OK-432 Reduces Mortality and Bacterial Translocation in Irradiated and Granulocyte-colony Stimulating Factor (G-CSF)-treated Mice

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Acute radiation induces bacterial translocation from the gut, followed by systemic infection and sepsis. In order to reduce the mortality after acute whole body irradiation, it is essential to control bacterial translocation. In this study, we established a bacterial translocation assay as a sensitive method to detect minor mucosal injury by radiation. By utilizing this assay, we evaluated the adverse effects, if any, of hematopoietic reagents on the mucosal integrity in the respiratory and gastro-intestinal tracts. Bacterial translocation to the liver and spleen occurred after whole-body irradiation if the dose exceeded 6 Gy. The administration of G-CSF unexpectedly increased the bacterial translocation in 8 Gy-irradiated mice. The pharmaceutical preparation of low-virulent Streptococcus pyogenes, OK-432, significantly reduced the endotoxin levels in peripheral blood without any reduction of bacterial translocation. A combined treatment with G-CSF and OK-432 decreased bacterial translocation and prevented death. This result indicates that the early administration of G-CSF has an adverse effect on bacterial translocation, and that a combined treatment of G-CSF and OK-432 attenuates the adverse effect of G-CSF and improves the survival rate after acute irradiation.

INTRODUCTION

High-dose irradiation induces bone-marrow failure and gastrointestinal injury. Recent advances in the treatment of bone-marrow failure in irradiated victims have raised the survival
rates into the range of radiation dose where the consequences of gastrointestinal injury limit survival. To reduce gastrointestinal injury, growth hormones or cytokines have been used in experimental animals\(^1\)–\(^7\). The aim of such a treatment is to facilitate the turnover rate of epithelial cells in the gastrointestinal tract. Another strategy for treating gastrointestinal injury is to stimulate hematopoiesis and lymphopoiesis\(^9\). It is well known that granulocytes and lymphocytes play important roles in the manifestation of radiation-induced gastrointestinal syndrome\(^8,9\).

Pro-inflammatory and hematopoietic cytokines are useful for treating radiation-induced bone-marrow failure\(^10\). However, cytokines generally have pleiotropic effects on not only hematopoietic precursors, but also other somatic cells\(^11\). Moreover, they induce other cytokines through the cytokine network\(^11\). Therefore, a cytokine that stimulates hematopoiesis may have unexpected effects on the gastrointestinal tract\(^12,13\). Cytokines in the immune system can be classified into three groups: pro-inflammatory\(^11\), type-1 and type-2 cytokines\(^14\). The pro-inflammatory and type-1 cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF)-\(\alpha\), IL-12 and interferon (IFN)-\(\gamma\), mediate inflammation and increase tissue injury\(^12,13,15,16\). In contrast, type-2 cytokines, such as IL-4 and IL-10, decrease inflammation\(^17\). In radiation-induced gastrointestinal syndrome, the integrity of the intestinal mucosa is lost\(^18\) and bacterial translocation occurs. Bacteria and serum endotoxin induce pro-inflammatory cytokines and cause regional and systemic inflammation. It was recently demonstrated that IL-12 rather promoted gastrointestinal injury by inducing IFN-\(\gamma\) in irradiated mice\(^13\), while IL-12 alone stimulated hematopoiesis\(^13\). These findings prompted us to investigate combined therapy with a hematopoietic cytokine and an inducer of type-2 cytokine in irradiated mice.

In a previous study\(^19\), we demonstrated that the pharmaceutical preparation of low-virulent \textit{Streptococcus pyogenes}, OK-432, prevented endotoxin shock in BDF1 mice. The mechanism of endotoxin-resistance was partly explained by IL-10, induced in macrophages by OK-432\(^19\). In the present study, we investigated the effect of G-CSF and OK-432 on bone-marrow failure and gastrointestinal injury. Although G-CSF facilitated the recovery of bone-marrow cells, it increased bacterial translocation through the mucous membrane. OK-432 in combination with G-CSF reduced both bacterial translocation and the levels of serum endotoxin in irradiated mice, and prevented death after 8 Gy-irradiation.

