Scorpion Venom Peptides Accelerate Hematopoietic Recovery of Myelosuppression in Irradiated Mice

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Abstract: Sublethally irradiated mice were administered with scorpion venom peptides (SVP) or with PBS in the saline control group, 3 days before and 7 consecutive days after irradiation. Hematopoietic recovery was assessed by bone marrow (BM) cell proliferation index (PI) and colony forming unit-granulocyte/macrophage (CFU-GM), spleen weight index (SI) and thymus weight index (TI), colony-forming unit-spleen (CFU-S) and peripheral leukocyte counts. In addition, IL-1α and SCF levels in BM, IL-6 and GM-CSF levels in serum were determined. In SVP treated groups, PI was improved dramatically versus control mice on day 22 after irradiation. The number of CFU-GM colonies in all SVP treated groups was higher than the control groups. The difference of the number of CFU-GM colonies between SVPV group (0.2 mg/kg) and the control was significant on day 5 and 10 after irradiation (p < 0.05). SVPIV (0.2 mg/kg) could activate the CFU-S formation on day 10 after irradiation. SI was in peak value on day 15 after irradiation in all groups and the SI value of SVPV treated group was higher than control group (p < 0.05). Our results suggest that SVP may be valuable natural peptides that relieve myelosuppression caused by radiation. The effect of SVP accelerating the hematopoietic recovery was potentially through a mechanism of stimulating the release of cytokines.

Keywords: Scorpion Venom; Myelosuppression; Hematopoietic Cytokines; Radiation.

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Introduction

Intensive radiotherapy and chemotherapy are widely used for advanced malignant tumors and neoplastic hematologic disorders. One of the major side-effects of radiotherapy is myelosuppression (Chen et al., 2007). The main strategies for improving therapeutic efficacy for these malignant disorders include hematopoiesis and immune reconstitution, which can be achieved by stimulating the proliferation and differentiation of preserved hematopoietic stem and progenitor cells (HSPC). The proliferation and differentiation of HSPC are influenced by hematopoietic growth factors (Herodin and Drouet, 2005). This is the rationale of using these growth factors for accelerating the recovery of hematopoiesis, especially by using cytokines immediately and continuously after irradiation (Drouet et al., 2004). But continuous use of cytokine combinations can cause proinflammatory and immunological response and other side effects (Li et al., 2001; Negrin, 1998).

Scorpion venom peptides (SVP) were separated and purified from scorpion Buthus Martti Karsch. The inhibiting effects of scorpion venom on tumors and its effects on antagonizing radiation injury and immune regulation have been widely investigated (Mamelak and Jacoby 2007). The growth of human tumor cell lines and animal transplanting tumors was evidently inhibited by SVP (Kong, 2004). Besides its anti-tumor effect, SVP can also stimulate the hematopoietic recovery after radiotherapy (Kong et al., 2000). After administrating SVP combined with irradiation, H22-tumor weights were decreased, and WBC count and spleen index (SI) were higher than those of the irradiated group. Interestingly, it has been found that scorpion venom could stimulate the release of cytokines. Tityus serrulatus venom (TSV) induced marked elevations of interleukin (IL)-1α, IL-1β, IL-6, IL-10 and interferon gamma (IFN-gamma) (Petricevich and Peña, 2002). TNF-α, IL-1β, IL-6 and IL-8 levels were significantly increased in individuals stung by Tityus serrulatus (Ts) scorpions (Fukuhara et al., 2003). The results herein provide evidence that SVP can promote hematopoietic recovery and elevate the cytokines release in mice after sublethal irradiation. SVP elevated the levels of cytokines SCF, IL-1α, IL-6 and GM-CSF in bone marrow (BM) and in serum, as well as increased the proliferating index (PI) and the colony-forming unit numbers of bone marrow cells in radiation-induced myelosuppression mice. It is suggested that SVP can be considered as a substituent for hematopoietic cytokine combinations to accelerate hematopoietic recovery after irradiation.

