Subcutaneous administration of rhIGF-I post irradiation exposure enhances hematopoietic recovery and survival in BALB/c mice

Shilei CHEN, Yang XU, Song WANG, Mingqiang SHEN, Fang CHEN, Mo CHEN, Aiping WANG, Tianmin CHENG, Yongping SU and Junping WANG*

State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Combined Injury of PLA, Research Center of Nanomedicine of Chongqing, Third Military Medical University, Chongqing, China
*Corresponding author. State Key Laboratory of Trauma, Burns and Combined Injury, Institute of Combined Injury of PLA, Research Center of Nanomedicine of Chongqing, Third Military Medical University, Chongqing, China, 400038. Tel/Fax: +86-23-68752283; Email: wangjunping@tmmu.edu.cn

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It is unclear how to effectively mitigate against irradiation injury. In this study, we studied the capacity of recombinant human insulin-like growth factor-I (rhIGF-I) on hematologic recovery in irradiated BALB/c mice and its possible mechanism. BALB/c mice were injected with rhIGF-I subcutaneously at a dose of 100 μg/kg twice daily for 7 days after total body irradiation. Compared with a saline control group, treatment with rhIGF-I significantly improved the survival of mice after lethal irradiation (7.5 Gy). It was found that treatment with rhIGF-I not only could increase the frequency of Sca-1+ cells in bone marrow harvested at Day 14 after irradiation, but also it could decrease the apoptosis of mononuclear cells induced by irradiation as measured by flow cytometry, suggesting that rhIGF-I may mediate its effects primarily through promoting hematopoietic stem cell/progenitor survival and protecting mononuclear cells from apoptosis after irradiation exposure. Moreover, we have found that rhIGF-I might facilitate thrombopoiesis in an indirect way. Our data demonstrated that rhIGF-I could promote overall hematopoietic recovery after ionizing radiation and reduce the mortality when administered immediately post lethal irradiation exposure.

Keywords: insulin-like growth factor-I; irradiation injury; hematopoietic recovery; apoptosis

INTRODUCTION

IGF-I, also known as somatomedin C, is a 70-amino-acid basic peptide. The biological action of IGF-I is mediated by insulin-like growth factor-I receptor (IGF-I-R), a tyrosine kinase receptor which comprises two extracellular alpha subunits that bind the hormone and two beta subunits that span the membrane and contain tyrosine kinase activity. After binding to its receptor, IGF-I causes phosphorylation of insulin receptor substrate (IRS-1 and IRS-2), which then activates the signal pathways such as MAPK/ERK and PI3K/Akt and results in a variety of biological effects [1, 2]. In previous studies recombinant human insulin-like growth factor-I (rhIGF-I) has been shown not only to enhance the proliferation of many cell lines [3–5], but also to protect cells from apoptosis induced by various factors in vitro [6–9]. It has been reported that rhIGF-I could preserve mucosal integrity by protecting intestinal cells from radiation-induced apoptosis and suppress salivary gland dysfunction by inhibiting salivary acinar cell apoptosis in vivo [9–12]. Moreover, rhIGF-I also plays important roles in the regulation of hematopoiesis. It has been demonstrated that rhIGF-I could enhance the in vitro proliferation of committed progenitor cells derived from umbilical cord CD34+ cells [13], and had stimulatory effects on lymphopoiesis, erythropoiesis and granulopoiesis either directly or indirectly [14–17]. However, there is a lack of understanding about the effect of rhIGF-I on thrombopoiesis and its overall role in hematopoietic recovery after irradiation injury. In this study, we investigated the effect of rhIGF-I on the reconstruction of the hematopoietic system in BALB/c mice following irradiation. The results indicate that rhIGF-I treatment significantly enhances overall hematologic recovery, especially for the erythrocytes and platelets, and mitigates the mortality of mice subjected to lethal irradiation doses.
MATERIALS AND METHODS

