The Effect of OK-432 upon Erythropoietic Recovery in Sub-lethally Irradiated Mice: A preliminary report

HISAMASA JOSHIMA¹, HIROSHI OHARA² AND YOSHIRO AOKI³

¹Training School, National Institute of Radiological Sciences, Chiba 263 Japan
²Biological Department, College of Arts and Sciences, Okayama University, Okayama 700 Japan
³Department of radiological Health, Faculty of Medicine, University of Tokyo, Tokyo 113 Japan

(Received March 4, 1992)
(Revision received July 23, 1992, Accepted August 24, 1992)

OK-432, a multicytokine inducer and clinically used as an immunopotentiating anti-cancer agent, is known to induce IL-1 and TNF-α. The suppressive effect of IL-1 and TNF-α on erythropoiesis could limit the clinical use of OK-432 in cancer treatment, especially when combined with radiotherapy. In this study, the effect of OK-432 on normal and X-ray impaired erythropoiesis was examined. C57BL/6J mice were injected with a single dose of OK-432 (5.0 KE). Erythropoietic activity was measured by ⁵⁹Fe incorporation into circulating erythrocytes and the heme iron fraction of erythropoietic tissue.

When irradiated with 662 cGy of X-rays, OK-432 prolonged the survival of mice. No significant change in erythropoiesis was observed when normal mice were treated with OK-432. When treated with OK-432, the recovery of erythropoiesis after irradiation was promoted as judged by the uptake of ⁵⁹Fe into erythrocytes. This promotion was observed when OK-432 was injected within 1 day before or within 3 hours after the irradiation with 284 cGy of X-rays. This promoting effect, however, appeared to be limited to the spleen. Whether the combination of OK-432 with radiotherapy has the potential to improve the treatment of malignant tumors is still a subject of controversy. The present results, nevertheless, suggest that when combined with radiotherapy, OK-432, at the very least, may have no adverse effects on erythropoiesis.

INTRODUCTION

When a potentially lethal or sub-lethal dose of radiation is given to humans, by radiation accident or radiotherapy, bone marrow failure such as granulocytopenia and thrombocytopenia is one of the major problems. In such cases, infection and bleeding are common and may be fatal¹,2). OK-432, a multicytokine inducer³⁻⁵) and immunopotentiating anti-cancer agent⁶⁻⁸), prevents radiation-induced bone marrow death of mice⁹,¹⁰). The ability of OK-432 to protect
mice from radiation induced-granulocytopenia and thrombocytopenia has led to trials using OK-432 in the human radiotherapy of medullo-blastoma, pinealoma and pons glioma patients.\textsuperscript{11}

On the other hand, OK-432 has been reported to have a biological activity to induce IL-1\textsuperscript{12} and TNF-\alpha\textsuperscript{13,14}. Recent studies have demonstrated that IL-1 and TNF-\alpha inhibit erythropoiesis in vivo and in vitro\textsuperscript{15–18}. Because of the prolonged life span of adult erythrocytes, changes in the cellular hemoglobin level after exposure to radiation are slow to take place. However, erythroid precursor cells are somewhat more sensitive to radiation than those of the granuloid system\textsuperscript{19}, and erythropoietic impairment following radiation was demonstrated by as little as 5 rads\textsuperscript{20}. In some instances, due to stem cell depletion, the erythropoietic damage induced by radiation may be irreversible, or it may take a long time before recovery takes place, and picture of anemia may develop during this period\textsuperscript{21,22,23}. Anemia reaches its maximum severity at about the 30th day, when platelets and granulocytes return toward normal values. Furthermore, because of decreases in platelets and resulting bleeding, red blood cell transfusions are sometimes given to exposed patients to maintain their hemoglobin level\textsuperscript{24}. However, in some patients, the cause of death was due to diffuse haemorrhage\textsuperscript{25}. The prognosis for life of exposed persons may depend, in part, on whether decreased erythropoiesis is involved. Therefore, the fact that OK-432 induces IL-1 and TNF-\alpha could limit its clinical use as a therapeutic agent in some circumstances, especially when combined with radiotherapy.

