Radioprotective Effects of Adrenochrome Monoaminoguanidine Methanesulfonate (AMM) on Irradiated C57BL Mice and the Survival of GM-CFC, a Hematopoietic Progenitor Cell

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Intraperitoneal administration of adrenochrome monoaminoguanidine methanesulfonate (AMM) enhanced recovery from radiation-induced leukopenia. The effects of AMM administered in vivo and in vitro on survival of GM-CFC, a committed progenitor cell, were examined using an in vitro colony formation method. The survival was increased when AMM was administered intraperitoneally to C57BL mice 30 min before irradiation and when bone marrow which included GM-CFC was isolated 4 h after irradiation to examine in vitro colony formation. On the other hand, incubation of isolated bone marrow cells with AMM from 30 min before and to 4 h after X-irradiation had no effect on the survival. These results suggest that some processes may be required to function in mice for AMM to have effect. When the serum prepared from rabbits which had been injected with AMM 4 h before irradiation was added to isolated bone marrow cells, the survival of GM-CFC was increased with each dose of irradiation. The activity was not lost after dialysis of the serum. It is concluded from these observations that some substances in the serum which enhance survival of GM-CFC may be induced or activated by intraperitoneal administration of AMM, thereby accelerating recovery from radiation-induced leukopenia.

INTRODUCTION

Sugahara and Tanaka reported the radioprotective effects of adrenochrome derivatives¹. Adrenochrome, derived by oxidation from epinephrine, exhibits physiological and pharmacological activities, including hemostatic, ACTH-like and antimitotic activities². Adrenochrome monoaminoguanidine methanesulfonate (AMM), an adrenochrome derivative has some of these activities³.

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The most effective group of protectors are the sulfhydryl compounds which scavenge radiation-induced free radicals. Some pharmacologically or physiologically active drugs also show radioprotective effects but function differently. Since AMM has no sulfhydryl groups to scavenge for the free radicals, the pharmacological activities of AMM may be related to radioprotection. AMM enhances the recovery from radiation-induced leukopenia in rabbits and in humans, and inhibits the increases in chromosome aberrations in peripheral lymphocytes of patients with cervical carcinoma under radiotherapy. In the above, it has been shown that the radiation-induced initial decrease in number of peripheral blood leukocytes (PBL) is not affected by AMM, but recovery from the decrease is enhanced, shortening the period of leukopenia. This suggests that AMM may not exert its effects by protecting PBL directly but by protecting stem and/or progenitor cells in hematogenesis which proliferate and differentiate to PBL after irradiation. Recently developed culture techniques enabled us to examine the proliferating ability of some of these cells in vitro. GM-CFC, one of the committed progenitor cells, which differentiates to granulocytes and macrophages, can form colonies in vitro when isolated bone marrow cells are cultured with supernatant of L cell culture. We used an in vitro colony formation method to test whether or not AMM has a protective effect on the survival of GM-CFC.

MATERIALS AND METHODS

Chemicals and animals
Adrenochrome monoaminoguanidine methanesulfonate (AMM) prepared by Shiraimatsu Shinyaku Co. Ltd., Shiga, according to the method of Yamanishi et al. was used. Agarose-LTG was obtained from Nakarai Chemicals Co. Ltd., Kyoto; RPMI 1640 medium and fetal bovine serum (FBS) from Gibco Laboratories; and Penicillin G potassium and Kanamycin sulfate from Banyu Pharmaceutical Co. Ltd., Tokyo. Six-week-old female C57BL mice purchased from Charles River Japan Inc., Kanagawa, and New Zealand White rabbits from Kari Co. Ltd., Osaka, were used. All animals were kept at 23°C and 55% relative humidity in separate rooms and provided tap water and chow ME and RC-4 (Oriental Yeast Co. Ltd., Tokyo) ad libitum.

X-irradiation
C57BL mice weighing about 20 g and bone marrow cells were irradiated with X-rays (180 KVP, 25 mA, 0.5 mm Cu + 0.5 mm Al filter for mice or 2.0 mm Al filter for cells, 0.4 Gy/min) using Toshiba KXC-18-2 X-ray equipment (Toshiba Co. Ltd., Tokyo).

Survival of whole-body irradiated C57BL mice and measurement of peripheral blood leukocytes (PBL)
To check the survival rates, C57BL mice that had been whole-body irradiated with 7 Gy were monitored daily \( \times \) 30. To measure the number of PBL, tail arterial blood was bled into heparinized capillary tubes from each mouse one day before and on days 7, 14, 21 and 28 after the irradiation. Leukocytes in a mixture of heparinized blood and Turk solution (1:3 to 1:10)
were counted using a standard hemocytometer (Erma Co. Ltd., Tokyo).

