In Vivo Efficacy of Recombinant Leukotactin-1 against Cyclophosphamide

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Abstract Leukotactin-1 (Lkn-1), a human CC chemokine, has been demonstrated to induce chemotaxis of neutrophils, monocytes, eosinophils and lymphocytes and has been shown to suppress colony formation of hematopoietic stem and progenitor cells (HSPC) *in vitro* and *in vivo*. The temporal suppression of HSPC by chemokines could potentially be applicable for various indications, such as the protection of HSPC from the several anti-proliferating chemotherapeutics in cancer treatments. In order to evaluate the protective effects on myeloid progenitor cells, the recombinant Lkn-1 was produced by *Pichia pastoris* and tested with cyclophosphamide, cytotoxic chemotherapeutics. The pretreatment of Lkn-1 increased the number of HSPC in bone marrow as well as the potency of resulting progenitor cells after the treatment of cyclophosphamide. After the first cycle of cyclophosphamide treatment these protections of HSPC correlated with the increased number of white blood cells and neutrophils in the peripheral blood. In lethal conditions created by the repeated administration of cyclophosphamide, the treatment of Lkn-1 enhanced the survival of mice, suggesting the potential use of Lkn-1 as the protective agent for HSPC from various cytotoxic insults.

Keywords: leukotactin-1, hematopoietic stem cell, progenitor cell, cyclophosphamide

INTRODUCTION

Chemokines are a family of chemotactic cytokines of 8-to 17-kDa with 20~70% percent homology in amino acid sequences [1]. These chemotactic cytokines can be divided into 4 groups (CXC, CC, CX3C, and C) according to the position of the first 2 closely paired and highly conserved cysteine residues [2,3]. From a functional viewpoint, chemokines may be classified with the inflammatory chemokines involved in the recruitment of leukocytes to inflammatory sites and also classified with the constitutive chemokines responsible for the trafficking and homing of leukocyte populations [4,5]. In addition to their primary role of recruiting leukocytes, chemokines have been shown to be involved in an increasing range of other functions, including the control of hematopoiesis, angiogenesis, oncogenesis, and HIV infection [6-9]. Lkn-1 belongs to the subfamily of CC chemokine which contains the conserved 6 cystein residues with 3 disulfide bonds [10]. Lkn-1 induces chemotaxis and calcium influx in human neutrophils, monocytes, eosinophils, and lymphocytes through binding to its receptors: CCR1 and CCR3 [10, 11].

In chemotherapy, hematological toxicity is one of the major complications resulting from anemia, neutropenia, and thrombocytopenia caused by the depletion of proliferating progenitor cells due to the use of cytotoxic drugs. In hematopoiesis, twenty-five chemokines have been demonstrated to possess the suppressive activity on the proliferation of myeloid progenitor cells [6]. The myelosuppressive activity of chemokines has been proposed to be applied in reducing the hematological toxicity of cell cycle active chemotherapy [12]. Among the various chemokines, MIP-α1 has been demonstrated to possess the protective activities on the myeloid progenitor cells showing an increased number of Colony Forming Units of Macrophage (CFU-M), Granulocyte (CFU-G), Granulocyte/Macrophage (CFU-GM) by placing normal stem and progenitors into a slowly cycling state against the specific cell cycle inhibitor of Ara-C and hydroxyurea [13-15]. Likewise, several chemokines, including Lkn-1, appear to suppress the progenitor cells in a transient manner and thus, protect against the cytotoxic agents, even though the distribution of the corresponding receptors may differ from each other. Nevertheless, the degree of myeloprotection seems to depend on the type of cytotoxic drugs. In case of the MIP-α1, the protection of the progenitor cells against the treatment of cyclophosphamide (Cy) was not evident as in the case of 5-FU and Ara-C because it showed only the temporal increment of the monocytes from the hematotoxicity [12]. Cy is known to block the multi-stage of the cell cycle in proliferating cells and is

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frequently used for several cancer chemotherapies [16]. In this study, we investigate the efficacy of the recombinant Lkn-1 on the bone marrow hematopoietic stem and progenitor cells against the treatment of Cy. We also evaluate the relative effects on the WBC and neutrophils in the peripheral blood.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice, aged 6~8 weeks, were purchased from the Jackson Laboratories and housed in our institute's animal facility.

Preparation of Recombinant Lkn-1 and Its Administration

The recombinant Lkn-1 was constructed to delete the 26 amino acids from the N-terminus of the intact form and was produced by methylotropic yeast, *Pichia pastoris*. The secreted rLkn-1 was purified from the cation exchange chromatography and reverse phase chromatography. The purified proteins were verified by reverse isoelectric focusing (IEF) using reverse phase HPLC and also by N-terminal sequencing using an automatic peptide sequencer (Procise, Perkin-Elmer, USA). The rLkn-1 was administered by a subcutaneous injection (8 μg/mouse) prior to the treatment of Cy.

