Lineage[−] cells stained with antibodies to detect LSK cells (Lineage-PE-Texas Red, c-Kit-PE-Cy5, Sca-1-PreP-Cy5.5) were sorted on a FACS Aria cell sorter (BD).

Expression of EP receptors

Total RNA and cDNA prepared from sorted LSKs was amplified by RT-PCR using the following primers: *EP2 Fw*: 5′ - ATG CTC CTG CTG CTT ATC GT -3′, *EP2 Rv*: 5′ - TAA

TGG CCA GGA GAA TGA GG -3', *EP4 Fw*: 5' - CCA TCG CCA CAT ACA TGA AG -3', *EP4 Rv*: 5' - TGC ATA GAT GGC GAA GAG TG -3'.

CFU-S Assays

BMMCs from sub-lethally irradiated mice treated with dmPGE2 or vehicle were harvested 24 hr post-radiation and used for CFU-S assays as previously described¹¹.

Competitive Repopulation Assays

BMMCs from sub-lethally irradiated CD45.2 mice treated with dmPGE2 or vehicle were harvested 72 hr post-radiation and mixed with naïve competitor BMMCs (CD45.1+) at a ratio of 10:1 (donor:competitor). The cell mixture was resuspended in sterile FACS buffer and a total of 1×10^6 cells in 150 μ L was injected into the tail veins of preconditioned CD45.1-expressing recipient mice and engraftment was quantified as previously described¹¹.

Expression of Apoptosis-related Genes

RNA was harvested from LSK cells sorted 24 hr post-TBI as described above. cDNA was synthesized using the RT2 First Strand Kit (QIAGEN). Relative expression of apoptosis-related genes was assayed by quantitative real-time PCR on LSK cDNA from control non-irradiated, and vehicle- and dmPGE2-treated mice post-TBI on pathway-focused gene expression profile arrays carrying out manufacturer's instructions (SABiosciences). Results are generated from 3 separate experiments with LSK cells harvested and pooled from 4–8 mice per group in each experiment. Heat maps were constructed using online data analysis software provided by SABiosciences (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

Colony-forming Unit Assays

For HPP/LPP assays, BMMCs from irradiated mice were resuspended at 4×10^6 cells/ml in IMDM (Stem Cell Technologies) + 20% FBS supplemented with CSF-1 (250ng/ml), SCF (50ng/ml), IL-1 (50 ng/ml) and IL-3 (50ng/ml). 0.25 ml cell suspension was mixed into 2.5 ml Methocult 03231 (Stem Cell Technologies) and 1 ml was plated into each of two 35 mm culture dishes. Dense colonies >0.5 mm (HPP) and <0.5 mm (LPP) were counted after 14 days at 37°C. For erythroid and myeloid colony assays, 5×10^4 BMMCs from irradiated mice obtained at the times indicated after TBI were plated in 1 ml of methylcellulose media consisting of IMDM (Invitrogen), 10% PDS (Animal Technologies, Tyler, TX), 20% BIT 9500 (StemCell Technologies, Vancouver, BC, Canada), 5% PFHM-II (Invitrogen), 2 mM glutamine (Invitrogen), and 55 nM 2-mercaptoethanol (Invitrogen) in 1% methylcellulose (StemCell Technologies) with 2 U/mL rhEPO, 5 ng/mL GM-CSF, 0.02 μ g/mL IL-3 and IL-6, and 0.12 μ g/mL SCF (Peprotech, Rocky Hill, NJ). Colonies were counted and scored as erythroid (BFU-E) or myeloid (CFU-G/M) after 7 days at 37°C. Colony counts were reported both per input BMMC and per 2 hindlimbs using BMMC counts from each experimental mouse.

Blood Cell Analysis

A 10–15 μ L droplet of blood was obtained by piercing the tail vein of individual mice with an insulin syringe and was collected into EDTA coated microtainer tubes (BD). Platelet and hematocrit counts were obtained by analyzing a 1:4 dilution (blood:PBS) using the CBC-DIFF Veterinary Hematology System (HESKA). For white blood cell analysis, 20 μ L of blood was obtained from mice in order to analyze blood without dilution. In all experiments individual mice were sampled at most every 3 days. Frequency and timing of sampling of individual mice was identical in vehicle- and dmPGE2-treated mice.

Quantification of bone marrow PGE2 levels

After TBI, sacrificed mice were placed in ice-cold ethanol. Marrow from both femora and one tibia per mouse was flushed into α -MEM with 10% FBS and 1% penicillin/streptomycin containing 25 μ M indomethacin (Sigma), centrifuged, and PGE2 levels in marrow supernatant quantified by ELISA (Cayman Chemicals).