Mice and irradiation
Male BALB/c mice, 8–12 weeks old and weighing 18–22 g., were purchased from the Center of Experimental Animals at our university. All procedures on these mice were approved by the Animal Care Committee of our university and carried out in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Mice were allowed 3 days to become acclimatized to the animal care facility prior to the study and had free access to standard mice chow and water. The mice were divided randomly into three groups according to the treatment they received: normal group (no irradiation and no treatment, n = 10); saline control group (irradiation plus saline solution treatment, n = 30); rhIGF-I treatment group (irradiation plus rhIGF-I treatment, n = 30). Mice were irradiated with a single dose of 7.5 Gy for probability of survival analysis and of 5 Gy for mechanism investigation at a rate of 93.1 cGy/min using a 60Co gamma ray irradiator.

rhIGF-I treatment
rhIGF-I was purchased from PeproTech (Rocky Hill, NJ, USA). For in vivo and in vitro studies, rhIGF-I was reconstituted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and stored at −80°C until use according to the manufacturer’s instructions. Mice were injected subcutaneously with a dose of 100 μg/kg rhIGF-I twice daily for 7 days. The first injection was given immediately after irradiation. The experiment was repeated three times.

Peripheral blood cell counts
A small aliquot of blood (20 μl) was collected on Days 0, 3, 7, 9, 11, 13, 17, 19 and 21 from the tail vein. Complete blood cells were quantified with an ABC Vet Hematology Analyzer (Heska Corp., Waukesha, USA).

Histology
Three mice from each group were killed on the 14th day after irradiation and biopsies were taken from tibia and stored in buffered formalin. Specimens were then embedded in paraffin, sectioned at 5 μm and then stained with hematoxylin-eosin. The stained slides were studied and images under microscope were acquired with a CCD camera (Olympus, Tokyo, Japan). All images used in this paper were original and unmodified.

Mononuclear cell isolation and cytometric analysis for hematopoietic stem cells
Mice were sacrificed at the designated time points. Bone marrow cells were flushed from femurs with Iscove’s Modified Dulbecco’s Medium (IMDM) medium (Hyclone, Logan, USA) containing 2% fetal bovine serum (FBS) (Gibco, Grand Island, USA). The suspension was washed and passed through a 200 mesh-per-inch stainless steel grid to produce a suspension of single cells. Erythrocytes were lysed with an ammonium chloride potassium buffer. The cells that were not lysed were mononuclear cells. The cells were then washed with PBS once and resuspended in 100 μl PBS. Lineage-negative(Lin−) bone marrow cells were isolated using a cocktail of lineage-specific antibodies (Stem Cell Technologies, Vancouver, Canada) with magnetic beads (Stem Cell Technologies, Vancouver, Canada). Lin− cells were then further labeled with c-kit+ magnetic beads and separated by MACS(Magnetic Activated Cell Sorting) for murine Lin−c-kit+ cells. Lin−c-kit+ BM cells were >92% positive for both Lin− and c-kit+.

Colonizing-forming unit assay
Hematopoietic colony-forming cells were enumerated in semisolid methylcellulose cultures. Mononuclear cells were obtained as described above. Colony-forming units was measured using methylcellulose medium (Stem Cell Technology) in 35-mm gridded dishes. After 10–12 days at 37°C, triplicate plates were scored for colony-forming units-granulocyte, macrophage (CFU-GM), burst-forming units-erythroid (BFU-E), and colony-forming units-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), using standard criteria for their detection with an inverted microscope.

Apoptosis assay for mononuclear cells induced by irradiation
The mononuclear cells obtained from normal mice were seeded at 1 x 10³/ml in IMDM containing 1% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were subjected to irradiation at a dose of 5 Gy. Cultures were then incubated in 5% CO₂ in humidified air at 37°C, in the presence or absence of rhIGF-1 (100 ng/ml). After 24 h in in vitro culture, mononuclear cells were harvested. As described by the kit manufacturer (Roche Applied Science, Penzberg, Germany), the cells were incubated with Annexin-V fluorescein (dilution 1:50) and propidium iodide (PI, dilution 1:50) in Hepes buffer (provided by the kit) for 15 min in the dark at room temperature.
Double-stained mononuclear cells were analysed immediately on a cytometer.