Methods and Materials

Animals

Congenic male BALB/c mice, 4–6 weeks old (weighing 16–20 g), were purchased from Guangdong Provincial Center of Laboratory Animals (Guanzhou, China). Mice were bred and maintained in our animal facilities for a minimum of 5 days prior to use. After irradiation, the mice were divided into the control group (saline) and treated groups (SVP). In each group, 5 mice were picked out randomly for testing after irradiation. Unirradiated mice were used as normal controls.
Irradiation Procedure

Whole body of mice was irradiated with 600cGy in a lucite chamber. This dose of X-rays is sublethal irradiation from a 6 M high-energy X-ray linear accelerator at a dose rate of 150 cGy/min.

SVP Treatment

SVP was separated from the venom of Buthus martensii Karsch (BMK) scorpion by gel filtration. SVPIV and SVPV were isolated from SVP by using ion exchange methods. HPLC and SDS PAGE were used for their purity analysis. SVPIV and SVPV were shown a single peak (purity surpassed by 96%) by HPLC and a single band with SDS PAGE. The molecular mass of the SVPIV was 6.35 KDa as determined by SDS PAGE, and SVPV was 6.14 KDa.

SVPIV and SVPV were diluted with sterile saline and filtered through a 0.22 μm filter before intraperitoneal (IP) injection. Mice were injected with SVPIV or SVPV once a day in two doses of 0.2 mg/kg and 0.4 mg/kg, respectively. It was given to mice everyday 3 days before irradiation and 7 consecutive days after irradiation. Negative control animals were injected with an equal volume of sterile saline in the same way.

Spleen Colony-Forming Unit (CFU-S) Assays and Lymphatic Organ Weight Index

Mice were killed by decapitation. Their thymuses and spleens were removed and weighed for lymphatic organ weight index calculation. The spleens were fixed in Bouin’s solution and the spleen colonies formed by the surviving endogenous hematopoietic cells (spleen colony-forming unit, CFU-S) were enumerated.

Granulocyte-Macrophage Colony-Forming Units (CFU-GM) Assay

Mice bone marrow cells were cultured for CFU-GM evaluation in 0.6% agar culture (40% L-DMEM, 20% equinum serum) by adding 30 ng/ml GM-CSF (ebioscience, San Diego, CA, USA) as a source of colony stimulation. Cultures were incubated at 37°C in a humidified atmosphere with 5% CO2. Colonies were scored at day 14. The average number of colonies and standard errors were calculated from triplicate.

Peripheral Blood Leukocyte Analysis

Leukocytes were counted in the blood collected from the tail of mice. Leukocyte analyses were performed every other day starting before irradiation and continuing for 30 days after irradiation.

Bone Marrow Cell Proliferation Index (PI)

The bone marrow cells were drawn by flushing mice femoral bones with PBS buffer and fixed by 75% cold alcohol for 48 hours. Cells were washed twice with PBS, suspended in
PBS buffer and preincubated with propidium iodide (PI) for 30 min at 4°C in dark. Cell cycle was detected by flow cytometry and PI was calculated.

**Cytokines Assay**

Secretion of IL-1α and SCF in BM was measured by using immunohistochemistry in tissue-array method. 80 samples were examined and scored based on the staining intensity. IL-6 and GM-CSF levels in blood serum were determined by enzyme linked immunosorbent assay (ELISA; eBioscience, San Diego, CA, American) following the protocol. The mixture of the serum of five mice in each group was used as samples in ELISA test. The data were obtained from duplicate determinations.

**Statistical Analysis**

All data were processed and plotted with the SPSS 11.0 software and the means were reported ± standard error to the mean. Significance levels: $p < 0.05$ (*compared to saline control group; #compared to the normal control group).

**Results**

**CFU-S and Spleen and Thymus Weight Index**

The numbers of CFU-S were counted in each group from day 1 to day 20 after irradiation every five days. There was almost no colony formed until day 10 after irradiation. On day 15 after irradiation, the spleens were apparently larger and heavier than earlier measurements and the colonies were hard to be discerned. The results of the colony enumeration on day 10 after irradiation showed that treatment with SVPIV (0.2 mg/kg) could activate the CFU-S formation compared to the control group ($p < 0.05$, Fig. 1).

The values of spleen weight index (SI) and thymus weight index (TI) dropped after irradiation and then both began to increase gradually (Table 1). SI was in peak on day 15 after irradiation; it was higher in SVPV treated groups than in the saline group at this time point ($p < 0.05$). TI was also higher in all SVP treated groups than in the saline control group on day 15 after irradiation, however, it is not significant.