Expression of Cox-2

Mouse femur and tibia were harvested and soft tissue was removed. Bones were fragmented (<1mm), vortexed in PBS cells and strained (40 μ m strainer) to separate the hematopoietic fraction. The bone fragments were digested with collagenase and CD45⁻ cells were further magnetically purified as described¹⁵. The hematopoietic fraction was further enriched for CD45⁺ cells in the same manner. Total RNA was extracted, reverse transcribed and amplified as described¹⁵ using the following conditions: 95°C for 3 min followed by 40 cycles consisting of 15 s at 95°C and 30 s at 60°C. Data were analyzed using the relative standard curve method with each sample being normalized to β -actin. Each sample was run in triplicate, averaged, normalized to β -actin and expressed as relative change as indicated. The sequences for primers used for amplification reactions are as follows: *Cox-2 Forward (Fw)*: 5' - AGA CTA CGT GCA ACA CCT GAG -3', *Cox-2 Reverse (Rv)*: 5' - GCA ATG CGG TTC TGA TAC TGG -3'.

Immunohistochemistry

Paraffin-embedded sections from mouse hind limbs were prepared as described¹⁵. All slides were deparaffinized and rehydrated to PBS (pH 7.4) and treated with aqueous 3% H₂O. For Cox-2 staining, slides were blocked in 5% normal goat serum for 30 minutes. Cox-2 antibody (1:200 dilution, Cayman Chemical 160126) was applied overnight at 4 °C. The biotinylated secondary antibody (goat anti-rabbit, 1:200 dilution, Vector BA-1000) was applied for 30 minutes at room temperature. HRP Streptavidin detection system (Jackson Immuno labs 016-030-084) was applied for 30 minutes followed by Vector DAB chromagen (Vector SK-4100) for 10–30 minutes. Slides were counterstained with hematoxylin, dehydrated and cover-slipped with cytooseal. For F4/80 staining, antigen retrieval was performed in 3% Triton X/PBS at 37 degrees for 20 min and slides were blocked with MaxHetero blocking solution (MaxHetero Rat on Mouse Polymer HRP Detection Kit, NaxVision TRM01-D) for 10 minutes. F 4/80 antibody (1:5000 dilution, Serotec MCA497GA) was applied for 60 min. The rat antibody amplifier was applied for 15 min followed by polymer HRP for 15 min, then incubated with TrueBlue (KPL 71-00-64) for 10 min. Slides were counterstained with Orcein (KPL 71-01-00), dehydrated and cover-slipped with cytooseal. For α -SMA staining, slides were incubated in Proteinase K (DAKO S3004) for 10 min after blocking and smooth muscle actin antibody (1:400 dilution, Abcam ab5694) was applied for 90 min. The rabbit antibody amplifier (MaxPoly-Two Polymer HRP Detection Kit, MaxVision PT03-D) was applied for 15 min followed by polymer HRP for 15 min, then incubated in DAB for 10 min. Slides were counterstained with hematoxylin, dehydrated and cover-slipped with cytooseal. Histology slides were imaged as described¹⁵.

Statistics

All data represent mean $\pm$ SEM except where otherwise noted. Results were analyzed by 2-tailed Student's *t* test, one way ANOVA with Dunnett's Multiple Comparison post-test, when multiple comparisons to control group were made, or two way ANOVA with Bonferroni's post test using the Graph Pad Prism program version 5.02. Statistical significance was defined as *P* <0.05.

Results

In vivo treatment with PGE2 decreases HSPC apoptosis

In vivo PGE2 could expand HSPCs in the bone marrow through several mechanisms, including decreased differentiation, increased proliferation and inhibition of apoptosis. In vivo PGE2 did not inhibit HSPC differentiation¹¹ or change HSPC proliferation (Supplemental figure 1). However, PGE2 has anti-apoptotic effects in several cell types, including dendritic cells¹⁶, cardiomyocytes¹⁷, as well as in many solid tumors^{18, 19}. Recently it has also been suggested that ex vivo treatment of HSPCs with dmPGE2 may decrease their apoptotic rates^{10, 20}. Therefore, we examined the effects of PGE2 on marrow HSPC apoptosis by Annexin V staining and detection of active-Caspase 3 (Fig. 1). Mice that were treated in vivo with PGE2 exhibited significantly lower levels of apoptotic HSPCs compared with vehicle-treated mice, as measured by Annexin V detection (Fig. 1B, $p < 0.0001$) as well as by Active Caspase 3 levels (Fig. 1C, $p < 0.0001$). The PGE2-mediated decrease in apoptosis rates was detected not only in the ST-HSC/MPP populations of cells, but also phenotypic LT-HSCs. These results suggest that inhibition of apoptosis may contribute to the expansion of HSPCs by in vivo PGE2 treatment.

PGE2 treatment is protective of HSPCs in the setting of hematopoietic injury

Since PGE2 treatment inhibits apoptosis in HSPCs in naïve mice, we tested PGE2 effects on HSPCs when apoptotic rates are increased by hematopoietic injury. BMMCs pretreated with either vehicle or PGE2 ex vivo for 90 minutes were treated with cytarabine (Ara-C) for 4 hours to induce cell death (Fig. 2A). The frequency of apoptotic LSK cells was significantly lower in the cultures pre-treated with PGE2 as compared to cultures pre-treated with VEH before Ara-C exposure (Fig. 2B). Therefore PGE2 inhibits apoptosis in HSPCs in the setting of cytotoxic injury.