**MATERIALS AND METHODS**

**Mice and Treatment**

Male BDF1 mice were purchased from Japan SLC, Shizuoka, Japan, and were kept in the conventional animal facility at the National Institute of Radiological Sciences, Chiba, Japan. Ten- to eleven-week-old mice were used for the experiments. In the bacterial translocation experiment, mice were given autoclaved food and acidified water supplemented with gentamycin sulfate (40 \(\mu\)g/ml) and tetracycline hydrochloride (110 \(\mu\)g/ml) from day –7 to day 0, in order to decrease the bacterial flora in the gut. The prior administration of antibiotics was essential, since without this maneuver all mice developed bacterial translocation after irradia-
tion, which could not be controlled by the treatment schedule described below. On day 0, mice received whole-body irradiation of indicated dose at a dose rate of 74 cGy/min using an X-ray irradiator (PANTAK HF-320 (Shimazu, Tokyo, Japan)) at 200 kV, 20 mA with 0.6 mm Cu and 0.1 mm Al filters. After irradiation, the mice were given conventional food and acidified water without prophylactic antibiotics to regenerate bacterial flora. With prophylactic antibiotics, LD50/30 of whole-body irradiation in BDF1 mice was 7.28 ± 0.18 Gy (unpublished observation).

After irradiation, mice received 1 µg of recombinant human granulocyte-colony stimulating factor (rhG-CSF, 1 × 10^8 U/mg, Chugai Pharmaceutical Co., and Tokyo, Japan), twice a day i.p. for 3 consecutive days, as has been demonstrated to be more effective than a once-a-day schedule in a previous study. After irradiation, on the indicated day, mice received 5 Klinische Einheit (KE, i.e., clinical unit; one KE was equivalent to 0.1 mg of dried S. pyogenes) OK-432 (Picibanil®, Chugai Pharmaceutical Co.) i.p. As a control, physiological saline was injected twice a day for 3 consecutive days instead of G-CSF.

All experiments followed the guidelines of the Institutional Committee for Animal Experiments.

**Bacterial translocation assay**

The liver was homogenized in 5 ml of physiological saline. The liver homogenate was diluted tenfold in autoclaved Trypto-Soya Broth (Nissui Pharmaceutical Co., Tokyo, Japan), and 100 µl of each diluted sample was cultured overnight in a 96 well-type round-bottomed culture plate at 37°C. To calculate the number of bacteria in the liver, the last dilution number (N) of the homogenate where bacterial growth was observed was used in the following formula:

\[(\text{Number of bacteria in the liver}) = 5 \times 10^{(2+N)}\]

Positive infection in the liver was arbitrarily judged when the liver contained more than 5 × 10^3 bacteria. The infection rate in the liver was determined by the following formula:

\[\% \text{Infection in the liver} = 100 \times \frac{(\text{Number of mice bearing infected liver})}{(\text{Number of mice in the experiment})}\]

**Measurement of Endotoxin**

Cardiac puncture was performed under anesthesia with diethyl ether. The serum was frozen and stored at −20°C. The level of serum endotoxin was measured with a micro-plate kit using the Lumilus test (Seikagaku Corp., Tokyo, Japan).

**Histology**

The duodenum, jejunum, ileum and colon were fixed with 10% formalin and embedded in a paraffin block. Ten µm-tissue sections were cut and stained with a hematoxylin-eosin solution.

**Statistical analysis**

Data were analyzed either by the Cox-Mantel test, Student-t test or χ² analysis, as indicated.
RESULTS

Bacterial translocation assay is a sensitive method to detect minor mucous membrane injury after irradiation

Without a prophylactic treatment with antibiotics, 73.4% of BDF1 mice died by 30 days after 8 Gy-irradiation (Fig. 1). A dose of 8 Gy, however, did not denude the intestinal mucosa (data not shown), while bacterial translocation occurred to the liver (Fig. 2) and spleen (data not shown) after irradiation. The minimum radiation dose that caused bacterial translocation was 6 Gy, and almost half of the mice developed liver infection on the 8th day after 8 Gy-irradiation (Fig. 2). The rate of liver infection and the number of bacteria in the liver increased with time, and 100% of 8 Gy-irradiated mice developed liver infection on the 14th day (Fig. 3). Thus, bacterial translocation assay is sufficiently sensitive to detect minor mucous injury where bacterial invasion is threatening after irradiation, e.g., in the gastrointestinal or respiratory tract.