In order to apply OK-432 to heavily irradiated people as a radioprotective agent, investigations including toxicological studies will be required\textsuperscript{10}. The present study was conducted to investigate whether treatment with OK-432 could have adverse consequences on erythropoiesis when it is combined with irradiation. We limited our measurement to the uptake of \textsuperscript{59}Fe into circulating erythrocytes, heme and nonheme iron fractions of the femoral marrow, spleen and liver, simply because these are very simple but highly sensitive and reproducible measurements of late stage erythropoiesis\textsuperscript{24–26}.

**MATERIALS AND METHODS**

**Animals**

Male C57BL/6J mice, 8–12 weeks old at the time of irradiation, were housed five per cage at 24±1°C and fed a standard cubed diet and acidified water ad libitum. All groups were matched for age and body weight and each experimental group consisted of eight or more mice. The present study was conducted according to the Guidelines for Animals Welfare and Experimentation issued by the National Institute of Radiological Sciences.

**Irradiation**

Whole body X-irradiation was delivered to mice with a therapeutic X-ray machine (Shimadzu Seisakusho Co. Ltd. Kyoto), operated at 200 kVp, 20 mA, with a dose rate of 75 cGy per minute at a skin target distance of 60 cm; HVL was 1.2 mmCu.
Injection of OK-432

OK-432, a lyophilized preparation of attenuated strain Su of Streptococcus haemolyticus manufactured by Chugai Pharmaceutical Co. Ltd, Tokyo, was diluted with saline immediately before use. The cell content of OK-432 was expressed as a Klinische Einheit (KE), i.e. 1 KE is equivalent to 0.1 mg of dried cells. When mice were given OK-432, all mice were intraperitoneally injected with a single dose of 5.0 KE, the same dosage previously used\(^9\). An equal volume of saline was injected to control mice.

Study of survival.

Mice were exposed to whole-body irradiation with 662 cGy (700 R) or 756 cGy (800 R) of X-rays. The mice were divided into three groups of 20 animals each. Of these, two treatment groups received an intraperitoneal injection of OK-432 1 hour before (pre-treated group) or 1 hour after (post-treated group) irradiation, while the remaining group serving as control received an equal volume of saline in the same manner. Mortality was examined daily for 60 days after irradiation.

Erythropoiesis.

Changes in erythropoiesis were examined by measuring \(^{59}\)Fe into circulating erythrocytes and erythropoietic tissue. Twenty-four hours after the injection of 37 kBq of \(^{59}\)Fe-citrate (Amersham, Japan), the rate at which \(^{59}\)Fe was incorporated into circulating erythrocytes was determined by the method described by Hodgson\(^{27}\). The activity in the erythrocytes was calculated as a percentage of the injected activity. After exsanguination, one femur, spleen and liver were excised and the uptake of \(^{59}\)Fe into the heme and nonheme iron fractions was measured by the method of Gresham\(^{24}\). In brief, tissues were homogenized in Drabkin’s solution. The homogenate was taken to pH 2 with 2 N HCl to separate heme from the hemoglobin molecules, and an equal volume of methyl ethyl ketone was added. Heme being extremely soluble in this solvent appeared in the organic phase, whereas nonheme iron remained in the aqueous phase. When investigating the effect of OK-432 on normal erythropoiesis, normal mice were injected with a single dose of OK-432 at various times before or after the injection of \(^{59}\)Fe. Twenty-four hours after the injection of \(^{59}\)Fe, the rate of erythropoiesis was measured. When investigating the effect of OK-432 on X-ray impaired erythropoiesis, OK-432 was injected 1 hour before or 1 hour after irradiation with 378 cGy (400 R) of X-rays. Changes in erythropoiesis were measured on the 1st, 3rd, 5th, 7th, 14th and 20th day after irradiation. In order to determine the time period at which OK-432 is effective, the mice were injected with OK-432 at various intervals before or after irradiation with 284 cGy (300 R).

The student’s t-test was used to assess the level of significance between the experimental groups.

RESULTS

The radiation dose of 756 cGy used in this experiment killed all mice within 26 days, and no
significant changes in survival rate were observed between the OK-432 treated groups and untreated group. However, when irradiated with 662 cGy of X-rays, the injection of OK-432 resulted in increased survivals (Fig. 1).

![Figure 1](image)

**Fig. 1.** A comparison of survival rates of mice between the control group (-----), the group treated with OK-432 1 hour before irradiation (------) and the group treated with OK-432 1 hour after irradiation (-----). Mice were irradiated with 662 cGy or 756 cGy of X-rays.