To test if PGE2 protects HSPCs in the setting of injury in vivo, we employed a sub-lethal TBI murine model. The LSK cell population was significantly depleted 24 hr post-TBI in a dose-dependent manner (Fig. 2C,D), however these injured LSK cells retain expression of the EP2 and EP4 receptors (Fig. 2E), which were reported to mediate the effects of PGE2 on HSCs in zebrafish and are essential for PGE2-dependent liver regeneration^{8–10}. Therefore, TBI-injured HSPCs would be expected to maintain competency to respond to PGE2 treatment. Since PGE2 is short-lived in vivo and the timing and duration of the apoptotic process in HSPCs in response to TBI is unknown, the longer acting dmPGE2 (rather than PGE2) or vehicle was administered immediately following 6.5 Gy TBI, and then daily until the time of sacrifice or for a maximum of 72 hours (Fig. 2F). Twenty-four hours post-TBI, dmPGE2-treated mice had significantly more bone marrow LSK cells than vehicle-treated mice (Fig. 2G). This effect was also demonstrated at a lower TBI dose (4 Gy) in which measurement of more rare subpopulations could be attained, where dmPGE2 treatment increased LSK and subsets ($p = 0.0305$, Fig 2H).

The immature nature and functional capacity of the increased HSPCs found in dm-PGE2-treated mice were supported by increased CFU-S₁₂ in marrows from dm-PGE2- compared to vehicle-treated mice (Fig. 3A). This increase in survival of functional HSPCs was also confirmed by competitive repopulation assays (Fig. 3B). Overall, BMMCs from injured mice displayed very low levels of engraftment in transplantation studies (Fig. 3B), consistent with previous data which have highlighted the severe functional damage induced by sub-lethal TBI on both murine and human HSPCs^{21–23}. However, there was superior repopulating ability of BMMCs from injured dmPGE2-treated mice compared with those from injured vehicle-treated mice at all time points assayed from 3 weeks until 22 weeks post-transplantation, a time period consistent with long-term HSC activity²⁴ (Fig. 3B,

$p=0.0059$ for treatment by 2-way ANOVA). Together, these data establish that dmPGE2 treatment shortly after radiation injury increases the survival of the pool of functional immature hematopoietic cells in the bone marrow.

If dmPGE2-induced HSC protection is dependent on apoptosis, we would expect relatively rapid changes in the expression of apoptosis-related genes induced by TBI. LSK cells were therefore sorted from BMMCs of non-injured as well as injured and dmPGE2-treated mice 24 hr post-TBI. RNA from the sorted LSK cells demonstrated that the global irradiation-dependent expression of apoptosis-related signals was largely reversed by dmPGE2 treatment (Fig. 3C). Overall, these data indicate that in HSPCs dmPGE2 treatment disrupts the apoptotic program initiated by TBI.

PGE2 treatment accelerates hematopoietic recovery after hematopoietic injury

Inhibition of apoptosis may result in damaged HSPCs with impaired ability to generate a progeny. Moreover, previous reports have demonstrated that PGE2 treatment can inhibit hematopoietic differentiation, particularly in the myeloid lineage²⁵. Therefore, in vivo dmPGE2 treatment may be expected to hinder hematopoietic recovery after injury. This would be an important caveat of dmPGE2 treatment post-TBI. To determine the consequences of in vivo dmPGE2 on hematopoietic recovery we investigated the effect of dmPGE2 post-TBI on downstream progeny of HSCs by assaying the more mature highly proliferating progenitors (HPPs), lowly proliferating progenitors (LPPs) and CFU-Cs. Mice were treated with vehicle or dmPGE2 immediately and at 24, 48 and 72 hours after injury. BM cellularity as well as hematopoietic progenitors and precursors were quantified at different time-points, including at 14 days after injury and 11 days after the last dmPGE2 dose. In vivo dmPGE2 treatment did not significantly change the total number of bone marrow cells (Fig. 4A). We next quantified marrow colonies derived from both immature progenitors with significant replating potential, referred to as HPP-CFC, and from more mature lineage-restricted myeloid progenitors called LPP-CFC²⁶. At 24 and 72 hours after TBI, in vivo dmPGE2 treatment increased HPP-CFCs ($p<0.05$), consistent with an anti-apoptotic effect of dmPGE2 on HSPCs (Fig. 4B). dmPGE2 significantly increased marrow LPP-CFC frequency at 72 hours (vehicle 45 ± 6 , dmPGE2 71 ± 8 LPP-CFC/ 5×10^5 BMMCs, $p<0.05$, $N=6$ mice per treatment group), although total numbers were not significantly increased (Fig. 4C). There was no detrimental effect of dmPGE2 treatment on HPP or LPP numbers at 14 days post-TBI (Fig. 4D). No significant inhibitory effects of dmPGE2 on myeloid progenitor/precursors were found at 24 hours (Fig. 4E) or 72 hours (Fig. 4F) after TBI. At 14 days post TBI, rather than inhibiting the generation of myeloid progenitors, in vivo treatment with dmPGE2 significantly increased myeloid colonies (Fig. 4G, $p<0.05$). In contrast, recovery of erythroid colonies was not significantly affected by dmPGE2 treatment (Fig. 4H). Since there was a sustained beneficial effect of dmPGE2 on myeloid progenitors but not erythroid progenitors several days after the last treatment dose, dmPGE2 may have additional hematopoietic effects, possibly microenvironmental, beyond its anti-apoptotic effect on HSPCs.