**Megakaryocyte culture and proliferation assay**

The human megakaryocyte cell line MO7e (ATCC, Manassas, VA, USA) was cultured in 1640 modified medium (Hyclone, USA) supplemented with 10% FBS and 10 ng/ml GM-CSF (PeproTech, Rocky Hill, USA) at 37°C with 5% CO₂. Cells in log-phase were seeded into 96-well plates (Costa, Corning, USA) at a density of 5 × 10⁴/well and cultured in medium deprived of GM-CSF, in the presence or absence of rhIGF-I, and 48 h later 10 μl CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well and plates were incubated at 37°C for 5 h. The absorbance was measured with a Bio-rad680 microplate reader (Bio-rad Corp., Hercules, USA) at 450 nm. Within the experiment, A450 values were averaged across six replicate wells.

**Statistical analysis**

Experimental data were represented as mean ± standard deviation. The data were evaluated for statistical significance using Student’s t test. Statistical significance was assumed at a P value of <0.05.

**RESULTS**

**rhIGF-I administration mitigates against lethal irradiation**

To study the capacity of rhIGF-I to mitigate against lethal irradiation in mice, BALB/c mice were whole-body irradiated with a single dose of 7.5 Gy. It has been shown this dose of radiation could induce severe bone marrow myelosuppression with a high degree of associated mortality in our laboratory. As shown in Fig. 1, nearly 17% of the mice in the saline control group survived over 40 days post radiation exposure, while about 40% of the mice treated with rhIGF-I survived over 40 days, demonstrating that rhIGF-I possesses a significant radio-protective effect.

**rhIGF-I promotes hematopoietic recovery post irradiation exposure**

Hematopoietic cells are highly sensitive to ionizing irradiation and the sequelae of hematopoietic failure is one of the main causes of death for irradiated mice [18]. To understand how rhIGF-I mitigates against total body irradiation, we monitored the recovery of circulating platelets, erythrocytes and leukocytes following irradiation through to Day 21 post irradiation at a dose of 5 Gy. As demonstrated in Fig. 2A, the platelet counts decreased rapidly in both groups and remained at a concentration close to 200 × 10⁹/l for several days, while treatment with rhIGF-I significantly accelerated the recovery of total platelets. The rapid increase in platelet counts in rhIGF-I-treated mice was observed between Days 11 and 17 post irradiation. Erythrocyte counts in rhIGF-I-treated mice were higher than those in the saline control group on Days 11–21 post irradiation, and the level reached near baseline, while that in the saline control group was still very low on Days 17–21 (Fig. 2B). With rhIGF-I treatment, the level of leukocytes was also higher than that in the saline control group from Day 7 post irradiation (Fig. 2C). Consistent with the data from the hemocyte counts assay, histological analyses (Fig. 3) at Day 14 post irradiation indicated that rhIGF-I treatment significantly increased the cellularity in bone marrow as compared with the saline control and there were clearly visible megakaryocytes at different stages, further confirming that rhIGF-I enhances hematopoietic recovery post irradiation exposure. Moreover, as demonstrated in Fig. 4, the bone marrow from the rhIGF-I-treated mice had statistically significant higher numbers of CFU-GM and BFU-E and CFU-GEMM compared with the saline control mice (Fig. 4).

**rhIGF-I facilitates hematologic recovery by increasing the amounts of Sca-1+ cells post irradiation exposure**

In order to further understand how rhIGF-I promoted hematopoietic recovery post irradiation, we measured the frequency of Sca-1+ cells post irradiation. At Day 14,
representative mice in each group were sacrificed and the mononuclear cells were flushed and isolated from bone marrow. The content of Sca-1+ cells was assayed by flow cytometry after Lin−c-kit+ cells isolation. The result showed that, following Lin−c-kit+ isolation, Sca-1+ staining accounted for 33.24 ± 3.86%, 50.35 ± 3.21% and 80.23 ± 1.98% of positive cells in the saline control group (A), the rhIGF-I-treated group (B) and the normal group (C), respectively. A representative datum is shown in Fig. 5. Compared with the saline control group, the bone marrow from the rhIGF-I-treated mice had a significant higher frequency of Sca-1+ cells. Owing to the fact that Sca-1+ cells can indicate the characteristics of hematopoietic stem cells (HSCs) after Lin−c-kit+ cells isolation [19] these results reflect that rhIGF-I treatment may promote the recovery of HSCs.

