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Accelerated hematopoietic recovery with angiotensin-(1–7) after total body radiation

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Abstract

Purpose: Angiotensin (1–7) [A(1–7)] is a component of the renin angiotensin system (RAS) that stimulates hematopoietic recovery after myelosuppression. In a Phase I/IIa clinical trial, thrombocytopenia after chemotherapy was reduced by A(1–7). In this study, the ability of A(1–7) to improve recovery after total body irradiation (TBI) is shown with specific attention to radiation-induced hematopoietic injury.

Materials and methods: Mice were exposed to TBI (doses of 2–7 Gray [Gy]) of cesium 137 gamma rays, followed by treatment with A(1–7), typical doses were 100–1000 µg/kg given once or once daily for a specified number of days depending on the study. Animals are injected subcutaneously via the nape of the neck with 0.1 ml drug in saline. The recovery of blood and bone marrow cells was determined. Effects of TBI and A(1–7) on survival and bleeding time was also evaluated.

Results: Daily administration of A(1–7) after radiation exposure improved survival (from 60% to 92–97%) and reduced bleeding time at day 30 after TBI. Further, A(1–7) increased early mixed progenitors (3- to 5-fold), megakaryocyte (2- to 3-fold), myeloid (3- to 6-fold) and erythroid (2- to 5-fold) progenitors in the bone marrow and reduced radiation-induced thrombocytopenia (RIT) (up to 2-fold). Reduction in the number of treatments to 3 per week also improved bone marrow recovery and reduced RIT. As emergency responder and healthcare systems in case of nuclear accident or/and terrorist attack may be overwhelmed, the consequence of delayed initiation of treatment was ascertained. Treatment with A(1–7) can be delayed up to 5 days and still be effective in the reduction of RIT or acceleration of bone marrow recovery.

Conclusions: The data presented in this paper indicate that A(1–7) reduces the consequences of critical radiation exposure and can be initiated well after initial exposure with maximal effects on early responding hematopoietic progenitors when treatment is initiated 2 days after exposure and 5

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Declaration of interest

Authors Kathleen E. Rodgers, PhD, and Gere S. diZerega, MD, are inventors on patents regarding reduced radiation-induced thrombocytopenia (RIT) held by the University of Southern California. Dr diZerega is the owner of US Biotest, Inc., which provided the drug used in these studies. The remaining authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

days after exposure for the later responding progenitors and reduced thrombocytopenia. There was some effect of A(1–7) even when given days after radiation exposure.

Keywords

Angiotensin; hematopoiesis; progenitor cells; TBI; radiation

Introduction

Extensive evidence points to a significant role for the renin angiotensin system (RAS) in the regulation of hematopoiesis and the development of hematopoietic progenitors. The centrality of this system in the regulation of early hematopoiesis was recently demonstrated (Jokubaitis et al. 2008, Zambidis et al. 2008, Slukvin 2009). Expression of angiotensin I converting enzyme (ACE; cluster of differentiation [CD]143) by cells derived from human pluripotent cells is currently the earliest marker that identifies the hemangioblast, an early cell found in the fetus that results in the development of the hematopoietic and vascular systems (Tavian et al. 2010, Roks et al. 2011). Furthermore, in vitro inhibition of the angiotensin type 2 [AT2] receptor or ACE blocked the development of these cells towards the hematopoietic pathways.

All the components of the RAS are contained within bone marrow-derived cells of the peripheral circulation including mRNA for angiotensinogen, renin, ACE, Angiotensin Type 1 receptor (AT1), AT2, and ACE2 (Haznedaroglu and Ozturk 2003, Strawn et al. 2004). A number of publications have shown that modification of the RAS will affect the bone marrow (Rousseau-Plasse et al. 1998, Chisi et al. 1999, 2000, Charrier et al. 2004, Davis et al. 2010, Barshishat-Kupper et al. 2011, Shen and Bernstein 2011). Inhibition of ACE using ACE inhibitors (ACEi) affects recovery following myelosuppression by modifying hematopoietic progenitor cell cycling. The timing of treatment relative to myelosuppression differentially affects cell viability and repopulation capacity (Davis et al. 2010). Suggested mechanisms for this modification include inhibition of the generation of Angiotensin II (A-II), reduction of hydrolysis of an acetylated tetrapeptide, AcSKDP, by ACE and modification of erythropoietin through activation of hypoxia-inducible factors (Barshishat-Kupper et al. 2011).

Our laboratory and others demonstrated that A-II mediates its action on hematopoietic cells through the AT1 receptor, affecting proliferation and differentiation of CD34+, erythroid and myeloid progenitor cells, the hematopoietic precursor of dendritic cells (Mrug et al. 1997, Rodgers et al. 2000, Peng et al. 2003). Bone marrow stromal cells release arachidonic acid, which affects hematopoiesis, upon A-II stimulation of the AT1 receptor (Rodgers et al. 2000, Richmond et al. 2004, Strawn et al. 2004). This may be one mechanism by which modification of the RAS, either through administration of ACEi or exogenous peptide, modulates hematopoiesis.

Previous studies from this laboratory (Rodgers et al. 2002) demonstrated administration of A-II and A(1–7), a non-hypertensive peptide of the RAS, and reconstitution of bone marrow progenitors in irradiated mice. Results from animal models and clinical trials show that recovery after myelosuppressive therapy, such as chemotherapy for the treatment of cancer,

in response to A(1–7) is multilineage, resulting in increases in peripheral blood concentrations of platelets, red blood cells and lymphocytes (Rodgers et al. 2002, 2003, 2006, Ellefson et al. 2004). Clinical studies also suggest that exposure to TXA127, a proprietary, injectable clinical formulation containing 2.5–100 µg/kg A(1–7) as the active pharmaceutical ingredient, mitigates the effects of chemotherapy on bone marrow by restoring the various hematopoietic parameters to baseline levels (Rodgers et al. 2006). Importantly, reduction in thrombocytopenia was the most sensitive response to TXA127 in this clinical study. The comparator arm, administration of Neupogen, a proprietary injectable formulation containing 300 µg/kg recombinant human granulocyte stimulating factor, resulted in a progressive decrease in the levels of all parameters. This consistent TXA127-mediated return to baseline may be due to an increase in the number of bone marrow progenitors that would allow the bone marrow to respond to the next insult in a manner comparable to bone marrow naïve of injury. These data from a human trial evaluating the efficacy of A(1–7) suggest that the drug may act on early progenitors in the hematopoietic system. Studies in this report demonstrate an optimized dosing schedule for A(1–7) in the reduction of radiation-induced thrombocytopenia (RIT). Furthermore, it was shown that these changes in RIT contributed to reductions in bleeding time and enhanced survival.