To confirm that dmPGE2 treatment does not impair the ability of HSPCs to produce the mature blood cells, recovery of blood counts was monitored in mice treated with vehicle or dm-PGE2 for 72 hr post-TBI. Blood counts reached similar nadir at the same time in both treatment groups (Fig. 5A–C) in a manner temporally consistent with previously published reports^{27, 28}, suggesting that the anti-apoptotic effect of dmPGE2 is likely limited to immature hematopoietic cells. A notable exception was the unexpected differences noted in hemoglobin delay, which may suggest direct dmPGE2 anti-apoptotic effects on erythroid progenitors and/or precursors (Fig. 5C). Consistent with the results from colony-forming assays, there was no dmPGE2-dependent inhibition in the post injury recovery of blood counts. In fact, there was accelerated recovery of platelets ($p<0.0001$, Fig. 5A), neutrophil

counts ($p < 0.05$, Fig. 5B) and hemoglobin levels ($p < 0.0001$, Fig. 5C) in dmPGE2-treated mice compared with vehicle-treated controls. dmPGE2-treated mice first reached normal platelet and neutrophil counts at days 13 and 14 post-TBI, respectively, whereas control mice remained cytopenic much longer, until days 21 and 23 post-TBI (Fig. 5A,B). As would be expected from the much longer lifetime of red blood cells compared to platelets and the likely Erythropoietin response in the setting of decreased hemoglobin, the decline in hemoglobin was more gradual and less severe than in other cells types (Fig. 5C). However, despite this, dmPGE2-treated mice first reached normal hemoglobin levels at day 13 post-TBI, whereas control mice remained anemic until day 16 post-TBI (Fig. 5C). Together, these data indicate that that in vivo dmPGE2 treatment early after bone marrow injury results in accelerated recovery of peripheral blood cell counts.

Sublethal TBI increases microenvironmental Cox-2, which is modulated by dmPGE2 treatment in vivo

Since not all populations of hematopoietic progenitor cells were increased at intermediate time points following injury, the quantitative and temporal changes in hematopoietic recovery are likely not due solely to the anti-apoptotic effect of dmPGE2 on HSPCs, suggesting additional effects of dmPGE2 on other marrow components including microenvironmental cells. Prostaglandins are known to be increased in the setting of injury; therefore TBI itself may increase marrow PGE2. Endogenous levels of PGE2 in the marrow microenvironment were quantified by ELISA at baseline and in response to TBI. Endogenous PGE2 was significantly increased by 8 hr post-TBI and this increase was sustained long after the time of injury (Fig. 6A). Notably, even low doses of radiation increased marrow PGE2 levels at 24 hours after TBI (Fig. 6B). The microenvironmental increase in PGE2 could be due to release from the injured cells, as has been recently reported²⁹, or by increased expression of cyclo-oxygenase 2 (Cox-2), a critical inducible enzyme that regulates PGE2 synthesis. Cox-2 is known to be increased in injury settings, such as in the CNS³⁰, in mammary epithelial cells³¹ and in the skin³². While Cox-2 is expressed by several cells in the bone marrow microenvironment, including osteoblasts³³, it is unknown whether its expression is increased by radiation injury. Therefore, we next established whether marrow Cox-2 expression is increased after TBI. The expression of Cox-2 was quantified in both CD45+ hematopoietic and CD45- non-hematopoietic cells from crushed and collagenase-treated long bones of adult mice. We recently demonstrated that CD45-cells obtained in this manner and enriched for osteolineage cells¹⁵. At baseline, there is a low level of Cox-2 expression that is mostly restricted to CD45- microenvironmental cells (Fig. 6C). Twenty-four hours after exposure to 6.5 Gy TBI, Cox2 expression was significantly increased in CD45- microenvironmental cells and remained unchanged in CD45+ hematopoietic cells (Fig. 6C). Cox-2 levels in the bone marrow microenvironment were also visualized by immunohistochemical analysis at 72 hr post-TBI. There were very few Cox-2+ cells in non-irradiated control mice, which were modestly increased following radiation injury (Fig. 6D). In agreement with the expression data, Cox-2 protein was detected on cells lining the endosteal or trabecular bone surfaces in the marrow of irradiated mice, suggesting again that radiation injury increases Cox-2 in microenvironmental, not hematopoietic cells. These data establish, for the first time, that sub-lethal radiation injury increases Cox-2 in the marrow microenvironment.