*In vitro colony formation of bone marrow cells*

In *vitro* colony formation of bone marrow cells was determined according to Robinson *et al.*\(^1\), with slight modifications. The femora of C57BL mice were removed following cervical dislocation. Both ends of the femur were excised and 2 ml of RPMI 1640 medium containing 10% FBS (FBS-RPMI) was forced through each femur using a sterile syringe and 21 G needle. The extruded marrow was pipetted and the cells diluted to 4 \( \times \) 10\(^6\) cells/ml with RPMI 1640 medium + 10% FBS. Incubation with or without AMM, or with serum prepared from rabbits (in place of FBS) was performed for 30 min before and 4 h after irradiation when required. The cells were then washed once with RPMI 1640 medium and an appropriate number of the cells was inoculated into each well of 24-well plates (Beckton Dickinson & Co., USA) with 0.5 ml of FBS-RPMI supplemented with 15% supernatant of L-929 cell culture as a colony stimulating factor (CSF)\(^8\),\(^9\) and 0.45% agarose-LTG for immobilization. The plates were kept at 4°C for 10 min to gel the medium and prevent damage. After incubation at 37°C in 5% CO\(_2\) humidified atmosphere for 7 days, colonies containing more than 20 cells were counted using an inverted microscope. In these conditions, colonies were formed in which we confirmed the presence of granulocytes and macrophages which were therefore thought to be derived from GM-CFC.

*Preparation of serum from AMM-injected rabbits (SAR) and dialysis of SAR*

AMM in saline in a dose of 100 mg/Kg was administered intravenously to three New Zealand White rabbits weighing about 3 Kg. Thirty minutes later blood samples from each rabbit were taken into sterile syringes using a 21 G needle. The blood was stood at room temperature for about 40 min and then centrifuged at 2,000 rpm for 20 min to obtain the serum. The sera from the three rabbits were mixed and used as SAR. Before giving AMM to each rabbit, blood was taken and used for preparation of the control serum. The sera were sterilized by passing them through a 0.22 \( \mu \)m Millipore membrane, and then added to the culture media. Two ml of SAR or control serum was dialyzed twice in 500 ml of RPMI 1640 medium at 4°C for 8 h, sterilized and added to the culture media.

**RESULTS**

The effects of AMM on the survival of C57BL mice irradiated with 7 Gy are shown in Fig. 1. C57BL mice were given intraperitoneally saline containing or not containing AMM 30 min before whole-body irradiation. In the control group, mice began to die after day 9 and the survival decreased gradually thereafter. One mouse was alive on day 30. On the other hand, in the AMM-injected group, no mouse died until day 18 and four mice were alive on day 30. These radioprotective effects of AMM were obtained at different doses of X-rays (data not shown). However, the effects were not statistically significant when examined by \( \chi^2 \) test using survival data on day 30. Statistically significant difference is reported by Sugahara and Tanaka\(^{11}\) using ddy mice.

The effects of the administration of AMM on the repopulation of peripheral blood leukocytes
(PBL) are shown in Fig. 2. AMM was injected into mice 30 min before irradiation of 5 Gy. The tail peripheral blood of mice injected with AMM or saline was sampled and the number of PBL examined before irradiation and on days 7, 14, 21 and 28 after irradiation. There was no effect of AMM on the number of PBL on day 7. AMM in doses of 25 mg/Kg and 100 mg/Kg significantly enhanced repopulation of PBL thereafter (Fig. 2). The drug was less effective when administered 15 min after irradiation.

The effects of intraperitoneal administration of AMM on the survival of GM-CFC were then examined using the in vitro colony formation method. AMM at 100 mg/Kg was given to mice 30 min before irradiation and the mice were killed 4 h after irradiation. A suspension of bone marrow cells was prepared from the femur of the mice, as described in Materials and Methods. AMM given to non-irradiated mice had no toxic effect on GM-CFC in terms of cloning efficiency. At each X-ray dose level survival of GM-CFC was greater from AMM-injected mice than that from controls (Fig. 3).