Materials and methods

Animals

The NIH Principles of Laboratory Animal Care were followed, and the IACUC of the Department of Animal Resources at the University of Southern California approved this study. Six- to eight-week-old female CD2F1 or C57Bl/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were quarantined for one week prior to initiation of treatment, and food and water were available *ad libitum*, and were kept on a 12-h light/dark cycle.

Materials

A(1–7) (prepared using Good Manufacturing Practices) was purchased from Bachem (Torrance, CA, USA). The medium and reagents used for hematopoietic progenitor cultures were purchased from Stem Cell Technologies, Incorporated (Vancouver, BC, Canada).

Methods

Radiation calibration and dosimetry

The whole animal irradiation was performed in a cesium 137 gamma irradiator (Atomic Energy of Canada Ltd [AECL], Mississauga, Ontario, Canada) located in the Norris Comprehensive Cancer Center on the University of Southern California (USC) Health Sciences campus. The nominal dose rate at the start of exposures for the study was 96 cGy per min. Cesium has a half-life of 30 years and thus the dose rate decreases by approximately 1 cGy every four months. Exposures times were corrected for this decay rate. Animals were irradiated while restrained and conscious in small Plexiglass containers placed in the exposure chamber. The Plexiglass containers have small holes in them to allow for

free flow of air while mice are exposed. The exposure chamber is in the dark during exposures and air flows through the exposure chamber while exposures are performed.

Animal treatments

The animals received injections of placebo or A(1–7) subcutaneously via the skin at the nape of the neck, on the schedule described in an individual study starting after total body irradiation (TBI). The day of TBI was designated as day 0. Therefore day 2 was 48 h after TBI. Mice were necropsied at day 30 after TBI. For the survival and bleeding time studies, no supportive care was given and no blood was drawn through day 30. These studies were performed in C57Bl/6 mice. In other studies, blood was harvested at days 3, 7, 10, 14, and 21 after TBI from the saphenous vein under 2% isoflurane inhaled anesthesia (Rodgers et al. 2002, 2003, Ellefson et al. 2004). Animals were euthanized by carbon dioxide overdose and bled by cardiac puncture. At necropsy, bone marrow was harvested to evaluate hematopoietic progenitors (Colony forming units, [CFU]–granulocyte erythroid megakaryocyte macrophage [GEMM], CFU megakaryocyte [Meg], CFU-granulocyte macrophage [GM], and burst forming units erythroid [BFU-E]). These studies were performed in CD2F1 mice.

Bleeding time

Prior to necropsy on day 30, the bleeding time (time to stop blood flow) from the site of blood harvest (saphenous vein) was examined. This was performed in a dose response study where A(1–7) was started 2 days after TBI.

Isolation of bone marrow

Bone marrow cells were harvested from the femurs of female CD2F1 mice by flushing the bones with Dulbecco's Minimal Essential Medium-High Glucose ('DMEM-HG') (Invitrogen, Carlsbad, CA, USA) containing 2% fetal bovine serum (Invitrogen, Grand Island, NY, USA) with a syringe (VWR, Irvine, CA, USA) and a 20-gauge needle (VWR, Irvine, CA, USA). The bone marrow was harvested on day 30 after TBI. The isolated cells were characterized with the listed progenitor assays.

White blood cell (WBC) and platelet evaluation

Twenty μ l of blood were mixed with 200 μ l of red blood cell (RBC) lysing solution (0.83% NH_4Cl , 10 mM Ethylenediamine tetra-acetic acid (EDTA), (Sigma Aldrich, St Louis, MO, USA), and 0.5% sodium bicarbonate (NaHCO_3) (Sigma Aldrich, St Louis, MO, USA). The mixture was then incubated for 10 min at 4°C. After this incubation, the sample was centrifuged, supernatant removed and the pellet was re-suspended in 100 μ l of phosphate buffered saline (PBS). To this, 100 μ l of 0.04% trypan blue was added. This solution was mixed on a vortex and the number of platelets evaluated by hematocytometer under phase contrast microscopy and the number of WBC (white blood cells) evaluated by hematocytometer under light microscopy.

Red blood cell evaluation

Five μ l of blood were mixed with 5 ml of phosphate buffered saline. The number of RBC (red blood cells) was determined by counting on a hematocytometer under light microscopy.

Evaluation of myeloid and erythroid progenitors in the bone marrow

After collection of bone marrow, the red blood cells were lysed with a hypotonic solution (described above), mixed with 0.04% trypan blue (Sigma Aldrich, St Louis, MO, USA) and the number of nucleated cells was assessed by hematocytometer under light microscopy. Aliquots of cells were then re-suspended at a density of 2×10^6 cells/ml (GM [normal levels 2800–4480 CFU per femur], GEMM [normal levels 709–913 CFU per femur]; and BFU-E [normal levels 736–1053 CFU per femur]). Fifty μ l of each suspension was added to 900 μ l of semisolid medium containing 0.9% methyl cellulose in Iscove's MDM, 15% fetal calf serum, 1% bovine serum albumin, 10 mg/ml bovine pancreatic insulin, 200 μ l/ml human transferrin, 10^{-4} M 2-mercaptoethanol, 2 mM glutamine, 10 ng/ml recombinant murine interleukin 3, 10 ng/ml recombinant human interleukin 6, 50 ng/ml recombinant murine stem cell factor and 3 units/ml erythropoietin (MethoCult, M3434, Stem Cell Technologies, Vancouver, BC, Canada). This mixture was then added to duplicate wells of a 24-well plate. The cultures were then placed at 37°C in a humidified atmosphere of 5% carbon dioxide (CO_2) in air. At day 14, the number of progenitor colonies formed was enumerated under phase contrast microscopy. Colonies that were measured were at least 20 cells in size and had usual characteristics of the appropriate colony type. No differences were noted between controls and treated in colony appearance.