In vivo Treatment of Cyclophosphamide

Cy powder (Sigma, USA) was dissolved in PBS, and a dose of 350 mg/kg was injected intraperitoneally (i.p.). Two different schemes for the Cy administration were used. To induce moderate effects, Cy was administered, once a week for 3 weeks, and to induce lethal effects, Cy was administered twice a week (day 0 and day 4). The peripheral blood and bone marrow progenitor cells were analyzed from the mice that were treated with Cy in moderate condition. In the lethal scheme, the gross appearance and body weight were analyzed until the recovery of body weight reached the normal level.

Stem/progenitor Cell Separation

To isolate hematopoietic stem and progenitor cells, negative selection was performed using the StemSep™ system according to the instructions of the manufacturer (StemCell Technologies, Canada). Briefly, total bone marrow cells were resuspended in PBS with 5% rat serum, incubated at 4°C for 15 min and then incubated for 15 min with a cocktail of antibodies: CD5 (Ly-1), CD45R (B220), CD11b (Mac-1), myeloid differentiation antigen (Gr-1), and erythroid cells (TER119). Antibody-bound cells were separated through centrifugation at 1,300 rpm for 10 min in a clinical centrifuge. The cells were washed twice and resuspended in PBS, and then, anti-biotin tetrameric antibody complexes were added. After incubating at 4°C for 15 min, a magnetic colloid was added and incubated for 15 min at 4°C. The antibody bound mature cells were removed using a magnetic particle concentrator (StemCell Technologies, Canada) resulting in a lineage-negative stem/progenitor cells.

Colony Forming Unit (CFU) -Assay

To estimate the effects of Lkn-1 on bone marrow progenitor cells, the colony forming units of progenitor cells from the Cy-treated animals were identified using the total bone marrow aspirate and the isolated HSPCs. Mouse bone marrow cells were obtained by aspiration from the femurs and were washed with Iscove's modified Dulbecco's medium. After total number of bone marrow cells was counted, 5×10^4 cells/mL were plated with the same volume of methycellulose medium containing growth factors such as rhEPO, rmGM-CSF, and rmSCF (R&D systems, USA). After incubating for 7 days at 37°C in an atmosphere of 5% CO_2, the individual colonies were counted through the inverted microscopy. For the counting of colony forming units from the purified stem cells, the cells were plated as 1,000 cells/mL with the same medium and counted after 7 days.

Peripheral Blood Analysis

The mouse blood was obtained by eyebleeding under anesthetic conditions and immediately placed in EDTA-treated CBC bottles. The whole blood cells counts were performed using the CBC test (MASCOT Automated Hematology System, CDC Technologies, USA). Mean values were obtained from five separate mice. Statistical comparison between treated and untreated controls was made using a paired t-test, and P values were stated where appropriate.

RESULTS AND DISCUSSION

High-dose chemotherapy is needed to eliminate the cancer cells but often causes side effects such as hair loss, nausea, vomiting, sterility and cytopenia. Usually, nonspecific chemotherapeutic agents present problems because they modulate general functions such as the proliferation present special problem. Normal hematopoietic cells in the bone marrow are particularly sensitive to many chemotherapeutic agents although this problem can be resolved by removing the bone marrow and returning it after the treatment is finished (transplantation). Alternatively, hematopoietic stem and progenitor cells of patients can be protected by chemotherapeutic adjuvant during chemotherapy to place them into a less proliferating stage to minimize the destruction of hematopoietic cells in the bone marrow and elsewhere. Most of the chemotherapeutic agents act at specific phases (phase-specific agents) of the cell cycle whereas Cy, one of the most used chemotherapeutic agents, functions at several phases and is referred to as cycle-specific [16].