Combined treatment with G-CSF and OK-432 increases the survival rate of mice after 8 Gy-irradiation

The treatment of mice with G-CSF for three consecutive days slightly improved the sur-
CONTROL OF BACTERIAL TRANSLOCATION BY OK-432

Fig. 2. Dose response of bacterial translocation after whole-body irradiation.
In order to investigate the bacterial translocation from intestinal flora, mice injected with physiological saline were bred without antibiotics after whole-body irradiation. Bacterial translocation into the liver was investigated on the 8th day after irradiation. Bacterial translocation parallelly occurred into the spleen (data not shown).

Fig. 3. Bacterial translocation starts to occur 6 days after 8 Gy irradiation.
Infection rate (A) and the number of bacteria in the liver (B) were investigated. The numbers in parenthesis represent the number of mice used.

vival rate (Fig. 1). The number of peripheral leukocytes decreased by 80% on the next day after 8 Gy-irradiation. G-CSF marginally increased the leukocyte number on the first and second days, but could not prevent a further decrease of leukocytes by more than 90% on the third day. The leukocyte numbers in peripheral blood became too low to count, even in mice treated with G-CSF or G-CSF and OK-432 on the 7th day. The administration of OK-432 alone on the third day did not significantly improve the survival rate (Fig. 1). On the contrary, a combined treatment with G-CSF with OK-432 on the 3rd or 4th day significantly improved
the survival rate after 8 Gy-irradiation. Delayed administration of OK-432 on the 5th or 6th day failed to increase the survival rate. Thus, an irreversible event seemed to occur between the 4th and 5th days. As shown in Figure 3, the extrapolated line of the %infection in the liver indicated the infection began at around this period.

**G-CSF increases bacterial translocation, which is suppressed by OK-432**

As mice began to die on day 10, we investigated bacterial translocation 8 days after irradiation in a following experiment (Fig. 4). Without any treatment, 7 of 13 mice developed liver infection, and the average bacterial number in the liver was $5 \times 10^4$. Surprisingly, G-CSF increased liver infection; 12 of 13 mice had liver infection in G-CSF treated mice (Fig. 4B). Also, the number of bacteria in liver significantly increased in G-CSF treated mice ($p<0.05$) (Fig. 4A). When G-CSF was administered for a prolonged period, i.e., 5 consecutive days, it did not improve the liver infection; 8 of 8 irradiated mice developed liver infection. Thus, G-CSF unexpectedly increased the bacterial translocation. This was not due to a rapid recovery of bacterial flora in the gut by G-CSF (data not shown). The numbers of bacteria in the liver and the rate of liver infection were almost the same in OK-432-treated and saline-injected mice (Fig. 4). In contrast, a combined treatment with G-CSF and OK-432 significantly decreased the infection rate ($p<0.01$) and the number of bacteria in the liver ($p<0.001$) (Fig. 4). This protective effect of OK-432 did not associated with the early recovery of intestinal mucosa (data not shown).

Finally, we investigated whether the bacterial translocation was accompanied by endotoxemia after irradiation. The levels of serum endotoxin increased from the 6th day after

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**Fig. 4.** G-CSF increases and OK-432 decreases bacterial translocation after irradiation.

BDF1 mice were irradiated and treated as described in Figure 1. The number of bacteria in the liver of each mouse (A) and the rate of bacterial translocation (B) were determined 8 days after irradiation. A G-CSF treatment for 3 consecutive days significantly increased the number of bacteria in the liver (v.s. saline: $p < 0.05$ by Student-\(t\) test). The combined treatment with G-CSF and OK-432 significantly improved both the number of bacteria (v.s. G-CSF: $p < 0.001$) and the infection rate (v.s. G-CSF 3 days and 5 days:**$p < 0.01$, v.s. saline 5 days: *$p < 0.05$ by \(\chi^2\) analysis). The number of mice used in the experiment were 13 for saline 3 days, 8 for saline 5 days, 13 for G-CSF 3 days, 8 for G-CSF 5 days, 10 for saline + OK-432, and 12 for G-CSF + OK-432.
CONTROL OF BACTERIAL TRANSLOCATION BY OK-432

irradiation (Fig. 5). Concomitantly with bacterial translocation data in Figure 4, G-CSF increased the endotoxin levels in peripheral blood (Fig. 5). Interestingly, the administration of OK-432 alone decreased the levels of endotoxin. Moreover, the combination of OK-432 and G-CSF significantly decreased the levels of endotoxin if compared with those in G-CSF treated mice. Thus, the radioprotective effect of OK-432 is in part due to the attenuation of any adverse effect of G-CSF by suppressing the endotoxin production and bacterial translocation after irradiation.