Megakaryopoietic progenitor number

Cells collected from the bone marrow were washed and re-suspended to a concentration of 2×10^6 cells/ml (Rodgers et al. 2003, Ellefson et al. 2004). Fifty μ l of this suspension was mixed with 2 ml of medium containing 1.1 mg/ml collagen, 1% Bovine Serum Albumin (BSA), 10 μ g/ml bovine pancreatic insulin, 200 μ g/ml human transferrin, 2 mM L-glutamine, 10 μ g/ml 2-mercaptoethanol, 50 ng/ml recombinant human thrombopoietin, 20 ng/ml recombinant human IL-6, 50 ng/ml recombinant human Interleukin 11, 100 ng/ml recombinant murine IL-3 in Iscove's MDM (MegaCult-C, Stem Cell Technologies, Vancouver, BC, Canada). The culture slide was placed in a humidified atmosphere and incubated at 37°C in an atmosphere of 5% CO_2 in air for 8 days, at which time the formation of megakaryocytes from megakaryocyte precursors (CFU-Meg; normal levels 3012–4387 per femur) was determined by staining for the expression of acetyl cholinesterase (Jackson 1973). The substrate solution (0.5 mg/ml acetylthiocholiniodide in 0.075 M sodium phosphate buffer, 0.01 M sodium citrate, 3 mM copper sulfate and 0.5 mM potassium ferricyanide solution [Sigma Aldrich, St Louis, MO, USA]) was then added to the fixed slides and allowed to incubate in a humid chamber for 3.5 hours and then counterstained. CFU-Meg were defined as colonies containing at least four megakaryocyte colonies.

Statistics

Results were analyzed by one-way analysis of variance followed by Tukey's test for multiple comparisons, where an alpha of 0.05 was set *a priori* as the threshold for statistical

significance. Area under the curve was calculated as the time that the platelet count was 60,000/ μ l or less by integration of regression line. The survival data was analyzed by a Chi-square test of Log Ranks.

Results

The results will be presented to define optimal parameters for recovery within a compartment of the hematopoietic system. As the hypothesized mechanism of action for A(1–7) is the recovery of bone marrow progenitors followed by development of those progenitors into formed elements in the blood, the bone marrow recovery is presented first. The studies to optimize the dosing schedule for A(1–7) were performed in a sequential manner. A dose-response study was performed for the endpoint of interest (bone marrow progenitor or formed element), followed by a dose frequency study using the optimal dose that was found. An evaluation of the interval after TBI to determine how long initiation of A(1–7) administration can be delayed and still be effective was then conducted for the endpoint of interest. Finally, the benefit of the pharmacological actions of A(1–7) on hematopoiesis was assessed through changes in bleeding time and survival. Results are presented for each end point of interest in the order of studies just outlined.

Bone marrow recovery

Dose response evaluation

In the first study to define optimal dose, A(1–7) administration (100, 300, 500 and 1000 μ g/kg/day) was initiated 24 h after the dose of TBI 200, 300, 400 or 500 cGy. At doses of radiation that did not result in mortality (up to 400 cGy), doses of A(1–7) increased progenitor concentrations in the bone marrow with a few exceptions (Figure 1A–D). While this study was intended not to result in mortality based upon the published LD₅₀ for CD2F1 mice (8.68 Gy [8.52–8.85; 95% CI] (Landauer et al. 2003) there was 100% mortality at 500 cGy in the saline-treated animals [95% survival in the animals treated with A(1–7)]. It is hypothesized that the mortality was a result of the increased stress placed on the mice due to blood harvesting. The number of CFU-GEMM (577%; p 0.05), CFU-GM (543%; p 0.05), BFU-E (412%; p 0.05) and CFU-Meg (435%; p 0.05) measured compared with 300 μ g/kg/day A(1–7) (percentage improvement in the parameter at 300 μ g/kg/day A(1–7) at 300 cGy TBI in parentheses).

It was hypothesized that at 300 cGy TBI and above, the radiation-induced injury was ongoing such that simultaneously proliferating bone marrow, which resulted from 500 and 1000 μ g/kg/day A(1–7), resulted in enhanced bone marrow injury. The percentage reduction of CFU-GEMM, CFU-GM, BFU-E and CFU-Meg by administration of 1000 μ g/kg/day A(1–7) compared with 300 μ g/kg/day A(1–7) at 500 cGy was 50, 53, 44 and 77%, respectively.

It was further hypothesized that delay of A(1–7) treatment would allow the effects of TBI to subside prior to initiation of progenitor proliferation by A(1–7). This was demonstrated when the same study was repeated with 500 and 1000 μ g/kg/day A(1–7) administration initiated 48 h after TBI (Figure 2). It should be noted that at doses of 400 and 500 cGy, there

was no difference in the remaining progenitor numbers in the saline controls. In this study, with initiation of drug at 48 h, A(1–7) continued to significantly increase the concentration of hematopoietic progenitors in a dose-dependent manner. An initial time course study was performed with 1000 µg/kg/day A(1–7) dosing starting 2 days after TBI of 500 cGy (Figure 3) with the evaluation of CFU-GM and CFU-GEMM numbers. The effects of A(1–7) on the recovery of these hematopoietic progenitors occurred early (day 7) after TBI (605% increase in CFU-GEMM and 451% increase in CFU-GM; $p < 0.05$), but did not affect the nadir in progenitor number at day 3 (136% increase in CFU-GEMM and 150% increase in CFU-GM; $p < 0.05$).

Effect of dosing frequency on efficacy of A(1–7)

This study evaluated the effect of dosing frequency on the recovery of bone marrow after TBI (Figure 4). Dosing was initiated 48 h after TBI using 1000 µg/kg (typical volume 0.1 ml per animal) as the optimal biological dose. The optimal biological dose is defined as the total dose of an agent that produces the desired effect. The studies were conducted at four radiation dose levels (200, 300, 400, and 500 cGy) and 10 treatment regimens (Table I). The study was designed to administer 1000 µg/kg each time (1 × /day, 2 × /day, 3 × /day, 2 × /week or 3 × /week) or to give a total dose of 7,000 µg/kg/week with the dose divided by the number of doses with a week (14 × /week; 21 × /week; 2 × /week or 3 × /week). Similar results to those shown were seen at all four radiation levels (only one [400 cG] is shown). This study was designed to determine if dosing more frequently than daily dosing would increase the hematopoietic recovery or if the peptide could be given less frequently than daily. Overall, daily dosing at 1000 µg/kg/day was found to be optimal and dosing 2 or 3 times per day did not increase efficacy (data not shown). The number of CFU-GEMM (1130%; $p < 0.05$), CFU-GM (900%; $p < 0.05$), BFU-E (687%; $p < 0.05$) and CFU-Meg (566%; $p < 0.05$) measured at 1000 µg/kg/day daily A(1–7) compared with saline-treated controls at 400 cGy (percentage improvement in the parameter in parentheses). However, A(1–7) dosed at 3 × per week after radiation exposure (TBI) also improved bone marrow recovery, while not to the same extent as daily administration. The number of CFU-GEMM (760%; $p < 0.05$), CFU-GM (611%; $p < 0.05$), BFU-E (687%; $p < 0.05$) and CFU-Meg (218%; $p < 0.05$) measured at 1000 µg/kg/day 3 × per week A(1–7) compared with saline treated controls at 400 cGy (percentage improvement in the parameter in parentheses).