Prostaglandins have been reported to stimulate expression of Cox-2³⁴, suggesting that PGE2 can increase its own local production. Therefore, exogenous treatment with PGE2 agonists may improve hematopoietic recovery after injury not only through direct effects on hematopoietic cells but also from stimulation of the microenvironment. Indeed, immunohistochemical studies demonstrated that dmPGE2-treated mice had markedly increased Cox-2+ cells in the bone marrow compared with vehicle-treated controls 72 hours

post-TBI (Fig. 7A). Moreover, quantification of Cox-2 mRNA levels from CD45-microenvironmental cells and CD45+ hematopoietic cells at 72 hr post-TBI revealed that exogenous dmPGE2 treatment increased Cox-2 expression specifically in the CD45+ cell fraction (Fig. 7B). This increase in Cox-2 in hematopoietic cells by dmPGE2 is distinct from the increase induced by radiation injury itself, which increases Cox-2 primarily in the microenvironmental cells (Fig 6C). The downstream isomerase Prostaglandin E2 synthase was not differentially expressed in either cellular compartment in the bone marrow, and was not significantly affected by dmPGE2 treatment (Supplemental Fig. 2), suggesting that most of the regulatory effects are at the level of Cox-2. These results uncover modulation of the injury-induced microenvironmental response to TBI by dmPGE2.

Monocytes and macrophages are known to be relatively radioresistant and to express Cox-2 and produce PGE2³⁵. These cells are also known targets of PGE2 and other inflammatory mediators, which can, in turn, further increase their expression of Cox-2^{35, 36}. Further, a recent report characterized a population of alpha-smooth muscle actin (α -SMA)-expressing macrophages located near primitive hematopoietic cells that increase their Cox-2 expression following sub-lethal radiation injury³⁷. Through Cox-2 production of PGE2, these cells maintained HSPC numbers and long-term repopulating ability following radiation injury. Consistent with these data, in our model, immunohistochemical studies revealed a drastic increase in F4/80 staining, a marker of macrophages, following radiation injury in both vehicle and dmPGE2-treated mice (Fig 7C), indicating that macrophages persist in the bone marrow post-TBI. Strikingly, there was also an increase in α -SMA staining in the bone marrow following radiation, and this was even further augmented in mice receiving dmPGE2 post-TBI (Fig. 7C). Flow cytometric quantification of the number of CD11b+ cells in the CD45+ fraction at 72 hr post-TBI revealed that the total number of macrophages in the bone marrow was similar between vehicle and dmPGE2-treated mice ($5.9 \times 10^5 \pm 2.9 \times 10^5$ vs. $7.7 \times 10^5 \pm 1.3 \times 10^5$; $p=0.60441$, data not shown). Taken together, these data suggest that, in addition to direct PGE2 effects on HSPCs, dmPGE2 may initiate microenvironmental changes in specific subpopulations of macrophages in the bone marrow leading to increased HSPC survival and hematopoietic recovery following radiation injury.

Discussion

In this study we tested whether the beneficial effects of in vivo PGE2 treatment, which induced bone microarchitectural changes and caused specific expansion of the ST-HSC and MPP populations without loss of LT-HSCs¹¹, are recapitulated in the setting of myeloablative injury in which the entire hematopoietic system, including HSPCs, are subjected to apoptotic stress. Our central hypothesis was that in vivo treatment, by targeting both the HSPCs and their niche, would afford additional benefits to the actions of PGE2 demonstrated on HSPCs ex vivo, which have been elucidated by studies in both murine models¹⁰ and non-human primates¹².

We first determined that PGE2 treatment in naïve mice inhibits apoptosis in HSPCs. This finding not only provides a mechanism for the observed PGE2-dependent HSPC expansion in vivo, since HSPC differentiation and proliferation rates were not altered, but also strongly anticipated a beneficial effect of in vivo PGE2 in instances of increased apoptotic stress. Apoptosis has been previously identified as a significant cellular fate of HSPCs, which can alter their pool size when manipulated³⁸. While apoptosis was decreased in both LT-HSCs and ST-HSCs/MPPs of PGE2-treated mice, we suspect that, in the absence of injury, PGE2 treatment decreases apoptosis only in cycling LT-HSCs, rather than in dormant LT-HSCs³⁹, both of which are contained in the populations we define as LT-HSCs in our studies.

To increase HSPC apoptosis in vivo, mice were exposed to radiation injury. In spite of their quiescence, HSPCs are susceptible to even low doses of acute radiation injury, which reduces the number of engraftable HSCs as demonstrated in murine models as well as in non-human primates and in humans^{40–42}. Prostaglandins have been implicated in protection of intestinal clonogenic cells when given prior to lethal radiation doses and when given in combination to bone marrow transplantation^{43,44,45}. However, to our knowledge, increased HSPC survival with in vivo stimulation of prostaglandin signaling following sub-lethal radiation injury has not yet been defined.