**CFU-GM Numbers and the Peripheral Blood Leukocyte Counts**

CFU-GM in mice sublethally irradiated alone or treated with SVP was examined (Fig. 2). The number of CFU-GM colonies was counted on day 5, 10, 15 and 20 after irradiation. There was a decreasing tendency of CFU-GM numbers after irradiation and the lowest point was reached on day 15 after irradiation. The numbers of CFU-GM colonies in all SVP treated groups were higher than the control group. It was significant on day 5 and day 10 after irradiation between SVPV treated group (0.2 mg/kg) and the control group ($p < 0.05$).
Figure 1. Influence of SVP on CFU-S after 6.0 Gy X-ray irradiation. It shows the CFU-S colonies enumeration, in saline, SVPIV, and SVPV treated groups in different doses on day 10 after irradiation. CFU-S numbers shown are means ± SD of five mice per group. *p < 0.05, with respect to saline values.

Table 1. Influence of SVP on Spleen Weight Index (SI) and Thymus Weight Index (TI) at 6.0 Gy X-Ray Irradiated BALB/c Mice

<table>
<thead>
<tr>
<th>Groups</th>
<th>SI (%)</th>
<th>TI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 5</td>
</tr>
<tr>
<td>Saline</td>
<td>0.13 ± 0.02</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>IV 0.4 mg/kg</td>
<td>0.10 ± 0.03</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>IV 0.2 mg/kg</td>
<td>0.19 ± 0.09*</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>V 0.4 mg/kg</td>
<td>0.14 ± 0.02</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>V 0.2 mg/kg</td>
<td>0.10 ± 0.01</td>
<td>0.12 ± 0.04</td>
</tr>
</tbody>
</table>

Leukocyte counts in the peripheral blood declined immediately after irradiation, followed by an increase on day 20 after irradiation and later. Leukocyte numbers in all SVP treated groups displayed the same trend, but there is no significant difference among SVP treated groups and the control group in the examined 30 days (data not shown).

**PI of Bone Marrow Cells**

The influence of SVP on PI of bone marrow cells on day 22 after irradiation is shown in Table 2. PI was calculated on the forum \((S + G2/M)/(G0/G1 + S + G2/M)\) with the
Figure 2. Influence of SVP on CFU-GM after X-ray irradiation. The number of CFU-GM colonies in saline and SVP treated groups on day 5, 10, 15 and 20 after irradiation is shown. Colonies consisting of at least 50 cells were scored. Data shown in means; bars, SD. *p < 0.05, with respect to saline values.

Table 2. Influence of SVP on PI of Bone Marrow Cells at Day 22 after 6.0 Gy X-Ray Irradiation

<table>
<thead>
<tr>
<th>Groups</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>77.37 ± 5.64</td>
<td>18.17 ± 4.69</td>
<td>4.5 ± 0.96</td>
<td>22.67 ± 5.65</td>
</tr>
<tr>
<td>IV 0.2 mg/kg</td>
<td>64.27 ± 3.19</td>
<td>28.47 ± 1.91</td>
<td>7.23 ± 1.78</td>
<td>35.7 ± 3.25*</td>
</tr>
<tr>
<td>IV 0.4 mg/kg</td>
<td>71.23 ± 4.58</td>
<td>23.17 ± 3.51</td>
<td>5.6 ± 1.64</td>
<td>28.76 ± 4.58</td>
</tr>
<tr>
<td>IV 0.2 mg/kg</td>
<td>71.77 ± 2.80</td>
<td>23.2 ± 2.30</td>
<td>4.97 ± 0.47</td>
<td>28.17 ± 2.75</td>
</tr>
</tbody>
</table>

*p < 0.05, compared to saline values.

percentage of bone marrow cells in each cell cycle. Although PI in all SVP treated groups was higher than that in the control group, PI was only significantly increased in SVPIV treated group (0.2 mg/kg, p < 0.05).

Cytokine Levels in BM and Blood Serum

SCF and IL-1α levels in mice BM on day 2, 5, 8 and 13 after irradiation are shown in Table 3 and Fig. 3. Compared to the normal control, SCF and IL-1α levels in BM increased on day 2 after irradiation in all irradiated groups and sustained for several days (p < 0.05). In SVPIV treatment groups, these two cytokines were almost at the same high levels as negative control group at the early 5 days of post-irradiation, while both were apparently higher than the negative control on day 8 after irradiation (p < 0.05).