How late after radiation exposure can A(1–7) be initiated?

In this study, the time after TBI when treatment could be initiated, and still have efficacy, was performed to determine the therapeutic window. In this study, two treatment regimens (1000 µg/kg/day daily and 1000 µg/kg/day 3 × per week) were tested. Treatment was initiated on days 2, 5, 7 or 10 after TBI. The day of administration varied with the time after initiation of treatment, but was every other day for dose one and two with two days in between dose two and three. Data from animals exposed to 400 TBI are shown (Figure 5). Starting A(1–7) on day 2 after 400 cGy TBI resulted in the largest increase in CFU-GEMM, the earliest progenitor measured, and CFU-GM (Figure 5A). The number of CFU-GEMM (345%; $p < 0.05$), CFU-GM (442%; $p < 0.05$), BFU-E (287%; $p < 0.05$) and CFU-Meg (218%; $p < 0.05$) measured at 1000 µg/kg/day daily A(1–7) compared with saline-treated controls at 400 cGy (percentage improvement in the parameter in parentheses). A(1–7)

therapy initiating on day 5 after TBI also increased the myeloid and erythroid progenitors in the bone marrow, but not to the same extent as day 2. The delay in treatment did not have as large an effect on platelet progenitors. The number of CFU-GEMM (222%; $p = 0.05$), CFU-GM (227%; $p = 0.05$), BFU-E (179%; $p = 0.05$) and CFU-Meg (254%; $p = 0.05$) measured at 1000 $\mu\text{g/kg/day}$ daily A(1–7) compared with saline treated controls at 400 cGy (percentage improvement in the parameter in parentheses). There was still an increase in concentration of myeloid and erythroid progenitor cells when the peptide was initiated at days 7 or 10, but the effect was reduced compared to earlier irradiation times as the study progressed. The number of CFU-GEMM (194%; $p = 0.05$), CFU-GM (202%; $p = 0.05$), BFU-E (167%; $p = 0.05$) and CFU-Meg (203%; $p = 0.05$) measured at 1000 $\mu\text{g/kg/day}$ daily A(1–7) compared with saline treated controls at 400 cGy at day 7 (percentage improvement in the parameter in parentheses).

Recovery of circulating formed elements

Leukopenia and anemia

Along with recovery of the bone marrow after TBI, we measured the ability of A(1–7) to reduce radiation-induced cytopenia. However in this model, RBC concentration was not severely diminished and was similar between the groups. As was found and reported in other publications (Rodgers et al. 2002, 2003, Wang et al. 2010), A(1–7) increased WBC number (data not shown) and the pattern of changes were comparable to that of platelets discussed below.

Thrombocytopenia

We described above the effect of TBI with and without administration of A(1–7) on bone marrow myeloid, erythroid and megakaryocytic progenitors. In this section, the effect of A(1–7) on radiation-induced thrombocytopenia (RIT) is presented. In our first study increasing doses of A(1–7) was administered starting 24 h after TBI. In this study, a threshold of 60,000 platelet/ μl was chosen to measure the area under the curve (that is the number of days an animal had a platelet count below 60,000/ μl). This threshold was chosen to reflect a level comparable to grade 4 thrombocytopenia in humans (mice have approximately three times the peripheral blood concentration of platelets compared to humans).

Treatment with 100 or 300 $\mu\text{g/kg}$ A(1–7) resulted in a decrease in the duration of nadir below 60,000 platelets/ μl to half (2.5 days on average) to one fifth (approximately one day) at these two doses, respectively (Figure 6). At 500 cGy, the time spent below 60,000/ μl was prolonged in the saline-treated controls (Figure 6). Administration of all doses of A(1–7) reduced the number of days below 60,000/ μl to a comparable level (approximately one-half to one-third the time that the controls were at this nadir). Furthermore, only one treated animal (at 1,000 $\mu\text{g/kg/day}$ dose) and all saline-control animals (saline control were treated with radiation but no A(1–7), succumbed to TBI after a 500 cGy dose. However mortality at 500 cGy, as noted earlier, was in part likely due to the blood harvesting of the animals during the recovery period. We hypothesize that: (i) This reduction in the dose of TBI that

resulted in mortality was due to the repeated bleeding (equivalent to a combined injury), and (ii) administration of higher doses of A(1–7) within 24 h of TBI reduced efficacy of A(1–7).

In support of the second hypothesis, when evaluating the platelet counts of individual animal responses, some platelet counts started to fall in animals that received 1000 µg/kg A(1–7) at day 21 and 30. This was observed in previous studies when the administration of A(1–7) occurred when the injury was still ongoing (Rodgers et al. 2002). Therefore, it is suggested that the higher dose of A(1–7) may have stimulated bone marrow progenitors during the time when there was still injury occurring to the bone marrow generated by the oxidative stress that results from radiation injury (as seen when comparing the results of Figures 1 and 2 above). This was tested in a second study when four doses of radiation (200, 300, 400 and 500 cGy) were used and the administration of A(1–7) (100 to 1000 µg/kg) was initiated 48 h after TBI.

We next examined platelet recovery after initiation of A(1–7) administration 48 h after TBI. As was shown in Figures 3–5, 48 h delayed administration of A(1–7) provided improved recovery of hematopoietic progenitors compared with administration 24 h after TBI. At only one dose of irradiation did the platelet count fall below 60,000/µl in control animals (400 cGy). At day 15 (where this occurred), no group treated with A(1–7) had an average nadir below 60,000/µl (Figure 7). Throughout the study, at all doses of TBI and time points, administration of 1000 µg/kg A(1–7) resulted in the most robust platelet recovery. At all doses of A(1–7) and radiation, administration of A(1–7) starting 48 h after irradiation also increased bone marrow and platelet recovery (Figure 7).

The next experiment evaluated the effect of dosing frequency on platelet recovery. Dosing was initiated 48 h after TBI using 1000 µg/kg as the optimal biological dose. The studies were conducted at four radiation dose levels (200, 300, 400, and 500 cGy) and 10 treatment regimens (Table I). The study was designed to administer 1000 µg/kg each time (1 × /day, 2 × /day, 3 × /day, 2 × /week or 3 × /week) or to give a total dose of 7000 µg/kg/week with the dose divided by the number of doses with a week (14 × /week; 21 × /week; 2 × /week or 3 × /week). As with hematopoietic progenitor cells in the bone marrow, increasing the frequency of A(1–7) dosing beyond daily did not increase the ability to increase platelet recovery (data not shown). Giving the equivalent of a daily dose over three different injections or giving the total dose three times in a day led to a lower increase in the number of circulating platelets than observed after giving the A(1–7) dose once daily.