In vitro effect of rhIGF-I on the proliferation of megakaryocytes

Previous published data have demonstrated that rhIGF-I has a stimulatory role on many cell lines as described above. Based on the results of platelet count recovery and increase

![Fig. 2.](image-url) rhIGF-I treatment enhances hematologic recovery post irradiation. BALB/c mice were irradiated with 5 Gy and treated with rhIGF-I for 7 days. Platelet (A), erythrocyte (B) and leukocyte (C) counts were determined using a hematology analyzer. Both the saline control group and the rhIGF-I-treated group contained 30 animals, while the normal group contained 10 animals. *P<0.05, **P<0.01, rhIGF-I vs. saline control as analyzed by Student’s t test.

![Fig. 3.](image-url) Histological analyses. Normal BALB/c mice (A) and irradiated mice treated with saline (B) and rhIGF-I (C) were sacrificed on Day 14, and the tibia slices were stained with hematoxylin and eosin. The magnification was 200×.

![Fig. 4.](image-url) The effect of rhIGF-I treatment on the numbers of CFU-GM, BFU-E and CFU-GEMM. BALB/c mice were irradiated with 5 Gy and treated with rhIGF-I for 7 days. Mice were sacrificed and bone marrow was harvested at Day 14 post irradiation. CFUs were determined after 10 days in culture. Each group contained three to five animals. This is a representative of three similar experiments. CFU-GM, colony-forming unit-granulocyte and monocyte; BFU-E, burst forming unit-erythrocyte; CFU-GEMM, colony forming unit-granulocyte, erythrocyte, monocyte, and megakaryocyte. *P<0.05, rhIGF-I vs. saline control; #P<0.05, normal BALB/c vs. rhIGF-I.
of megakaryocytes in bone marrow cavity after rhIGF-I treatment, we hypothesized that rhIGF-I may facilitate platelet recovery following irradiation by promoting the proliferation of megakaryocytes. So we studied the effect of rhIGF-I on the GM-CSF-dependent human megakaryocytic leukemia cell line MO7e using in vitro assays. The MO7e cells were cultured with rhIGF-I at concentrations of 10 ng/ml and 100 ng/ml for 48 h, paralleled with rhTPO treatment. However, as demonstrated in Fig. 6, no stimulatory role was observed with the rhIGF-I treatment when measured 48 h later, which suggests that rhIGF-I may not directly promote the proliferation of megakaryocytes.

rhIGF-I protects mononuclear cells from apoptosis induced by irradiation in vitro

Cells die either through apoptosis or mitotic death following irradiation. It has been demonstrated previously that rhIGF-I could protect many cell lines from apoptosis induced by various factors [6–9]. To test whether hematopoietic recovery after the treatment of rhIGF-I was also partly attributable to the protection of bone marrow cells from apoptosis following irradiation, the mononuclear cells from normal mice were irradiated at a dose of 5 Gy as previously described [20] and then incubated with various concentrations (10, 50 or 100 ng/ml) of rhIGF-I in 95% air, 5% CO2 at 37°C for 24 h. After being stained with annexin V-FITC and PI, the apoptotic rate of mononuclear cells was assessed by flow cytometry. It was found that the frequency of apoptotic cells was 76.64 ± 0.46, 59.15 ± 1.63 and 29.44 ± 1.43% in the absence (A) or presence (B) of 100 ng/ml rhIGF-I, paralleled with the normal control (C), respectively. A representative datum is shown in Fig. 7. This result demonstrated that mononuclear cells treated with 100 ng/ml of rhIGF-I had a significantly lower apoptotic rate than that of untreated control cells (P < 0.05). These data indicate that rhIGF-I may augment hematopoietic stem/progenitor cell activity by reducing apoptosis of mononuclear cells.

**DISCUSSION**

Experimental animals and human beings subjected to large doses of gamma ray irradiation often suffer from haemorrhage, infection and metabolic disorders caused by hematopoiesis dysfunction [21–22]. In this study, rhIGF-I showed a significant protective effect on BALB/c mice subjected to a lethal irradiation dose of 7.5 Gy. To reveal the mechanism and obtain statistically analyzable data, BALB/c mice were subjected to a dose of 5-Gy irradiation. We found that the bone marrow in mice subjected to 5-Gy irradiation displayed severe injury as
shown by histological assay, meanwhile the peripheral blood platelet, erythrocyte and leukocyte counts dropped to very low levels, which was in accordance with previous reports.