The levels of IL-6 and GM-CSF in the blood serum on day 1, 5, 10 and 15 after irradiation were detected by using an ELISA, which showed a different expression pattern from the tissue microarray assay results of SCF and IL-1α in BM. IL-6 and GM-CSF were decreased right after irradiation and increased several days later. In SVP treated group (0.4 mg/kg),
Table 3. Effect of SVPIV on Bone Marrow SCF and IL-1α Levels at 6.0Gy X-Ray Irradiated BALB/c Mice (Immunohistochemistry, Positive Unit Value)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before Irradiation</th>
<th>SCF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>Normal control</td>
<td>22.0 ± 2.81</td>
<td>—</td>
</tr>
<tr>
<td>Saline</td>
<td>—</td>
<td>24.0 ± 6.56</td>
</tr>
<tr>
<td>SVPIV 0.4 mg/kg</td>
<td>—</td>
<td>32.6 ± 3.82*#</td>
</tr>
<tr>
<td>Normal control</td>
<td>24.9 ± 3.89</td>
<td>—</td>
</tr>
<tr>
<td>Saline</td>
<td>—</td>
<td>40.2 ± 3.39*#</td>
</tr>
<tr>
<td>SVPIV 0.4 mg/kg</td>
<td>—</td>
<td>39.6 ± 6.89*#</td>
</tr>
</tbody>
</table>

*p < 0.05, compared to saline values. #p < 0.05, compared to values obtained from mice not receiving irradiation (normal control).

Figure 3. Effect of SVP on bone marrow SCF and IL-1α levels in irradiated BALB/c mice on day 5 after irradiation. Bone marrow tissues from normal control, saline control and SVPIV 0.4 mg/kg treated mice were shown. (A) HE stains. (B) Immunohistochemistry of SCF. (C) Immunohistochemistry of IL-1α (×400).
Figure 4. Effect of SVP on IL-6 and GM-CSF levels in blood serum of irradiated BALB/c mice on day 1, 5, 10 and 15 after irradiation. The pool of the serum of five mice in each group was used in ELISA test. The data were obtained from duplicate determinations.

IL-6 and GM-CSF levels on day 5 and day 10 after irradiation were respectively higher than the control (Fig. 4).

Discussion

Radiation-induced myelosuppression is a severe complication that limits the use of radiotherapy in malignant tumor treatment. Hematopoietic and immune reconstitution can be achieved by stimulating the proliferation and differentiation of preserved hematopoietic stem and progenitor cells (HSPC). In this study, we explored the possibility that administration of scorpion venom peptide (SVP) would accelerate the hematopoiesis recovery in mice which were given a sublethal dose of X-rays. It was shown that SVP can significantly enhance hematopoietic function in sublethally irradiated mice. SVP treatment groups had
more hematopoietic cells than the control group. In SVPIV treatment group, primitive cell
count (CFU-S) was increased markedly on day 10 after irradiation and mature (CFU-GM)
progenitor cells were higher from day 5 to 20 after irradiation. Although there were no sig-
nificant differences of absolute neutrophil counts (ANC) in the peripheral blood from 8 to 10
post-irradiation days, there was a higher level of neutrophil progenitors in the bone marrow
of SVP treated mice. It was also shown that SVP enhanced the bone marrow cell cycling by
accelerating the cells into S phase. In SVPIV 0.2 mg/kg treated group, PI of bone marrow
cells was significantly increased, strongly suggesting that after irradiation, these cells would
repopulate the bone marrow and that hematopoietic recovery would follow. In conclusion,
it is reasonable to hypothesize that administration of SVP pre- and post-irradiation may
accelerate the hematopoietic recovery of bone marrow by protecting HSPC from radiation-
induced injury, as well as by stimulating its proliferation after irradiation.