We then looked at platelet recovery in a study that tested the time after TBI when treatment could be initiated. We tested two treatment regimens, 1000 µg/kg/day daily and 1000 µg/kg 3 × per week. Treatment was initiated on day 2, 5, 7 or 10 after TBI. Treatment regimens initiated on day 2 or 5 after TBI were equally effective at stimulating platelet recovery (400 cGy, day 14: 172% or 180% for 1000 µg/kg 3 × per week, started day 2 or 5; 191% or 171% for 1000 µg/kg daily, starting at day 2 or 5; $p < 0.05$ compared to saline; $p > 0.05$ compared between dosing groups and day 21: 198% or 181% for 1000 µg/kg 3 × per week, started day 2 or 5; 164% or 176% for 1000 µg/kg daily, starting at day 2 or 5; $p < 0.05$ compared to saline; $p > 0.05$ compared between dosing groups) (Figure 8), but were not effective if started on day 7 or 10 after TBI (data not shown).

Benefits due to improvement of hematopoiesis by A(1–7) in C57Bl/6 mice

The studies reported in this paper so far have showed that administration of A(1–7) accelerated bone marrow and platelet recovery after TBI and improved survival in a model of combined vascular and radiation injury. Two additional studies were conducted in a model of TBI only to ascertain the clinical relevance of the improvement in bone marrow and platelet recovery. In the first study, the effect of A(1–7) treatment starting on day 2 after TBI on the bleeding time of a nick to the saphenous vein at day 30 was tested. In saline-treated irradiated animals, the bleeding time was doubled compared to non-irradiated controls (Figure 9). There was a decrease in bleeding time after treatment with A(1–7). The bleeding time for mice that received 300 or 1000 µg/kg/day A(1–7) was not significantly different from non-irradiated controls ($p = 0.05$).

In the second study, the ability of A(1–7) to improve survival was examined after TBI (7 Gy) in C57Bl/6 mice (Figure 10). Mice were euthanized when they became moribund, stopped eating or were not responsive. Treatment with A(1–7) in this study starting 48 h after TBI and where no bleeding was done, improved survival at all doses of A(1–7) tested (Figure 10). The LD_{50/30} for survival after TBI is in the range of 7.75–8.3 Gy in this strain (Day et al. 2008, Brown et al. 2010). We found that 40% of TBI mice succumbed by 30 days if no A(1–7) was administered and this is consistent with the data of Brown and of Day mentioned above (or 60% of mice survived). When A(1–7) was administered the survival was 92–97% as shown in Figure 10). So A(1–7) improved survival at this dose of TBI ($p < 0.025$ overall; 0.0011 at 300 µg/kg/day; 0.0438 at 500 µg/kg/day and 0.098 at 1000 µg/kg/day).

Discussion

Current radioprotective strategies attempt to reduce the radiation-mediated tissue damage either by reducing cellular proliferation to prevent the immediate consequence of DNA breakage or through reducing the formation or increasing the scavenging of oxygen radicals that cause damage during and after the ionizing radiation. To be effective, currently identified radioprotective agents must be administered within a restricted time frame, typically just before radiation exposure to protect against rather than mitigate the effects of the early effects of radiation (Hosseinimehr 2007). In situations when this is impractical (such as a nuclear accident or attack), therapies that accelerate repair of radiation-induced injury and mitigate the consequences of radiation exposure must be developed that can begin after exposure. This is an active area of development with novel mechanisms undergoing evaluation (Guinan et al. 2011).

A(1–7) is a seven-amino acid peptide that stimulates hematopoietic and epithelial recovery after irradiation and following exposure to anti-neoplastic chemotherapy (Rodgers et al. 2002, 2003, 2006, Hosseinimehr 2007). Data from animal studies, which were supported in human clinical trials (Rodgers et al. 2006), demonstrated that A(1–7) can accelerate recovery of hematopoietic progenitor cells in the bone marrow and epidermal stem cells in the dermis thereby enhancing regeneration of damaged hematopoietic and dermal tissue after exposure to irradiation (Rodgers et al. 2001, 2002, 2003, Ellefson et al. 2004).

The studies in this report focused on development of A(1–7) as a therapeutic drug to mitigate RIT by defining the most effective dose-schedule and characterizing the window of effectiveness following radiation for A(1–7) therapy. We showed (Figures 7 and 8) that starting treatment with 1000 µg/kg A(1–7) daily or 1000 µg/kg 3 × per week starting 48 h after radiation exposure produced the most robust platelet recovery. However significant recovery of platelets was still observed when A(1–7) treatment was started 5 days after TBI, but not when started at day 7 or 10 following TBI. Bone marrow recovery was observed in treatment regimens of A(1–7) that did not increase platelet recovery suggesting that additional mechanisms may be involved in A(1–7)-induced platelet recovery after TBI. Furthermore, treatment with 1000 µg/kg/day A(1–7) could be started later than day 2 and have slightly better effects on megakaryocyte progenitors. This indicates that the kinetics of the response of megakaryocyte progenitors to TBI and to A(1–7) may differ from the erythroid and myeloid lineages. This higher concentration of A(1–7) may increase the expression of factors involved in megakaryocyte differentiation, such as thrombopoietin. Additionally, since A(1–7) has activity that leads to the stimulation of proliferation of endothelial and epithelial stem cells (Rodgers et al. 2001, Wang et al. 2010), it is conceivable that the lack of platelet reduction may be, in part, a consequence of enhanced tissue repair that may lead to lower platelet consumption.

Studies of the effects of A(1–7) on hematopoietic recovery early after TBI (days 3 and 7) show that the peptide did not affect the nadir at day 3, but rapidly increased the number of multipotential progenitors in the bone marrow at day 7. The lack of an effect on the nadir suggests that A(1–7) stimulates proliferation rather than reducing apoptosis.

This report also showed that treatment with A(1–7) reduced the pathological and physical consequences of TBI by reducing bleeding time (showing the platelets generated are functional) and improving survival. This is consistent with the observation that RIT is a hematological change that is a hallmark of reduced survival after TBI (Dicarlo et al. 2011).

