Article

Recombinant Human Prolactin Protects against Irradiation-Induced Myelosuppression

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Prolactin is a multifunctional hormone that exerts many separate functions and acts as an important connection between the endocrine and immune systems. There are increasing researches implicating the role of prolactin in hematopoiesis. Enhanced erythropoiesis in pregnant women and direct erythropoietic effects *in vitro* of plasma either from pregnant or lactating mice have been reported. Furthermore, regression of erythroblastic leukemia has been observed in a significant number of rats after hypophysectomy. In this study, the effects of recombinant human prolactin (rhPRL) on hematopoiesis were assessed in irradiated mice. Mice were treated with rhPRL for five consecutive days after exposure to a lethal dose or a sub-dose irradiation. Prolonged survival rate and increased erythropoiesis were observed in the irradiation-induced myelosuppressive mice. It was concluded that rhPRL might act on erythropoiesis and could be a potential candidate for the treatment of irradiation-induced myelosuppresion in clinic. *Cellular & Molecular Immunology*. 2005;2(5):379-385.

Introduction

Prolactin (PRL), a 200 amino acid peptide hormone in human, is one of a hormone family including growth hormone (GH) and placental lactogen (PL), which is linked to a still more extended family of proteins, referred to as hematopoietic cytokines (1). PRL is synthesized and secreted by the anterior pituitary to increase its amounts during pregnancy and suckling, and acts primarily on the mammary gland by initiating and maintaining lactation in the postpartal phase. The mRNA and protein of PRL have been identified at many other extra-pituitary sites including human deciduas, mammary tissue, myometrium, and regions of the brain. Extra-pituitary prolactin is also secreted by immune cells (2, 3) as well as detected in the bone marrow environment (4). The receptor for prolactin is a single membrane-bound protein that belongs to class I cytokine receptor superfamily

(5). Prolactin and growth hormone receptors share several structural and functional features despite their low (30%) sequence homology (6), which contain extracellular, transmembrane, and intracellular domains (7). In addition to the membrane-bound receptors, soluble prolactin-binding proteins have also been described in mammary epithelial cells (8) and milk (9). These soluble forms contain 206 N-terminal amino acids of the extracellular domain of the PRL receptor. The soluble PRL binding proteins are also products of the same PRL receptor gene, but it is still uncertain whether they are results of alternative splicing of the primary transcript or products of proteolytic cleavage of the mature receptors (10). PRL receptors are presented in a wide range of peripheral organs like the pituitary gland, skeletal muscle, skin uterus, heart, lung, spleen, liver, pancreas, kidney, and thymus (11). PRL receptors have also been described to be expressed on diverse bone marrow-derived human cell types including B cells, T cells, monocytes (12), NK cells and CD34^{+} human stem cells (13).

In conventional view, PRL is a mammal gland and milk production stimulator, but except for its anterior function, PRL has also been shown to have cytokine-like activities and to have important immunoregulatory activities and immuno-hematopoietic function. Recently, it has been described that some hyperprolactinemic patients develop autoimmune

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rheumatic diseases and some women develop natural autoantibodies during pregnancy probably due to the hyperprolactinemic state (14, 15). Other evidence has been found that PRL protects against development of type 1 diabetes induced by multiple injections of streptozotocin (STZ) in mice (16). Administration of rhPRL into HT-29 tumor-bearing SCID mice promotes the antitumor effects of adoptively transferred NK cells has been reported (17). At present, accumulating experimental data demonstrate the multiplicity of PRL actions and over 300 different functions of PRL have been reported and organized into categories related to water and electrolyte balance, growth and development, endocrinology and metabolism, brain and behavior, reproduction, and immunoregulation and protection, which highlights the importance of this pituitary hormone (18, 19).

Myelosuppression, a potential side effect after radiotherapy or chemotherapy that causes the bone marrow to slowly product white blood cells (WBC), red blood cells (RBC) and platelets (PLT), is a major limiting factor in the clinical treatment of cancers. Therefore, promotion of hematopoiesis remains an extremely important challenge in cancer therapy. A large number of drugs and substances of natural and chemical origin have been screened for evaluation of their radioprotective potential. The administration of cytokines including granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and stem cell factor (SCF), following exposure to sub-lethal doses of irradiation, typically enhances the survival of hematopoietic progenitor cells in the bone marrow and accelerates the recovery of peripheral blood cells (20, 21). Widespread expression of PRL receptors on hematopoietic and immune cells implies PRL also has a direct role in hematopoietic system development. Murphy et al. demonstrate that mice lack of PRL and other pituitary hormones exhibit immunologic and hematologic defects. Mice present with an atrophied thymus and peripheral lymphoid organs as well as a wide range of immunologic abnormalities including suppressed number of splenic hematologic progenitor cells (22). Pituitary transplantation or exogenous prolactin restores humoral and cellular immune deficiencies in hypophysectomized rats. Hypophysectomy combined with neutralization by anti-PRL antibody leads to severe anemia and death from hematological failure in rats (23). These studies provide the evidence that PRL administration could benefit the hemopoeisis. The purpose of our investigation is to study the effects of recombinant human PRL on erythropoeisis and aim to clarify whether rhPRL has possible utilization on irradiation-induced myelosuppression mice