The Lkn-1 has been demonstrated to suppress the pro-

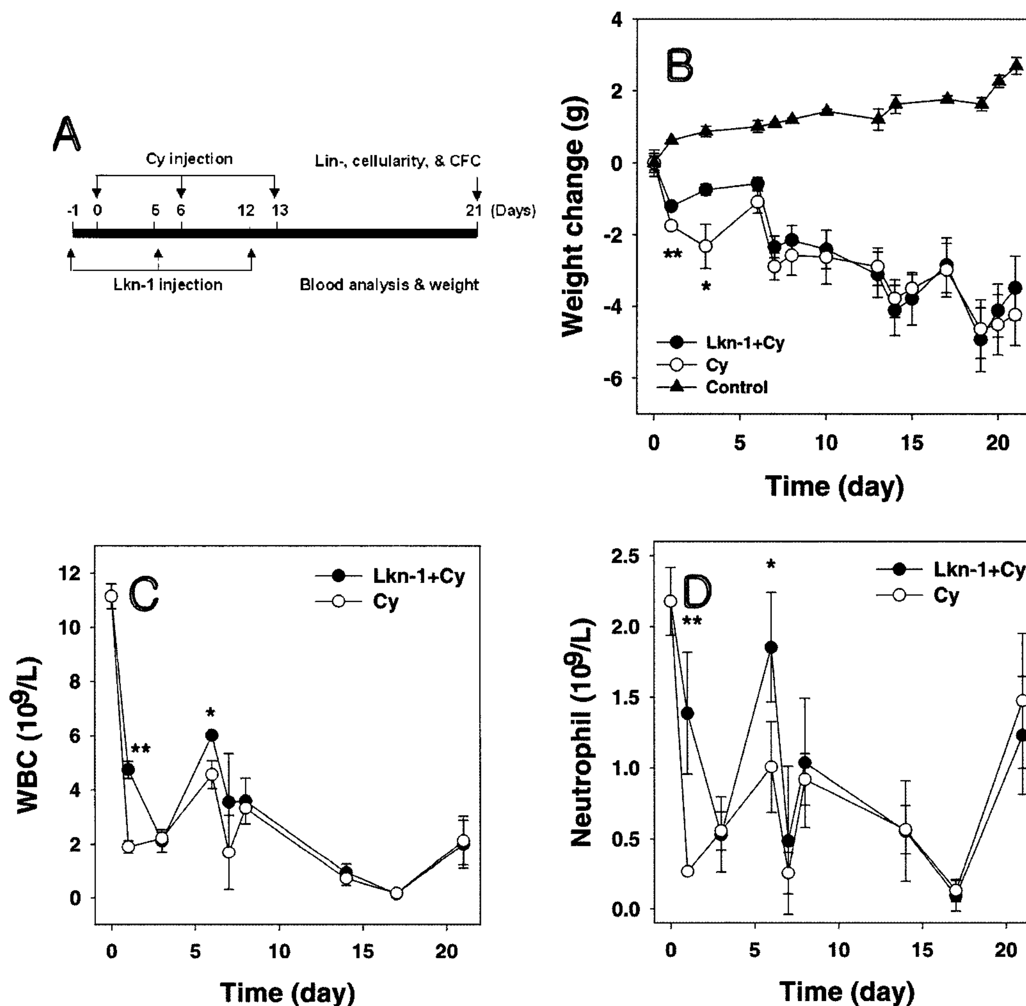

Fig. 1. Effects of Lkn-1 on mouse condition over a period of 21 days: Treatment scheme (A), Weight change (B), WBC in peripheral blood (normal WBC average number: 10.7 × 10^9/L) (C), and neutrophil (normal neutrophil average number: 1.4 × 10^9/L) (D). Cy (350 mg/kg body weight) was given intraperitoneally on day 0, 6, and 13, while Lkn-1 (400 μg/kg body weight) was administered subcutaneously 24 h before the Cy injection in the group receiving both of these drugs. There were 5 animals in the group receiving Cy alone or Cy plus Lkn-1. STD at each mean value is shown by the error bar. (** p = 0.01, *p = 0.05)

liferation of HSPC in reversible fashion, which in turn, protects the myeloid progenitor cells against the cytotoxic effects from the cell cycle specific drugs such as 5-FU and Ara-C [17]. But the translation of the protection of the progenitor cells into *in vivo* therapy has been difficult due to the variations of *in vivo* response against the different cytotoxic chemotherapeutics. In order to evaluate the beneficial effects of Lkn-1 *in vivo*, we examined the HSPC, peripheral blood, and the recovery of mice during the course of Cy treatment. With the Cy treatment performed once a week (Fig. 1A), the cytotoxiciy seems to be moderate without showing any lethality. Lkn-1 was treated prior to the Cy administration because the post treatment of Lkn-1 showed negative effects presumably due to the myelosuppressive activity (data not shown). The body weights from both Lkn-1 pre-treated and Cy alone groups were decreased immediately after the first day and started to increase after the final treatment of Cy (Fig 1B). The degree of weight loss and recovery showed the difference between the Lkn-1 treated and the control mice during the initial period (day 2~6), which is a clinically important stage for the recovery from chemotherapy. We also examined the peripheral blood and found that the pattern of the decrease in myeloid cells (WBC and neutrophil) was similar to that of the body weight (Fig. 1C and D). Especially on day 6, the recovery of WBC and neutrophil reached the normal level in the case of Lkn-1 treated mice. Since the decrease of myeloid cells after chemotherapy is one of the major complications leading to anemia, neutropenia and thrombocytopenia, the pro tective effects shown in the early period (Fig. 1B) suggest to be applicable in clinical settings.