In these studies, we detected a general reversal in the overall expression pattern of apoptosis-related genes in response to TBI with dmPGE2 treatment, indicating activation of a robust pro-survival program in HSPCs. Notably, in the setting of sublethal TBI, dmPGE2 did not selectively protect a population of HSPCs with limited self-renewal, since superior engraftment of BMMCs from dmPGE2-treated irradiated mice persisted for at least 22 weeks post-transplantation. This difference between the injured and non-injured in vivo models may be due to the use of the more sustained PGE2 analogue, dmPGE2.

In addition to decreased apoptotic rates, the increase in repopulating activity of HSPCs from dmPGE2-treated mice post-TBI could also be explained by further qualitative effects of dmPGE2 on HSPCs, such as changes in homing or retention in the niche, especially since some reports have suggested that ex vivo exposure to dmPGE2 prior to transplantation may increase homing of HSCs^{10, 20}. Further studies are needed to determine if dmPGE2 in our model improves HSPC homing.

Several early reports demonstrated that PGE2 treatment can specifically inhibit myelopoiesis. However, there was no decrease in myeloid or erythroid bone marrow progenitors with in vivo dmPGE2 treatment at any time point analyzed. In fact, our data identified an increase in hematopoietic progenitors, particularly at 14 days post-TBI, 11 days following the final dmPGE dose. The lack of PGE2-dependent progenitor inhibition may be specifically due to the injury setting. Another explanation may be that the injected dmPGE2 would be expected to have dissipated by the time in which the progenitors are expanding.

The accelerated hematopoietic recovery afforded by in vivo dmPGE2 treatment suggests that PGE2-dependent inhibition of apoptosis in the context of injury preserves HSPCs that are not severely damaged and are thus able to properly differentiate. Moreover, the short term benefits of in vivo dmPGE2 treatment are not at the expense of long term-repopulating cells, as demonstrated by superior long-term engraftment of BMMCs from dmPGE2-treated mice.

Since we observed anti-apoptotic effects of PGE2 on HSPCs in vitro, a component of the beneficial action of PGE2 in vivo is likely to be directly on the HSPCs. However, the unexpected acceleration of hematopoietic recovery with its delayed effects strongly suggests a contribution of PGE2-induced microenvironmental changes as well. Thus, we next focused on TBI-induced microenvironmental changes in PGE2 regulation and how they are modulated by in vivo dmPGE2 treatment.

Following TBI, bone marrow PGE2 is rapidly increased, likely by up-regulation of Cox-2 in CD45⁺ bone marrow microenvironmental cells, and remains elevated for at least 6 days after injury. This observation provides a scenario in which increased PGE2 could be an endogenous physiologic signal protecting injured HSPCs. This hypothesis is in line with data implicating PGE2 in a central evolutionarily conserved mechanism for tissue repair after injury²⁹. Moreover, these results are consistent with the previous analysis of Cox2^{-/-} mice, which based on our data would be expected to have defects in marrow PGE2 production in response to injury. Mice with global lack of Cox-2 have in fact decreased rates

of hematopoietic recovery after treatment with the chemotherapeutic agent 5-Fluorouracil⁴⁶. In this context, the observed increase in endogenous PGE2 post-TBI in our model, even in the setting of low dose radiation, would caution against the inhibition of cyclo-oxygenases via anti-inflammatory therapies during marrow recovery.

The induction of microenvironmental Cox-2 following sub-lethal TBI was modulated by dmPGE2, particularly in CD45+ cells. This result demonstrates that 1) PGE2 produced in response to marrow injury can act via the microenvironment to amplify and prolong its own effects and 2) that the microenvironmental response to injury can be further augmented by treatment with PGE2 agonists with the goal of accelerating hematopoietic recovery. Macrophages in the bone marrow are known to be relatively radioresistant, and to up-regulate Cox-2 expression in response to PGE2 signaling³⁵. Thus, we examined the prevalence of macrophages in the bone marrow before and following radiation injury by immunohistochemistry. The increase in F4/80+ cells after TBI suggests that macrophages could be a microenvironmental population mediating the delayed effects of dmPGE2 after injury. Further, the α -SMA-expressing population of macrophages was specifically increased in the bone marrow of dmPGE2-treated mice post-TBI. This cell population was recently shown to support HSCs following sub-lethal radiation injury via up-regulation of Cox-2³⁷. Taken together, the microenvironmental effects of dmPGE2 treatment following radiation injury may be mediated by a specific subpopulation of macrophages in the bone marrow. This hypothesis, if confirmed, may add to the role of marrow macrophages, which have lately been implicated as a regulatory component of the HSPC niche⁴⁷⁻⁴⁹. This dmPGE2-dependent activation of CD45+ cells could explain some of the delayed beneficial effects of dmPGE2 on hematopoietic recovery. Additional studies are required to further define the contribution of specific subsets of marrow macrophages to the dmPGE2 effect on marrow recovery, and to determine if other populations within the bone marrow are participating as well.