As discussed earlier, multiple publications evaluated the ability of modifications of the RAS to modify responses to radiation injury, including pulmonary function and hematopoiesis. Early publications focused on the ability of ACEi to modify late effects to radiation through modification of endothelial dysfunction (Ward et al. 1988, 1989, Molteni et al. 2000). Two focused on the ability of ACEi to mitigate the effects of radiation exposure (Charrier et al. 2004, Davis et al. 2010). ACEi, depending on the site in ACE inhibited (N or C terminus site or both), have multiple effects on the RAS including inhibition of the conversion of angiotensin I (AI) to A-II. This results in decreased A-II, but because of increased AI, levels of circulating A(1–7) increase, (Iyer et al. 1998). However, if both enzymatic sites are blocked, there is an increase in plasma levels of AcSDKP, a peptide hydrolyzed by ACE and that inhibits hematopoiesis (Wei et al. 1991, Azizi et al. 1996, Rousseau-Plasse et al. 1998, Chisi et al. 1999, 2000). In the publication of Charrier, the authors showed that radioprotection of the hematopoietic system due to ACEi was not due to increased AcSKDP, but was mimicked by blockade of AII receptors, suggesting the ACEi may act through reduced production of AII. Therefore, treatment with an ACEi could cause alterations in bone marrow activity after radiation due to a balance in generation or reduced degradation of

A(1–7) or AcSDKP, or reduced production of AII. Further, the use of captopril, an ACEi, modified the production of erythropoietin through increased in hypoxia-induced factors.

The most sensitive hematopoietic lineage to A(1–7) therapy after myelosuppression is the megakaryocytic lineage (Rodgers et al. 2006). In a Phase I/IIa clinical trial, peripheral platelet concentrations remained within normal range despite the patients receiving three consecutive courses of myelosuppressive chemotherapy when treated with A(1–7) (Rodgers et al. 2006). In addition, A(1–7) was shown to enhance bone marrow recovery in preclinical animal studies after myelosuppression and bone marrow transplantation (BMT) (Heringer-Walther et al. 2009). In conclusion, the animal data presented support that A(1–7) is a good candidate for additional study as a drug to reduce thrombocytopenia after radiation exposure for a number of reasons including stability, safety and the ability to delay therapy initiation until up to 5 days after radiation exposure with maximal effect when treatment is started two days after exposure.

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References

- Azizi M, Rousseau A, Ezan E, Guyene TT, Michelet S, Grognet JM, Lenfant M, Corvol P, Menard J. 1996 Acute angiotensin-converting enzyme inhibition increases the plasma level of the natural stem cell regulator N-acetyl-seryl-aspartyl-lysyl-proline. *Journal of Clinical Investigation* 97:839–844. [PubMed: 8609242]
- Barshishat-Kupper M, Mungunsukh O, Tipton AJ, McCart EA, Panganiban RA, Davis TA, Landauer MR, Day RM. 2011 Captopril modulates hypoxia-inducible factors and erythropoietin responses in a murine model of total body irradiation. *Experimental Hematology* 39:293–304. [PubMed: 21146580]
- Brown AP, Chung EJ, Urick ME, Shield WP, 3rd, Sowers AL, Thetford A, Shankavaram UT, Mitchell JB, Citrin DE. 2010 Evaluation of the fullerene compound DF-1 as a radiation protector. *Radiation Oncology* 5:34. [PubMed: 20459795]
- Charrier S, Michaud A, Badaoui S, Giroux S, Ezan E, Sainteny F, Corvol P, Vainchenker W. 2004 Inhibition of angiotensin I-converting enzyme induces radioprotection by preserving murine hematopoietic short-term reconstituting cells. *Blood* 104:978–985. [PubMed: 15105290]
- Chisi JE, Briscoe CV, Ezan E, Genet R, Riches AC, Wdzieczak-Bakala J. 2000 Captopril inhibits in vitro and in vivo the proliferation of primitive haematopoietic cells induced into cell cycle by cytotoxic drug administration or irradiation but has no effect on myeloid leukaemia cell proliferation. *British Journal of Haematology* 109:563–570. [PubMed: 10886205]
- Chisi JE, Wdzieczak-Bakala J, Thierry J, Briscoe CV, Riches AC. 1999 Captopril inhibits the proliferation of hematopoietic stem and progenitor cells in murine long-term bone marrow cultures. *Stem Cells* 17:339–344. [PubMed: 10606162]
- Davis TA, Landauer MR, Mog SR, Barshishat-Kupper M, Zins SR, Amare MF, Day RM. 2010 Timing of captopril administration determines radiation protection or radiation sensitization in a murine model of total body irradiation. *Experimental Hematology* 38:270–281. [PubMed: 20116413]
- Day RM, Barshishat-Kupper M, Mog SR, McCart EA, Prasanna PG, Davis TA, Landauer MR. 2008 Genistein protects against biomarkers of delayed lung sequelae in mice surviving high-dose total body irradiation. *Journal of Radiation Research (Tokyo)* 49:361–372.
- Dicarlo AL, Poncz M, Cassatt DR, Shah JR, Czarniecki CW, Maidment BW. 2011 Medical countermeasures for platelet regeneration after radiation exposure. *Radiation Research* 176:134–137. [PubMed: 21545289]