DISCUSSION

In the present study, we detected radiation-induced minor injury on the mucous membrane by a bacterial translocation assay. Although 8 Gy-irradiation transiently decreased the height of the intestinal micro-villi (data not shown), bacterial translocation occurred in irradiated mice. G-CSF facilitated the recovery of hematopoiesis in bone marrow, but increased both the rate of liver infection and the number of bacteria in the liver. The combined treatment with G-CSF and OK-432 suppressed bacterial translocation, decreased the levels of serum endotoxin, and prevented death after 8 Gy-irradiation. The present study demonstrated the therapeutic effectiveness of a combined treatment with G-CSF and OK-432 in high-dose irradiated mice.
Murine intestinal epithelial cells are regenerated from stem cells in the crypt every 3–4 days, and the D₀ value of the crypt cells is about 0.25 Gy and the total cellularity of the crypt decreased by 44% after 8 Gy-irradiation. Thus, the dose which we delivered was too low to denude the intestinal mucosa. The intestinal permeability is regulated through loosening the tight junction between epithelial cells by zonulin-dependent protein kinase C activation. Radiation alone loosens the tight junction and facilitates bacterial translocation (A. Fasano, personal communication). In the present study, although 8 Gy-irradiation only transiently decreased the thickness of the intestinal mucosa, bacterial translocation increased and the levels of serum endotoxin increased in 8 Gy-irradiated mice. Therefore, these indicators are useful to detect minor injury in the mucosal membrane after irradiation.

Pro-inflammatory cytokine, such as IL-1, TNF-α, and IL-12, and type-1 cytokine IFN-γ promote inflammation and tissue damage after septic infection. In contrast, type-2 cytokine, such as IL-4 and IL-10, decrease the activity of pro-inflammatory cytokines. IL-10 and other regulatory cytokine, transforming growth factor (TGF)-β, suppress the cytokine cascade in the animal model of endotoxin shock. Both IL-10 and TGF-β are abundantly produced in the gut, and may control inflammation of the intestinal mucosa. Therefore, it is reasonable to assume that combined therapy with a hematopoietic cytokine and biological response modifier (BRM) capable of inducing IL-10 is beneficial for radiation-induced gastrointestinal injury.

G-CSF preferentially stimulates growth and differentiation of the neutrophil precursor. G-CSF also activates the neutrophil function, i.e., phagocytosis and respiratory burst, and is clinically used for treating of acute infectious disease in non-neutropenic patients. It was therefore unexpected that G-CSF would enhance bacterial translocation in irradiated mice. Since it is thought that neutrophil damages host tissue by producing reactive oxygen species, the interaction of G-CSF-activated neutrophil with radiation-damaged cells must be investigated in future studies.

OK-432 has been clinically applied in cancer patients in Japan for more than 20 years. It is also currently under clinical trial in the United States. OK-432 induces several pro-inflammatory and hematopoietic cytokines. In addition, it induces a regulatory cytokine, IL-10, and prevents endotoxin shock in BDF1 mice. Since endotoxin produced by Gram-negative bacteria in the gut is a major factor initiating inflammation of the mucous membrane, we speculate that OK-432 suppresses inflammation caused by serum endotoxin and controls bacterial translocation. It is also plausible that OK-432 augments the phagocytosis of macrophage, or may induce cytokines capable of controlling the protein kinase c activity in the mucous membrane. These issues are beyond our scope in the present study and will be investigated in future studies.

In summary, acute radiation syndrome could be modified after irradiation. Although G-CSF enhanced bacterial translocation, a combinatory treatment with G-CSF and OK-432 suppressed bacterial translocation, reduced plasma endotoxin levels, and improved the survival rate after acute irradiation.
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REFERENCES