Although the body weight and the myeloid cells did not show any significant differences at day 21, we examined

Table 1. Effect of Lkn-1 on hematopoictic cells in mice treated with Cy

	Cellularity 10^6cells/femurs	Lin- cell 10^5cells/femurs	CFC 10^4cells/femurs
Lkn-1 + Cy	9.8±2.4	6.6	1.23±0.23
Cy	10.1±3.2	3.7	0.99±0.05
Normal	19.2±1.3	5.1	4.12±0.76

the number and the potentials of the HSPC in order to measure the reserved repertoire of progenitor cells in the bone marrow. When we analyzed the bone marrow aspirates, we did not find differences in the number of total nucleated cells from each group (Table 1). From the nucleated bone marrow cells, the lineage negative cells (Lin-) representing HSPC were separated using antibody cocktails for the mature myeloid cells. In the case of the Lkn-1 treated mouse, the number of Lin- cells was 1.8 times higher than that of the control. The number of colony-forming cells (CFC) representing HSPC was also higher in Lkn-1-pretreated mice by 1.3-folds suggesting the presence of protective effects of Lkn-1 on HSPC in the mouse bone marrow. HSPCs are a heterogeneous population with multiple potentials to differentiate into several lineages of the mature blood cells. Although the myeloid suppressive chemokines are similar in their action through the chemokine receptors (CCR), the distribution and the level of CCRs in the individual progenitor cells are different, and thus, the myeloprotective effects of the chemokine could vary [18]. Gilmore *et al.* reported that the MIP-1α analog, BB-10010 treatment did not significantly alter the total white cell count or the number of circulating lymphocytes in Cy-treated mice, but enhanced the recovery of myeloid lineages, both neutrophils and monocytes [12]. The treatment of MIP-1α showed no detectable effects on the number of nucleated cells from the bone marrow as well as CFU from Cy-treated mice, while those effects were evident in case of Ara-C treatment [19]. The basis of the different spectrum of myeloid protective chemokines on diverse cytotoxic drugs has to be further elucidated in order to be applied clinically.

The degree of cytotoxicity by Cy varies depending on the dosing schedules, and the degree of protection by Lkn-1 could show various aspects. Instead of the moderate cytotoxic conditions by once a week administration of Cy, the treatment of Cy with short intervals of double treatment on day 0 and day 4 induced the lethality of mice. Using this condition, we tested the effects of Lkn-1 on the survival of mice (Fig. 2). Until 10-day from the second treatment of Cy, the survival rate was similar between the two groups with a continuous decrease in body weight. While the lethality of the control group was continued until day 25, the Lkn-1 treated group showed no additional lethality (Fig. 2, closed circle) as shown with the remaining mice reaching full recovery status to normal conditions. In addition, the appearance of the Lkn-1 treated mice reached the normal condition faster than the mice of the control group. The precise basis for the difference in lethality is not clear, but could be related to the

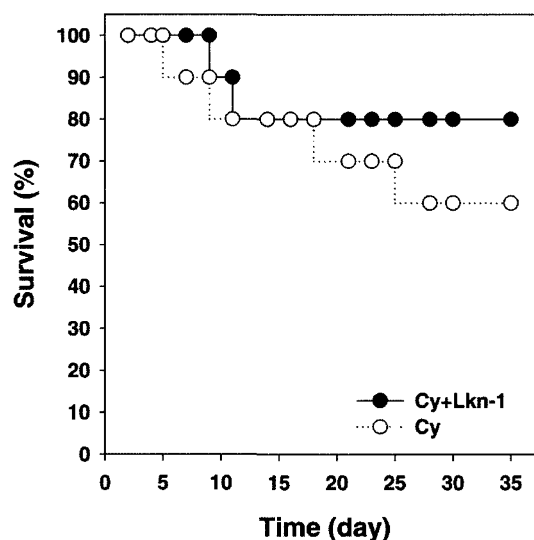

Fig. 2. Effect of Lkn-1 on the survival of Cy-treated mice One group (10 mice) was injected with cyclophosphamide on day 0 and 4, and the other group (10 mice) was treated with cyclophosphamide and injected with Lkn-1 on day -2, -1, 2 and 3. Until the recovery of the mouse's normal weight, the survival rate was observed.

protection of HSPCs in mouse bone marrow.

High-dose chemotherapy is unavoidable to enhance the survival rate of cancer patients, but it accompanies the potential complications for the increased cytotoxicity, especially on the hematopoietic cells. The protection of HSPCs by adjuvant, such as Lkn-1, may be proved to be of significant clinical benefit. Since chemokines, including Lkn-1, have a diverse spectrum of action, the basis of myelo-protective effects might be complicated. Recently, Lkn-1 has been reported to induce the transient expression of TNF-α, IL-8, and MCP-1 from the THP-1 cells [20], and IL-8 and MCP-1 are known to be myelosuppressive chemokines [6]. Therefore, the detailed studies on the action mechanism and the elucidation of the target cell will be of importance in order to develop the myeloprotective activity of Lkn-1 as a useful tool for chemotherapy.

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