Materials and Methods

Animals and reagent
C57BL/6 mice, female, aged 8 to 12 weeks, weighing 25 ± 2 g, were obtained from the Animal Production Area (National Institute of Experimental Animal, Beijing, China). All mice were kept under specific pathogen-free condition. Animal care, handling and experimental procedures were conducted in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals".

Recombinant human PRL (> 99% pure, by RP-HPLC) was provided by Dr. Williams J. Murphy from National Cancer Institute-Frederick, NIH, and the administration schedule of which has been verified both *in vitro* and *in vivo* in previous publications (17, 24).

Irradiation and treatment
The rhPRL (10 μg) was suspended in 0.2 ml PBS. Mice received *i.p.* injection of rhPRL for one time before or daily treatment for five consecutive days after irradiation, and control mice were received the same amount of PBS alone. In irradiation process，γ-ray sources had been charged with a cesium 137 (^{137}Cs) isotope producing a relatively softer γ irradiation. Use of cesium 137 yielded better results since, unlike cobalt 60, its half-life was longer (some 30 years), making frequent recharges unnecessary. Admittedly, the energy of β rays emitted by cesium 137 was lower (0.51 MeV). For radioprotection studies, mice were placed in a Lucite box and were given whole-body irradiation at a lethal dose (850 cGy total body irradiation from a ^{137}Cs irradiation source) under unanesthetized conditions and fresh air was continuously circulated in the irradiation chamber to avoid hypoxic conditions. To access the therapeutic effects of rhPRL on irradiation-induced myelosuppressive mice, the mice were exposed to a sub-lethal dose (500 cGy total body). The mice were divided into three groups as followed: 1) before exposure to a lethal dose irradiation, rhPRL or PBS was injected intraperitoneally (*i.p.*) to observe the protective roles of rhPRL; 2) after exposure to a lethal dose irradiation, rhPRL or PBS was injected *i.p.* to evaluate the therapeutic effects of rhPRL; 3) after exposure to a sub-lethal dose irradiation, rhPRL or PBS was injected *i.p.* to confirm the effects of rhPRL on hematopoiesis.

Hematologic examinations
Blood samples were collected from the tail veins of mice weekly for 6 weeks. Blood was transferred into EDTA-coated tubes (EDTA-Na$_2$ 1.5 mg/ml) quickly for ensuring adequate mixing of blood to prevent blood clot formation and immediately diluted in PBS. Peripheral WBC, RBC, PLT counts and hematocrit (HCT) were analyzed. Hematology was monitored using the Hematology Analyzer (Danam, USA).

Survival study
Irradiated mice were observed to see mortality for 25 days after exposure. Data was expressed as % survival. All data collected from repeated experiments were combined for analysis.

Statistical analysis
All experiments were performed more than three times. Data are presented as the mean ± SEM. Student's *t*-test was
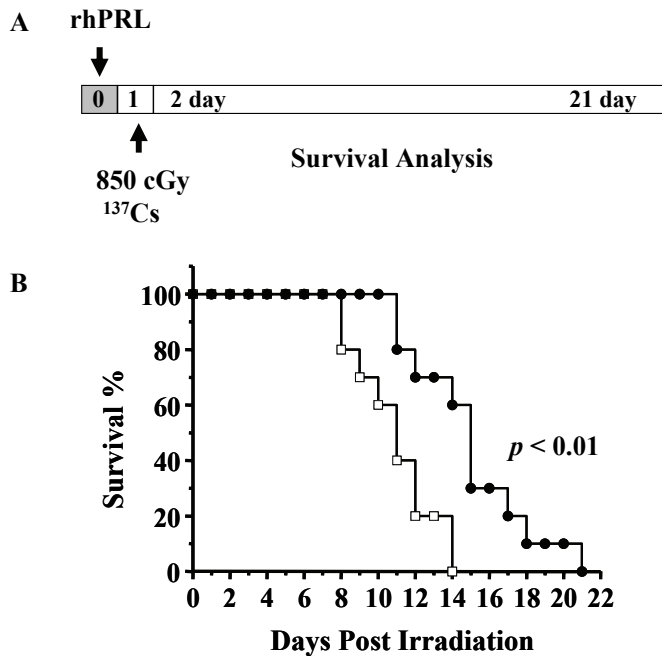
Figure 1. Recombinant human PRL protects mice from an exposure to lethal-dose irradiation. (A) Outline of the experimental protocol. Mice were *i.p.* injected with 10 μg rhPRL in 200 μl PBS or 200 μl PBS prior to irradiation, then a dose of 850 cGy radiation was given and survival was monitored daily for 21 days. (B) The percentage of surviving over time is shown (n = 10 mice in each group). Statistical analyisis revealed that rhPRL significantly improved the survival rate of the mice irradiated at 850 cGy, as assessed by the Gehan-Wilcoxon test (*p* < 0.01). [●] rhPRL treatment group; [□] PBS control group.