- Ellefson DD, diZerega GS, Espinoza T, Roda N, Maldonado S, Rodgers KE. 2004 Synergistic effects of co-administration of angiotensin 1–7 and Neupogen on hematopoietic recovery in mice. *Cancer Chemotherapy Pharmacology* 53:15–24. [PubMed: 14569417]
- Guinan EC, Barbon CM, Kalish LA, Parmar K, Kutok J, Mancuso CJ, Stoler-Barak L, Suter LA, Russel JD, Palmer CD, Gallington LC, Voskertchian A, Vergilio J-A, Cole G, Zhu K, D'Andrea A, Soiffer R, Weiss JP, Levy O. 2011 Bactericidal/Permeability-Increasing Protein (rBPI21) and fluoroquinolone mitigate radiation-induced bone marrow aplasia and death. *Science Translational Medicine* 3: 111–118.
- Haznedaroglu IC, Ozturk MA. 2003 Towards the understanding of the local hematopoietic bone marrow renin-angiotensin system. *International Journal of Biochemistry and Cell Biology* 35: 867–880. [PubMed: 12676173]
- Heringer-Walther S, Eckert K, Schumacher SM, Uharek L, Wulf-Goldenberg A, Gembardt F, Fichtner I, Schultheiss HP, Rodgers K, Walther T. 2009 Angiotensin-(1–7) stimulates hematopoietic progenitor cells in vitro and in vivo. *Haematologica* 94:857–860. [PubMed: 19377080]
- Hosseinimehr SJ. 2007 Trends in the development of radioprotective agents. *Drug Discovery Today* 12:794–805. [PubMed: 17933679]
- Iyer SN, Ferrario CM, Chappell MC. 1998 Angiotensin-(1–7) contributes to the antihypertensive effects of blockade of the renin-angiotensin system. *Hypertension* 31:356–361. [PubMed: 9453328]
- Jackson CW. 1973 Cholinesterase as a possible marker for early cells of the megakaryocytic series. *Blood* 42:413–421. [PubMed: 4125240]
- Jokubaitis VJ, Sinka L, Driessen R, Whitty G, Haylock DN, Bertoncello I, Smith I, Peault B, Taviani M, Simmons PJ. 2008 Angiotensin converting enzyme (CD143) marks hematopoietic stem cells in human embryonic, fetal and adult hematopoietic tissues. *Blood* 111: 4055–4063. [PubMed: 17993616]
- Landauer MR, Srinivasan V, Seed TM. 2003 Genistein treatment protects mice from ionizing radiation injury. *Journal of Applied Toxicology* 23:379–385. [PubMed: 14635262]
- Molteni A, Moulder JE, Cohen EF, Ward WF, Fish BL, Taylor JM, Wolfe LF, Brizio-Molteni L, Veno P. 2000 Control of radiation-induced pneumopathy and lung fibrosis by angiotensin-converting enzyme inhibitors and an angiotensin type II type 1 receptor blocker. *International Journal of Radiation Biology* 76:523–532. [PubMed: 10815633]
- Mrug M, Stopka T, Julian BA, Prchal JF, Prchal JT. 1997 Angiotensin II stimulates proliferation of normal early erythroid progenitors. *Journal of Clinical Investigation* 100:2310–2314. [PubMed: 9410909]
- Peng R, Li Y, Brezner K, Litherland S, Clare-Salzler MJ. 2003 Abnormal peripheral blood dendritic cell populations in type 1 diabetes. *Annals of New York Academy of Sciences* 1005:222–225.
- Richmond RS, Tallant EA, Gallagher PE, Ferrario CM, Strawn WB. 2004 Angiotensin II stimulates arachidonic acid release from bone marrow stromal cells. *Journal of Renin Angiotensin Aldosterone System* 5:176–182.
- Rodgers K, Xiong S, DiZerega GS. 2003 Effect of angiotensin II and angiotensin(1–7) on hematopoietic recovery after intravenous chemotherapy. *Cancer Chemotherapy and Pharmacology* 51: 97–106. [PubMed: 12647010]
- Rodgers K, Xiong S, Felix J, Roda N, Espinoza T, Maldonado S, Dizerega G. 2001 Development of angiotensin (1–7) as an agent to accelerate dermal repair. *Wound Repair Regeneration* 9:238–247. [PubMed: 11472620]
- Rodgers KE, Oliver J, diZerega GS. 2006 Phase I/II dose escalation study of angiotensin 1–7 [A(1–7)] administered before and after chemotherapy in patients with newly diagnosed breast cancer. *Cancer Chemotherapy Pharmacology* 57:559–568. [PubMed: 16096787]
- Rodgers KE, Xiong S, diZerega GS. 2002 Accelerated recovery from irradiation injury by angiotensin peptides. *Cancer Chemotherapy Pharmacology* 49:403–411. [PubMed: 11976835]
- Rodgers KE, Xiong S, Steer R, diZerega GS. 2000 Effect of angiotensin II on hematopoietic progenitor cell proliferation. *Stem Cells* 18: 287–294. [PubMed: 10924095]
- Roks AJ, Rodgers K, Walther T. 2011 Effects of renin angiotensin system on vasculogenesis-related progenitor cells. *Current Opinion in Pharmacology* 11:162–174. [PubMed: 21296616]

- Rousseau-Plasse A, Wdzieczak-Bakala J, Lenfant M, Ezan E, Genet R, Robinson S, Briscoe T, Melville J, Riches A. 1998 Lisinopril, an angiotensin I-converting enzyme inhibitor, prevents entry of murine hematopoietic stem cells into the cell cycle after irradiation in vivo. *Experimental Hematology* 26:1074–1079. [PubMed: 9766448]
- Shen XZ, Bernstein KE. 2011 The peptide network regulated by angiotensin converting enzyme (ACE) in hematopoiesis. *Cell Cycle* 10:1363–1369. [PubMed: 21441775]
- Slukvin II. 2009 Renin angiotensin system and hemangioblast development from human embryonic stem cells. *Expert Review in Hematology* 2:137–143.
- Strawn WB, Richmond RS, Ann Tallant E, Gallagher PE, Ferrario CM. 2004 Renin-angiotensin system expression in rat bone marrow haematopoietic and stromal cells. *British Journal of Haematology* 126:120–126. [PubMed: 15198742]
- Tavian M, Biasch K, Sinka L, Vallet J, Peault B. 2010 Embryonic origin of human hematopoiesis. *International Journal of Developmental Biology* 54:1061–1065. [PubMed: 20711983]
- Wang Y, Qian C, Roks AJ, Westermann D, Schumacher SM, Escher F, Schoemaker RG, Reudelhuber TL, van Gilst WH, Schultheiss HP, Tschope C, Walther T. 2010 Circulating rather than cardiac angiotensin-(1–7) stimulates cardioprotection after myocardial infarction. *Circulation Heart Failure* 3:286–293. [PubMed: 20103774]
- Ward WF, Kim YT, Molteni A, Solliday NH. 1988 Radiation-induced pulmonary endothelial dysfunction in rats: Modification by an inhibitor of angiotensin converting enzyme. *International Journal of Radiation Oncology, Biology and Physiology* 15:135–140.
- Ward WF, Molteni A, Kim YT, Ts'ao C. 1989 Structure-function analysis of angiotensin-converting enzyme inhibitors as modifiers of radiation-induced pulmonary endothelial dysfunction in rats. *British Journal of Radiation* 62:348–354.
- Wei L, Alhenc-Gelas F, Corvol P, Clauser E. 1991 The two homologous domains of human angiotensin I-converting enzyme are both catalytically active. *Journal of Biological Chemistry* 266:9002–9008. [PubMed: 1851160]
- Zambidis ET, Park TS, Yu W, Tam A, Levine M, Yuan X, Pryzhkova M, Peault B. 2008 Expression of angiotensin-converting enzyme (CD143) identifies and regulates primitive hemangioblasts derived from human pluripotent stem cells. *Blood* 112:3601–3614. [PubMed: 18728246]

Figure 1.