When normal mice were injected with $^{59}$Fe, about one-half of the $^{59}$Fe was incorporated into circulating erythrocytes 24 hours after the injection. No significant changes in $^{59}$Fe uptake into erythrocytes were observed even when a single dose of OK-432 was injected into normal mice at various intervals before or after $^{59}$Fe injection (Fig. 2). The injection of OK-432 into normal mice also failed to cause any significant changes in $^{59}$Fe uptake into heme and nonheme iron fractions of the spleen, femoral bone marrow and liver at any time tested (data not shown).

Serial changes in $^{59}$Fe incorporation into erythrocytes after irradiation with or without OK-432 treatment, as well as the data of unirradiated control, are shown in Fig. 3. During the first 5 days after irradiation, there was no significant difference in $^{59}$Fe uptake into erythrocytes between the OK-432 treated groups and untreated group. $^{59}$Fe incorporation into erythrocytes significantly increased ($p<0.01$) in the OK-432 treated groups on the 7th to 10th days after irradiation. For example, $^{59}$Fe into erythrocytes was $21.3\pm5.4\%$, $52.1\pm10.6\%$ and $38.4\pm6.7\%$, respectively, in OK-432 untreated, pre-treated and post-treated groups on the 10th day after irradiation with 378 cGy of X-rays. Significant increase ($p<0.05$) was still observed on the 14th day after irradiation.

During the first 5 days after irradiation with 378 cGy of X-rays, there was no significant
difference in spleen weight, \(^{59}\)Fe uptake into the heme iron fraction of the spleen and bone marrow, and \(^{59}\)Fe uptake into the nonheme iron fraction of the liver between OK-432 treated and untreated groups (Fig. 4). After that, the uptake of \(^{59}\)Fe into the heme iron fraction of the spleen was much higher and rose much more rapidly when treated with OK-432. For example, \(^{59}\)Fe in the heme iron fraction of the spleen was significantly higher (p<0.01) on the 7th and 10th days in the OK-432 treated groups than in the untreated group. Changes in spleen weight almost paralleled the changes in splenic uptake of \(^{59}\)Fe into the heme iron fraction. However, \(^{59}\)Fe in the heme iron fraction of bone marrow was almost the same or less than untreated mice when treated with OK-432. Decrease in \(^{59}\)Fe uptake into the heme iron fraction of femoral bone marrow by OK-432 on the 5th day after irradiation was partial but significant (p<0.05). \(^{59}\)Fe in the nonheme iron fraction of the liver significantly decreased on the 7th day after irradiation when treated with OK-432.

Fig. 5 shows the effectiveness of OK-432, administered at various intervals before or after irradiation, on \(^{59}\)Fe uptake into erythrocytes. When OK-432 was injected 3 days before irradiation with 284 cGy, no difference was observed between OK-432 treated and untreated groups. When OK-432 was injected 1 day after irradiation or later, OK-432 had no effect on erythropoiesis. However, \(^{59}\)Fe into erythrocytes was significantly high when OK-432 was injected within 1 day before or within 3 hours after irradiation.
DISCUSSION

Aoki and Kurishita demonstrated that treatment with OK-432 could prevent radiation-induced bone marrow death in BALB/C and ICR-MCH mice\(^9,10\). In the present experiment, survival rate was studied in order to ascertain whether an effective dose of OK-432 was being given to C57BL/6J mice. The significant radioprotection by OK-432 for lethally irradiated C57BL/6J mice observed in the present study confirmed the previous results, indicating that OK-432 is also effective for C57BL/6J mice, and that a sufficient amount of OK-432 was being given in this experiment.