Figure 2. Recombinant human PRL exhibits therapeutic roles and improves the survival rate of mice from irradiation-induced death. (A) Outline of the experimental protocol. Mice were injected *i.p.* with 10 μg rhPRL in 200 μl PBS or 200 μl PBS after a single lethal-dose (850 cGy) irradiation for five consecutive days. Survival was monitored daily for 23 days. (B) The lines represent mice survival rates for 10 mice per treatment and control group. Statistical analyisis revealed that rhPRL significantly improved the survival rate of the mice as assessed by the Kaplan-Meier method (*p* < 0.01). [●] rhPRL treatment group; [□] PBS control group.

performed on data as appropriate to determine statistical significance of differences between experimental groups and control groups.

Kaplan-Meier Product-Limit method was used to estimate the cumulative probability of survival; Gehan-Wilcoxon test was applied to compare survival in different treatment groups.

Results

Pretreatment with rhPRL protects mice from lethal-dose irradiation

To investigate the radioprotective properties of rhPRL, a single dose of rhPRL (10 μg) injection was administered prior to lethal-dose irradiation (850 cGy). Control mice were received a single injection of PBS (group 1). In three independent experiments, all control mice died within 14 days after exposure to irradiation, while 60% of rhPRL treated mice survived for up to 21 days (Figure 1). Pretreatment with rhPRL significantly prolonged the survival period of irradiated-mice. The mean of survival time was increased from 10.9 days (control mice) to 14.9 days. These results indicated that pretreatment with 10 μg rhPRL was
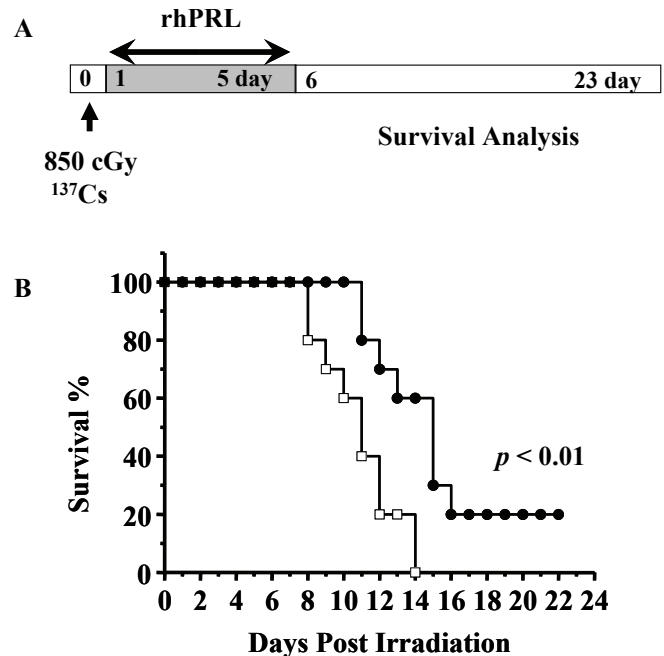
sufficient to protect mice from irradiation-induced lethality.

Treatment with rhPRL prolongs the survival rate of mice after exposure to lethal-dose irradiation

After exposure to lethal-dose irradiation, neutrophil, total mononuclear cell, platelet and red blood cell numbers were markedly decreased within several days, and mice will be dead soon. Because rhPRL had been reported to increase hematopoietic progenitor cells in normal C57BL/6 mice (24), we wanted to determine whether rhPRL acts as a potential therapeutic protein to improve the hematopoietic recovery. After exposure to a lethal-dose irradiation, mice were administrated with 10 μg rhPRL in 200 μl PBS or 200 μl PBS peritoneatally for five consecutive days. The 14-day survivals for rhPRL-treated mice (group 2) were 30%, whereas all the control mice died within this period. The mean of survival time for rhPRL treated mice was 15.2 days, whereas the mean of survival time for control mice was 10.9 days. Furthermore, there were 20% of rhPRL-treated mice survived for up to the end of observation period (Figure 2). These differences on survival rate were statistically significant (*p* < 0.01) based on analysis by Gehan-Wilcoxon test. The data demonstrated that mice treated with rhPRL survived for a significantly longer time compared with