Numerous studies (Thierry et al., 1995) have shown the efficacy of hematopoietic growth
factors (HGFs) in stimulating hematopoietic recovery after myeloablation. IL-1 seems to be
a pivotal hematopoietic cytokine in the therapy for radiation injury (Neta and Oppenheim,
1988). SCF is potent hematopoietic growth factor acting on both early stem cells and the
ones already differentiated in the bone marrow (Zsebo et al., 1990). IL-6 plays a primary role
in hematopoiesis as it regulates the differentiation of B cells, megakaryocytes and platelets
(Kishimoto et al., 1994). The prolonged administration of GM-CSF or G-CSF is the criterion
standard of cytokine therapy for irradiated victims in spite of their granulocyte-restricted
activity (Gale and Butturini, 1990). The hypothesis was suggested that the protection of mice
from radiation-induced hematopoietic death is mediated by cytokines and chemokines (Singh
et al., 2006). Serum cytokines were measured by using a sandwich ELISA, Luminex, and
cytokine array in lethal gamma irradiated mice treated with various drugs which administered
subcutaneously 24 hours before irradiation and found that these drugs could stimulate the
release of IL-6, GM-CSF and other cytokines and chemokines in irradiation treated mice.
A similar finding was also emerged from our study that SVP elevated the levels of cytokines
(SCF, IL-1α in BM and IL-6, GM-CSF in serum) in irradiated mice. In our study, on day 5,
10 and 15 after irradiation, IL-1α and SCF levels in BM and IL-6 and GM-CSF levels in
serum were increased by administration of SVP. Together with the effect of SVP to promote
bone marrow cell proliferation which emerged on day 5 after irradiation or even later, it is
therefore presumed that SVP may accelerate the hematopoietic recovery by stimulating the
release of cytokines.

Many studies have demonstrated that cytokine as a substitution in the neutropenic period
after radiotherapy has a protective effect from irradiation, and that the optimal proliferation
and differentiation of HSPC require the co-stimulation by two or more growth factors. It has
been demonstrated that the co-administration of multilineage cytokines during the treatment
of radiation-induced myelosuppression was very helpful (Frasca et al., 2000). The survival
rate of 10 Gy γ ray irradiated mice was markedly higher in the combination of IL-11 and
TPO treatment group than IL-11 or TPO treated group only (Van der Meeren et al., 2002).
In another report, B6D2F1 mice were treated with recombinant murine cytokines soon after
total body irradiation, and all treatments induced 30-day survival rates significantly higher
than control, 4F (SFT3) and 5F(4F SDF-1) being the most efficient combinations, which was
better than 3F, TPO and SDF-1 alone (Thierry et al., 1995). However, repeated and prolonged co-administration of cytokines to radiotherapy patients has been reported to induce adverse effects such as proinflammatory and immunogenic activity (Li et al., 2001; Negrin, 1998; Karlin et al., 2005). SVP, fortunately, could both antagonize radiation injury and present immunoregulating effects at the sametime (Mamelak and Jacoby, 2007). Earlier in 1986, it was found that the fragment of scorpion venom can improve the survival rate of LD98/30 γ ray irradiated mice to 65% (Vernigorova and Lebedev, 1986). SVP can elevate the cytokine levels in serum and bone marrow in irradiated mice, as well as stimulate the proliferation of their bone marrow cells. The accelerating effects of SVP on hematopoietic recovery suggest that SVP can be considered a substituent for hematopoietic cytokine combinations to accelerate hematopoietic recovery after irradiation.

In order to avoid the adverse effects of cytokine treatment, single administration, short-term administration and early administration of cytokines after irradiation were widely studied. Single administration of thrombopoietin 2 hours after TBI proved to be significantly more efficacious than treatment at 24 hours (Neelis et al., 1998). Herodin et al. (2003) studied the capacity of antiapoptotic cytokines to rescue mice from radiation when it was given as an early treatment to counteract cell death, especially in potential lethal lesions, as a combination to ensure multilineage protection, and as a short-term treatment to avert adverse effects. They found that the multiple cytokine combinations given 2 hours after TBI and the short-term treatment is efficient. The regimens of our study, injecting SVP each day starting 3 days before irradiation until 7 consecutive days after irradiation determined in our previous experiments, were proven efficient and had no adverse effects on mice. It might be a potential therapeutic regimen against radiosensitive tumors. However, the exact mechanism of how SVP accelerate the proliferation of hemopoietic cells and elevate cytokine levels in bone marrow is still to be explored.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

References

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