Optimal dose finding study. CD2F1 mice underwent TBI and 24 hours later various doses of A(1–7) given by subcutaneous administration were started and continued daily until necropsy. The animals were euthanized on day 30 and their bone marrow harvested. The bone marrow cells were cultured as described in the Methods section. Each of four progenitors is represented in the graph (Panel A: CFU-GEMM; Panel B: CFU-GM; Panel C: BFU-E; Panel D: CFU-Meg). Normal levels of these colonies are: GM: 2800–4480 CFU per femur, GEMM: 709–913 CFU per femur, BFU-E: 736–1053 BFU per femur, and Meg: 3012–4280 CFU per femur. These results represent the mean and standard error of the mean (SEM) of data from 5 mice per group. Bars marked with * are significantly different from saline-treated control ($p < 0.05$).

Figure 2

. Optimal dose finding study. CD2F1 mice underwent TBI. 48 hours later, various doses of A(1-7) given by subcutaneous injection were initiated and continued daily until necropsy. The animals were euthanized on day 30 and their bone marrow harvested. Each of three progenitors is represented in the graph (Panel A: CFU-GEMM; Panel B: CFU-GM; Panel C: BFU-E). Normal levels of these colonies are: GM: 2800–4480 CFU per femur, GEMM: 709–913 CFU per femur; and BFU-E: 736–1053 BFU per femur]. These results represent the mean and SEM of data from 5 mice per group. Bars marked with * are significantly different from saline-treated control ($p < 0.05$).

Figure 3.

Early effects of A(1-7) on hematopoietic recovery. C57Bl/6 mice underwent 5 Gy TBI. 48 hours later, 1000 $\mu\text{g/kg/day}$ A(1-7) given by subcutaneous injection was initiated and continued daily until necropsy. The animals were euthanized on day 3 or 7 and their bone marrow harvested. Each of two progenitors is represented in the graph (Panel A: CFU-GEMM; Panel B: CFU-GM). Normal levels of these colonies are: GM: 2800–4480 CFU per femur, and GEMM: 709–913 CFU per femur. These results represent the mean and SEM of data from 5 mice per group. Bars marked with * are significantly different from saline-treated control ($p < 0.05$).

Figure 4.

Impact on dosing frequency on BM progenitor recovery. CD2F1 mice underwent TBI.

Administration of A(1–7) in various schedules (Table I) was initiated at day 2 and continued until necropsy. The animals were euthanized on day 30 and their bone marrow harvested.

Each of four progenitors are represented in the graph (Panel A: CFU-GEMM; Panel B: CFU-GM; Panel C: BFU-E; Panel D: CFU-Meg). Normal levels of these colonies are: GM: 2800–4480 CFU per femur, GEMM: 709–913 CFU per femur, BFU-E: 736–1053 BFU per femur, and Meg: 3012–4280 CFU per femur. Animals were exposed to 400 TBI. These results represent the mean and SEM of data from 5 mice per group. Bars marked with * are significantly different from saline-treated control ($p < 0.05$).

Figure 5.

Impact on time to initiation of treatment on BM progenitor recovery. CD2F1 mice underwent TBI daily administration of 1000 µg/kg A(1–7), daily or three times per week was initiated at days 2, 5, 7 or 10 and continued until necropsy. The animals were euthanized on day 30 and their bone marrow harvested. Each of four progenitors are represented in the graph (Panel A: CFU-GEMM; Panel B: CFU-GM; Panel C: BFU-E; Panel D: CFU-Meg). Normal levels of these colonies are: GM: 2800–4480 CFU per femur, GEMM: 709–913 CFU per femur, BFU-E: 736–1053 BFU per femur, and Meg: 3012–4280 CFU per femur. Animals were exposed to 400 TBI. These results represent the mean and SEM of data from 5 mice per group. Bars marked with * are significantly different from saline-treated control ($p < 0.05$).

Figure 6.

Impact of A(1–7) on duration of thrombocytopenia. CD2F1 mice underwent TBI and 24 hours later, daily administration of various doses of A(1–7) by subcutaneous injection was initiated and continued daily until necropsy. The animals were bled prior to TBI, and on days 3, 7, 10, 14, 21 and 30. These results represent the mean and SEM of data from 5 mice per group. Bars marked with * are significantly different from saline-treated control (p 0.05).

Figure 7.

Impact of A(1-7) treatment on platelet recovery after TBI. CD2F1 mice underwent TBI and 48 hours later, daily administration of various doses of A(1-7) by subcutaneous injection was initiated and continued daily until necropsy. The animals were bled prior to TBI, and on days 3, 7, 10, 14, 21 and 30. In panel A and B, the animals received 400 or 500 cGy TBI, respectively. These results represent the mean and SEM of data from 5 mice per group. Bars marked with * are significantly different from saline-treated control ($p < 0.05$).

Figure 8.

Impact of time to initiation of treatment on platelet recovery after TBI. CD2F1 mice underwent TBI. Two or five days later, daily or 3 $\times$ /week 1000 $\mu\text{g/kg}$ A(1–7) was administered by subcutaneous injection was initiated and continued until necropsy. The animals were bled prior to TBI, and on days 3, 7, 10, 14, 21 and 30. The radiation doses given were 400 cGy TBI (Panel A) or 500 cGy TBI (Panel B). These results represent the mean and SEM of data from 5 mice per group. Bars marked with * are significantly different from saline-treated control ($p < 0.05$).

Figure 9.

Impact of treatment with A(1–7) on bleeding time after TBI. C57Bl/6 mice underwent TBI and 48 hours later daily administration of various doses of A(1–7) by subcutaneous injection and continued daily until day 30. These results represent the mean and SEM of data from 8–10 mice per group for irradiated animals, 5 per group for non-irradiated controls. Bars marked with * are significantly different from saline control ($p < 0.05$). Bars marked by ^ are significantly different from non-irradiated control.

Figure 10.

Improved survival after TBI with A(1-7) administration. C57Bl/6 mice underwent 7Gy TBI and daily administration of various doses of A(1-7) (started days 2-4 after TBI, combined for each dose) by subcutaneous injection and continued daily until day 30. These data are the combination of two studies of 5 animals per group per study ($n = 10$ mice per group for control; $n = 30$ mice per group for each dose).

Table I.

Experimental design for dose frequency study.

Dosing Frequency	Dose per injection µg/kg
Once per day	Saline
Twice per day	500
Thrice per day	333
Twice per wesh	3500
Three times per week	2333
Once per day	1C00
Twice aer day	1000
Thrice per day	1C00
Twice per week	1C00
Three times per week	1C00