Noticeably, treatment with rhIGF-I significantly accelerated the recovery of peripheral blood platelet, erythrocyte and leukocyte counts (Fig. 2). This notion was also supported by histological analysis because the cellularity in the bone marrow of rhIGF-I-treated mice significantly enhanced compared with that in the saline control mice (Fig. 3). Since Sca-1+ cells can represent the characteristics of hematopoietic stem cells (HSCs) after Lin-c-kit+ cells isolation [19], the frequency of Sca-1+ was used to reflect the number of HSCs. The increase of Sca-1+ positive cells in the bone marrow on Day 14 (Fig. 5) indicates that rhIGF-I may work at the stem cell level and result in an increase of the numbers of HSCs and committed progenitor cells after irradiation. Bone marrow from rhIGF-I-treated mice had significant higher numbers of CFU-GM, BFU-E and CFU-GEMM than the saline control, which also proved that rhIGF-I could enhance the recovery of all lineages of hematopoietic progenitor cells (Fig. 4).

Moreover, it has been demonstrated that rhIGF-I could decrease apoptosis and promote survival of cells in a PI3-kinase-dependent manner. IGF-I activates the serine/threonine kinase Akt through PI3-kinase. This activation phosphor-ylates and inhibits caspase 3, resulting in decreased apoptosis [23–25]. Our data shown in Fig. 7 indicate that rhIGF-I can protect isolated mononuclear cells from irradiation-induced apoptosis because the frequency of apoptosis decreased after rhIGF-I treatment. These observations suggest that rhIGF-I not only promotes the proliferation of hematopoietic stem and progenitor cells, but also protects mononuclear cells from apoptosis. Both pathways should contribute to the accelerated recovery of peripheral blood counts post irradiation, which is crucial for the survival of radiation victims.

It has been well elucidated that rhIGF-I can stimulate lymphopoiesis, erythropoiesis and granulopoiesis by colony formation or flow cytometry in vitro as described above [14–17]. In this study we further found that rhIGF-I could accelerate platelet recovery when it was administered post radiation exposure. The platelet counts dropped to a very low level after radiation injury and showed rapid recovery from Day 11 to Day 17 after treatment with rhIGF-I (Fig. 2), demonstrating that rhIGF-I has significant protective effects against platelet decrease after irradiation exposure. The increase in megakaryocyte numbers in bone marrow cavity after rhIGF-I administration also supports this notion as measured by histological analysis (Fig. 3). However, it is not clear how rhIGF-I works. We first hypothesized that rhIGF-I may increase the proliferative activity of megakaryocytes, therefore we cultured human megakaryocytic cell line MO7e in vitro and detected its proliferation response to rhIGF-I. However, our result showed that there were no stimulatory effects of rhIGF-I on the proliferation of megakaryocytes (Fig. 6), which indicates that rhIGF-I could not directly facilitate megakaryocytopoiesis. It has been previously demonstrated that rhIGF-I could enhance the secretion of hematopoietic cytokine IL-3 by an average of 5.9-fold from fibroblast-like (CD45−CD10+) stroma cells and sporadically increase the expression of other hematopoietic cytokines such as IL-6 and SCF [26]. It has been proven that these factors alone or their combination acts on different stages of megakaryocytopoiesis [27–30]. Based on these published data, we speculate that rhIGF-I treatment after irradiation promotes thrombopoiesis most likely through the secretion of thrombopoietic cytokines by other cell types such as stromal cells in the hematopoietic niches.

In conclusion, in this report we have demonstrated that rhIGF-I can mitigate against lethal irradiation in BALB/c mice by enhancement of overall hematopoietic recovery when it is administered post radiation exposure. In addition, we have also found rhIGF-I has beneficial effects on thrombopoiesis. Further studies are needed to evaluate the...
detailed mechanism for thrombopoiesis with the treatment of rhIGF-I following irradiation.

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