Based on the current results, our working model summarizing the observed effects of in vivo dmPGE2 after TBI includes both direct and indirect cellular mechanisms (Supplemental Fig. 3A). Expression of EP2 and EP4 receptors during injury supports direct actions of PGE2 on HSPCs, and therefore all the mechanisms implicated in the HSPC response to PGE2 ex vivo are likely at play (Supplemental Fig. 3A). dmPGE2 administration in vivo also modulates the bone marrow microenvironmental response to injury likely through specific subpopulations of macrophages persisting in the bone marrow (Supplemental Fig. 3B,C), accelerating recovery of hematopoietic progenitors and blood counts. Therefore, in vivo manipulation of HSPC function may provide an exciting potential treatment strategy to remedy myelosuppression.

This PGE2-dependent improvement of hematopoietic recovery has significant therapeutic implications. While bone marrow injury from radiation exposure or chemotherapeutic treatment, which can cause significant anemia, thrombocytopenia and leukopenia, can be treated with G-CSF and GM-CSF, megakaryopoiesis is minimally stimulated by these treatments⁵⁰, thus thrombocytopenia continues to be a serious clinical issue. In this context, our studies raise the prospect that the use of PGE2 agonists may represent a novel approach to meaningfully accelerate recovery of peripheral blood counts in patients following myelosuppressive treatments or injuries during a vulnerable time when few therapeutic options are currently available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. In vivo PGE2 treatment decreases the percentage of HSPCs undergoing apoptosis
A) Representative flow cytometric plots demonstrating the identification of LSK cells and HSPC subsets using cell surface markers. B) Representative plot demonstrating gating strategy for Annexin V, and quantification of the percentage of apoptotic HSPCs after 3 days of vehicle or PGE2 treatment, as measured by cell surface Annexin V. C) Representative plot demonstrating gating strategy for active-Caspase-3 analyses, and quantification of percentage of apoptotic HSPCs after 3 days of vehicle or PGE2 treatment, as measured by active Caspase-3 expression. N=6 to 7 per treatment group, 2 separate experiments. * $P < 0.05$, ** $P < 0.01$

Figure 2. PGE2 decreases LSK cell loss after ex vivo and in vivo myeloablative injury

A) Schematic of experimental design. BMMCs were incubated in the presence of PGE2 or vehicle for 90 minutes. After this pre-treatment, Ara-C was added to cultures to induce cell death. Four hours later, cells were harvested and assayed for Annexin V on the cell surface. B) The percentage of apoptotic LSK cells as measured by Annexin V surface expression 4 hours after Ara-C exposure. BMMCs pooled from 3 animals per group in each experiment, 3 separate experiments. C) Representative flow cytometric plots of Lin⁻ BMMCs from non-irradiated control mice and irradiated mice 24 hr post-6.5 Gy TBI. LSK populations are denoted by ovals. D) The effect of TBI dose on the percent of LSK cells remaining in the bone marrow at 24 hr post-TBI as determined by flow cytometric analysis (n = 3–6 mice/group). E) RT-PCR determination of EP2 and EP4 expression in LSK cells sorted from mice 24 hr post-6.5 Gy TBI. DNA ladder fragment length labeled in bp. F) Schematic of experimental procedure. Mice were treated with vehicle or dmPGE2 immediately following TBI and then every 24 hr for a maximum of 72 hr. Animals were sacrificed for analyses at 24 hr or 72 hr post-TBI. G) Total bone marrow LSK cells in mice at 24 hr post-TBI. Dots/circles represent individual mice from four independent experiments. H) Quantification of LSK and subsets from marrow of mice 24 hours post 4 Gy TBI. P=0.03 by 2-way ANOVA for treatment, with Bonferroni post test. Mean and SEM are indicated (n = 8–9 mice/group), **P<0.01 ***P<0.005.

Figure 3. In vivo dmPGE2 administration enhances survival of functional HSPCs and reverses changes in apoptotic gene expression induced by radiation injury
 A) Effect of dmPGE2 treatment post-TBI on CFU-S₁₂ in recipient mice (n=3 donors/group; 5 recipients/group for each time-point). * $P<0.05$. B) Competitive repopulation assays using donor (CD45.2⁺) BMMCs from irradiated vehicle- or dmPGE2-treated mice 72 hr post-TBI mixed with non-irradiated competitor (CD45.1⁺) BMMCs in a 10:1 ratio. Donor-derived (CD45.2⁺) cells in the peripheral blood of CD45.1⁺ recipient mice (n=4 donors, 3 recipients/group) were quantified at indicated time points ranging from 3 to 22 weeks post-transplantation. $p=0.0059$ for treatment by 2-way ANOVA. C) Heat map representing global expression levels of apoptosis-related genes in sorted LSK cells control non-irradiated mice and from mice 24 hr post-6.5 Gy TBI and vehicle or dmPGE2 treatment. Control group represents one experiment with expression determined from LSK cells sorted from 6 mice. The average expression levels from three individual experiments per treatment group in irradiated mice are shown, 3 separate experiments with LSK cells sorted from 5–8 mice per experiment. * $P<0.05$.