A

B   **C**

Figure 3. Recombinant human PRL promotes the peripheral blood recovery of irradiation-induced myelosuppressive mice. (A) Outline of the experimental protocol. Mice were injected *i.p.* with 10 μg rhPRL in 200 μl PBS or 200 μl PBS after a sub-dose (500 cGy) irradiation for five consecutive days. Peripheral blood was analyzed for irradiation mice on days 7, 14, 21, 28 and 35 after irradiation. (B) Total erythroid cells (RBC) and (C) haematocrit (HCT). The data were shown as mean ± SEM. [●] rhPRL treatment group; [□] PBS control group.

PBS-treated mice.

The effect of rhPRL on irradiation-induced myelosuppression in mice

We were interested in the possible hematopoietic effects of rhPRL on sub-dose irradiated mice. In this experiment, mice were irradiated with a sub-lethal dose of 500 cGy γ-ray to produce severe myelosuppression. Four to six hours after the irradiation, mice were injected *i.p.* with 10 μg rhPRL or PBS daily from day 0 to day 4. Blood samples were collected from the tail veins of mice weekly. Hematologic examinations showed that irradiated mice exhibited rapid and continuous decreases in the WBC, RBC, and platelet counts with maximal reduction in peripheral blood cells. Recombinant human PRL significantly ameliorated the decrease of peripheral RBC and HCT percentage of irradiated myelosuppressive mice on days 14, 21 and 28. In the irradiated control mice, the total RBC count ($5.0 \pm 0.4 \times 10^{12}$/L, Figure 3B) and HCT percentage ($32.6 \pm 1.2\%$, Figure 3C) were decreased maximally at day 28, and similar observations were obtained for the WBC count, platelet and haemoglobin content (data not shown). However, in irradiated mice treated with rhPRL (group 3), the values of haematological components were decreased more slowly compared with the irradiated control mice. Whereas the total RBC count ($7.5 \pm 0.1 \times 10^{12}$/L), and HCT percentage ($43.0 \pm 2.1\%$) were decreased maximally at day 28. The difference were statistically significant between rhPRL treated mice and control mice at day 14 ($p < 0.05$), day 21 ($p < 0.01$) and day 28 ($p < 0.01$). Administration of 10 μg rhPRL also restored peripheral WBC and platelet counts of irradiation-induced myelosuppressive mice to some extent, but there was no

statistic difference when compared to the control (data not shown).

Discussion

Hematopoiesis is controlled by a broad range of polypeptide ligands which signal through a structurally related set of transmembrane receptors to regulate the lineage-specific development of progenitor cells (25). These molecules induce specific signaling pathways to promote viability, proliferation and differentiation of hematopoietic cells. The receptors for PRL belong to the member of the class 1 cytokine receptor superfamily, which have been grouped by related structure, including IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, G-CSF, M-CSF, GH, and erythropoietin. PRL receptors are able to substitute for the erythropoietin receptor (EPOR) in erythroid development (26, 27).

The effects displayed by PRL are clearly associated to the receptor activation. Signal transduction by this receptor is mediated, at least in part, by two families of signaling molecules: Janus tyrosine kinases (JAK) and signal transducers and activators of transcription (STATs) (28). The initial step of PRL action is the binding to the receptor of PRL. Ligand binding leads to dimerization of the receptor and activation of JAK2, which in turn phosphorylates the PRLR and the transcription factor, STAT-5. Because the JAK2/STAT-5 signaling pathway is shared by both PRL and IL-3 receptors (10, 29), STAT-5 tyrosine phosphorylation was similarly induced by PRL and IL-3. It was demonstrated that IL-3 promotes myeloid cell development by inducing inhibitor of DNA-binding protein 1 in hemopoietic progenitor cells (30, 31).