For estimating erythropoiesis in hematopoietic tissues using \(^{59}\)Fe, it is more useful to use the method involving the measurement of \(^{59}\)Fe into the heme iron fraction than into the total iron fraction\(^{24-26}\). Serial changes in \(^{59}\)Fe uptake into erythrocytes and the heme iron fraction of the femoral bone marrow and spleen in 378 cGy irradiated mice in this study were similar to the
endogenous erythropoietic recovery from radiation impairment as reported by other investigators. The ability of OK-432 to induce IL-1 and TNF-α was considered to limit its clinical use because IL-1 and TNF-α suppress the late stage of erythropoiesis. The result that the treatment of normal mice with a single dose of OK-432 alone had no effect on erythropoiesis indicates that the amount of IL-1 and TNF-α produced by OK-432 was not sufficient to suppress normal erythropoiesis. Surprisingly, in sub-lethally irradiated mice, the rate of appearance of $^{59}$Fe into erythrocytes was accelerated following the injection of OK-432. Decreased uptake of $^{59}$Fe into the nonheme iron fraction of the liver by OK-432 on the 7th day after irradiation was also observed. It can be stated with some degree of certainty that recovery of X-ray impaired erythropoiesis was promoted by the injection of OK-432 in C57BL/6J mice. Furthermore, OK-432 seemed effective even when the application is started a few hours after exposure.

OK-432 is known as one of the biological response modifiers because it activates cellular
effector cells such as natural killer cells, macrophages and cytotoxic T lymphocytes\(^{30}\). OK-432 stimulates growth of myeloid progenitor cells in the bone marrow and increases granulocytes in sub-lethally irradiated mice\(^9\). It has also been reported that the radioprotective effect of OK-432 may be attributed partly to the prevention of post-irradiation infections\(^9,10\).

The reason why OK-432 stimulates erythropoiesis in sublethally irradiated mice is not clear. In vitro, OK-432 has been shown to accelerate not only CFU-GEMM, CFU-C and CFU-Meg, but also BFU-e colony formation\(^{31}\). However, there is no data available which would indicate that OK-432 induces EPO, the normal regulator of erythroid differentiation. As OK-432 is known as a multicytokine inducer, some of the factors active on the immature stem cells would increase erythrocytes as well as granulocytes and platelets. For example, GM-CSF, which is induced by OK-432, may promote the recovery of the erythropoietic cell line because GM-CSF potentiates erythroid and multipotential precursors\(^{32}\). However, the increased erythropoiesis appeared to be limited to the spleen, as judged by the uptake of \(^{59}\)Fe into the heme iron fraction. A marked divergence between spleen and bone marrow was also observed in lethally irradiated mice when given a syngeneic marrow injection\(^{33}\). To explain the depressed \(^{59}\)Fe uptake in marrow together with the accelerated \(^{59}\)Fe uptake in the spleen by a syngeneic marrow injection, several possibilities have been proposed\(^{33}\). In this respect, one that was particularly interesting was that
OK-432 could generate suppressors that are effective primarily in the marrow but not in the spleen.

In normal mice the spleen is an erythropoietic organ, although its overall contribution to basal erythropoiesis is minor. However, when accelerated erythropoiesis is required, the murine spleen becomes a major source of red cell production. Then it is not surprising that in sub-lethally irradiated mice splenic erythropoiesis increased when treated with OK-432. Unlike the murine spleen, the human spleen rarely plays a major hematopoietic role. Furthermore, there is no evidence to indicate that radiation causes human spleen to be an active site of erythropoiesis in cancer patients. Therefore, whether the combination of OK-432 with radiotherapy decreases the radiation-induced erythropoietic impairment in human cancer patients is uncertain. OK-432 has already been used clinically in Japan for cancer therapy⁶,⁷. Further investigations will be required to ascertain whether the use of OK-432 together with radiotherapy may have the potential of improving the radiotherapy for cancer, and also whether OK-432 could be used as a radioprotective agent after radiation accidents. A study of the effect of OK-432 on the survival and erythropoiesis of splenectomized and irradiated mice is currently in progress. Studies are also under way in order to determine the radiation dose range where OK-432 would be protective for radiation impaired erythropoiesis.

Whatever the mechanism is, the present study has demonstrated that erythropoiesis in irradiated mice recovered more rapidly than controls when treated with OK-432, and it may suggest that OK-432 has no negative effects on erythropoiesis even when combined with radiotherapy.

ACKNOWLEDGEMENTS

We thank Miss Kumiko Fukutsu for her excellent technical assistance. We sincerely wish to express our appreciation to Mr. Jeffrey Knoll for his invaluable assistance in the writing of this manuscript. This work was supported in part by a grant from the Association of Radiation Effects.

REFERENCES

between total $^{59}$Fe uptake and the uptakes into heme and nonheme fractions of spleen and bone marrow in irradiated mice and mouse radiation chimeras. Blood. 38: 343–352.