Figure 4. In vivo administration of dmPGE2 does not inhibit differentiation of hematopoietic progenitor cells

A) Total number of BMNCs harvested from 2 hind limbs from each donor (n=7–11 donor mice per experimental group). B–H) Quantification of hematopoietic progenitor by colony assays from mice treated for and sacrificed at 24 hr, 72 hr post-TBI, or from mice treated for 72 hr and sacrificed at day 14. Two to three experiments per time-point, n=3 to 4 mice mice/experiment. Data are represented as total colonies per mouse (2 hind limbs each) as colony assay frequency results were multiplied by individual BMNC counts. B) HPP-CFCs at 24 and 72 hrs post 6.5 Gy TBI (p=0.0108 for treatment by 2-way ANOVA with Bonferroni post test); C) LPP-CFCs at 24 and 72 hrs post 6.5 Gy TBI, D) HPP-CFCs and LPP-CFCs at 14 days post-6.5 Gy TBI, E–G) Myeloid colonies at 24hrs, 72 hrs and 14 days post 6.5 Gy TBI (CFU-G:CFU-Granulocyte, CFU-M:CFU-Macrophage, CFU-GM:CFU-Granulocyte Macrophage). For panel G, p=0.0205 by 2-way ANOVA with Bonferroni post test. H) Burst Forming Unit-Erythrocyte (BFU-E) colony quantification. *P<0.05 for column comparison.

Figure 5. In vivo administration of dmPGE2 accelerates hematopoietic recovery
 A–C) Peripheral blood platelets (A), neutrophil counts (B) and hemoglobin levels (C) from mice subjected to 6.5 Gy TBI and treated with dmPGE2 (red squares) or vehicle (blue circles) daily for 72 hr post-radiation. Open diamonds indicate timing of dmPGE2 treatment, gray regions represent normal ranges for each measurement, broken line indicates the lower range of age and sex-matched control non-irradiated mice. $n = 3$ –12 mice per data point; data generated from 3 separate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$

Figure 6. Radiation injury induces bone marrow microenvironmental Cox-2 expression and local production of PGE2

A) Quantification of bone marrow PGE2 levels by ELISA at indicated times after exposure to 6.5 Gy TBI. Bars represent mean, SEM is indicated ($n = 3-12$ mice/time point). Data were analyzed by 1-way ANOVA with Dunnett post test. The gray box indicates the range of the baseline levels based on $t=0$ data. B) Dose-dependent changes in bone marrow PGE2 levels by ELISA 24 hours after exposure to a single dose of 0 to 6.5 Gy TBI as indicated. Bars represent mean, SEM is indicated ($n = 3-12$ mice/time point). Data were analyzed by 1-way ANOVA with Dunnett post test. The gray box indicates the range of the baseline levels based on 0Gy data. C) Relative expression of Cox-2 in CD45⁺ and CD45⁻ cells from non-irradiated control mice and mice 24 hr post-6.5 Gy TBI. Levels are expressed as fold change over the expression in CD45⁺ cells from non-irradiated controls. All samples were performed in triplicate, and results are normalized to β -actin expression levels. Two separate experiments per time point with bone marrow cells pooled from 3–5 mice per treatment group in each experiment. D) Cox2 expression (in brown) in the bone marrow microenvironment following radiation injury. Representative images obtained with a 40x objective of sections from the distal femur in a non-irradiated control animal and an animal 72 hr post-6.5 Gy TBI in which immunohistochemical staining using a specific anti-COX-2 antibody was performed. Images were counterstained with hematoxylin. Arrows indicate Cox-2 expressing cells and inserts are magnified 4x. Scale bars represent 100 μ m, inset shown to the right. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Figure 7. dmPGE2 treatment post-TBI further increases microenvironmental and hematopoietic cell Cox-2 expression above that induced by radiation injury alone

A) Cox-2 expression (in brown) in the bone marrow microenvironment following radiation injury and treatment with vehicle or dmPGE2 at 72 hr post-TBI. Immunohistochemical staining of representative 40x images of sections from the distal femur using anti-Cox-2 antibody. Images were counterstained with hematoxylin. Scale bars represent 100 μ m. B) Relative expression of Cox-2 in CD45⁺ and CD45⁻ cells from irradiated mice treated with vehicle or dmPGE2 at 72hr post-6.5 Gy TBI. Levels are expressed as fold change over the expression in CD45⁺ cells from non-irradiated controls (dashed line). All samples were performed in triplicate, and results are normalized to β -actin expression levels. Two separate experiments per time point with bone marrow cells pooled from 3–4 mice per treatment group in each experiment. C) Detection of F4/80⁺ cells (top panels, in blue) and α -SMA⁺ cells (bottom panels, in brown) in representative images obtained on a 40x objective of sections from the distal femur in a non-irradiated control animal and in mice treated with vehicle or dmPGE2 for 72 hr post-6.5 Gy TBI. Immunohistochemical staining using specific anti-F4/80 and anti- α -SMA antibodies was performed and images were counterstained with hematoxylin. Arrows highlight representative F4/80⁺ bone marrow cells in the non-irradiated control sample. Scale bars represent 50 μ m. * P <0.05 ** P <0.01, *** P <0.005.