Chemotherapy- or irradiation-induced myelosuppression results in apoptosis of cycling hematopoietic cells and induces regression of bone marrow hematopoietic stem cells and hematopoietic progenitor cells. Moreover, timely regeneration of bone marrow hematopoietic cells is essential for reconstitution of hematopoiesis. With regard to the role of PRL in myelopoiesis, it has been shown that about 80% of bone marrow cells, which include all hematopoietic lineage precursors, express high levels of PRL receptors. Enhanced erythropoiesis in pregnant women and direct erythropoietic effect *in vitro* of plasma from pregnant and lactating mice have been observed (32, 33). Conversely, regression of ery-throblastic leukemia has been observed in a significant number of rats after hypophysectomy. Furthermore, *in vivo*, the potential function of PRL to reverse the anemia and myelosuppression induced by azidothymidine (AZT) has been examined (24). These results suggest that PRL may act on myeloid progenitors in resting, normal animals and those that have undergone myelosuppressive. Following sub-lethal irradiation, hematopoiesis experiences the transient ablation of nearly all hematopoietic progenitor cells then recovers after a long period.

Previous studies have shown that cytokine-induced radioprotection is dependent on both the cytokine dose and the administration schedule. Cytokine treatment given after irradiation exposure typically enhances hematopoietic recovery following sub-lethal doses of irradiation but has relatively modest effects on recipient survival after bone marrow lethal doses of irradiation. In our experiments, a significant increase in the survival rate is observed both in irradiated mice followed by pretreatment rhPRL and treatment rhPRL after a lethal dose irradiation. Respectively, the mean of survival time ranges from 10.9 days of control group to 14.9 days (rhPRL pretreatment group) (Figure 1) and to 15.2 days (rhPRL treatment group). In rhPRL treatment group, 20% of mice survive for a long-term after a lethal dose irradiation (Figure 2). A remarkable feature of rhPRL-induced radioprotection is the high degree of protection of the erythroid cell lineage (Figure 3). RBC count levels are only modestly decreased compared to control mice. Taken together, these findings indicate that rhPRL can up-regulate erythropoiesis *in vivo* as it does *in vitro*.

In the irradiation-induced myelosuppressive mice studied here, the administration of rhPRL prevents the rapid decline in the peripheral RBC counts and HCT percentage. rhPRL treatment given after irradiation exposure enhances hematopoietic recovery following sub-lethal doses of irradiation and has distinct effects on recipient survival after bone marrow lethal doses of irradiation.

For the minimal toxicity of systemic rhPRL administration, rhPRL may be an attractive agent to reverse myelosuppresion induced by irradiation, chemotherapy or other ablative therapies. Cytokines including GM-CSF, IL-1 and EPO have been observed to be contributed to the restoration of hematopoietic function following bone marrow transplantation (34). IL-1 administration improves platelet recovery after high-dose carboplatin therapy, but it also has significant toxicities (35). Except for systemic toxicity,

rhPRL has been shown to improve T cell and natural killer cell function (36, 37). Clinical use of rhPRL after cancer radiotherapy or chemotherapy may not only contribute to the hematopoietic recovery but also offer other benefits including improved immune response.

Several decades ago, the survival-promoting effects of lipopolysacharides (LPS) in irradiated animals were described by Ainsworth EJ et al., who provided the new sight for considerations in radioprotection (38, 39). It is well demonstrated that LPS elicit an inflammatory response by inducing cytokine productions, such as IL-1, IL-6, TNF, IFNs, and transforming growth factor, all shown to have some radioprotective activity of their own. Based on these effects, Neta and Oppenheim (40) postulated that an inflammatory response would promote the removal of damaged tissues and assist in recovery from irradiation. Later, it was postulated that growth factors induce or enhance repair mechanisms and, thus, may protect stem cells from an irradiation injury (41, 42). PRL receptors are expressed on T cells, B cells, NK cells, neutrophils, monocytes, macrophages and thymic epithelial cells (43, 44). PRL is also implicated in lymphoproliferation, antibody secretion and cytokine pro-duction (45, 46). As an important regulator of cytokine production, both Th1 cytokine IFN-γ and Th2 cytokine IL-6 are up-regulated by PRL. In addition to the direct effects on $CD34^+$ stem cells and progenitor cells (13), these evidence suggests that PRL protecting mice from challenge by irradiation may be responsible for its modulate functions of the immune system.

In general, the recovery of the blood cell number in irradiated animals depends on the survival of stem cells and their derivatives. The results from the present study suggest that the rhPRL has a radioprotective role in stimulating/ protecting the haematopoietic system. Hence, enhanced survival and an increase in the haematological constituents of peripheral blood of mice against lethal γ irradiation are observed.

Our data illustrate the radioprotective effects of rhPRL and extend information from previous studies concerning the potential of cytokine therapy in the treatment and prevention of irradiation injury. The clinical goal is to design optimized radioprotective methods to allow high-dose irradiation for cancer patients without the need for stem cell rescue. The results of this study open the way for further analyses of radioprotective effects and potential clinical agent of rhPRL.

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