The effects of the direct addition of AMM to isolated bone marrow cells on the survival of GM-CFC are shown in Fig. 4. Isolated bone marrow cells were incubated for 30 min before and 4 hr after irradiation. We decided to use 25 and 100 μg/ml as the doses of AMM, since the concentration of AMM in rabbit serum 30 min after injection of the drug at a dose of 50 mg/Kg was 46 μg/ml. The cloning efficiency of non-irradiated cells was not affected by AMM treatment. The effects of AMM on survival were nil at each dose.

The lack of any distinct effect of in vitro administration of AMM on the survival of GM-CFC suggests that certain processes functioning after the intraperitoneal administration of AMM may increase the survival of GM-CFC. To test this postulate, the effects of serum from AMM-injected rabbits (SAR) on the survival of GM-CFC were examined. Rabbits were chosen as the blood
Fig. 2. Effects of AMM on radiation-induced leukopenia. □, control; △, 25 mg/Kg AMM; ●, 100 mg/Kg AMM.

Table 1. Effects of dialyzed SAR on the survival of irradiated GM-CFC

| Serum  | Surviving fraction (3 Gy) |
|--------|---------------------------|
| SAR    | 0.079 ± 0.016*            |
| Control| 0.021 ± 0.013             |

a: Mean ± SE (n = 6)
donors because a large volume of serum was required for the experiment. Bone marrow cells from normal C57BL mice were prepared by adding to SAR or control serum (10%), and incubated at 37°C for 30 min before irradiation. After postirradiation incubation for 4 h the cells were cultivated for colony formation. The survival of GM-CFC treated with SAR was higher than that of those with control serum, at each dose of X-rays, as shown in Fig. 5. SAR showed less effects on survival when added 15 min after irradiation.

The SAR was dialyzed extensively in the culture medium to test whether or not the protective activity in the serum is dialyzable. The control serum was also dialyzed. Bone marrow cells were incubated with each dialyzed serum for 30 min before and 4 h after irradiation. The dialyzed SAR increased survival of irradiated GM-CFC, as shown in Table 1.

DISCUSSION

It is reported that AMM delayed deaths of whole-body irradiated mice and rabbits. In the present study, we found that AMM enhanced repopulation of PBL (Fig. 2), shortening radiation-induced leukopenia. The shortening of leukopenia by AMM after irradiation may explain
the increase in survival of irradiated mice. As shown in Fig. 2, AMM had no effect on a radiation-induced initial decrease in the number of PBL. This indicates that AMM does not protect PBL directly from radiation damage. The repopulation of PBL after irradiation is shown to depend on the proliferation and differentiation to PBL of stem and progenitor cells in the hematopoietic system\textsuperscript{12}). Since repopulation was accelerated by AMM injection (Fig. 2), AMM may protect the proliferating and differentiating ability of stem and/or progenitor cells. Recent progress in clonal cell culture has provided methods for studying multilineage hemopoietic colonies\textsuperscript{13}). Suda et al. proposed a model for the progressive and random differentiation of stem cells\textsuperscript{14}). Among the various progenitors which can form simple- or multiple-lineage hematopoietic colonies, we examined progenitors for GM-colonies consisting of neutrophils and macrophages, because neutrophils play main roles in protection against bacterial invasion which may be one of the reasons of bone marrow death. As expected we found that the administration of AMM to whole-body irradiated mice resulted in an increase in the survival of GM-CFC which is able to differentiate to granulocytes and macrophages (Fig. 3). Since the granulocyte is one of the major components of PBL, the increase in survival of GM-CFC would be reflected by at least some accelerated repopulation of PBL. In the present study, there is no information on the repopulation of

\begin{figure}[h]
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\caption{Effects of \emph{in vitro} administration of AMM on the survival of GM-CFC. □, control; ▲, 25 \textmu g/ml AMM; ●, 100 \textmu g/ml AMM.}
\end{figure}
lymphocytes, the other major component of PBL. However, previous studies have revealed that the repopulation of lymphocytes is enhanced by AMM\(^5\), although the mechanism involved has not been clarified yet. Other blood components also could play roles in radioprotection by AMM. However, the effects seem to be more specific on the hematogenesis of leukocytes, since AMM has little effect on erythropoiesis\(^5\). The effects of AMM on thrombopoiesis have not been performed yet.

In the present study, it is shown that certain processes occurring in vivo are needed for the protection by AMM (compare Figs. 3 & 4). There are at least three possibilities: 1. Intraperitoneal administration of AMM induces, activates or inactivates other intrinsic substances. 2. AMM has to be metabolized to be effective. 3. AMM induces physiological or pharmacological change(s) such as decreases in blood flow rate, thereby the oxygen supply to tissue is restricted and radiosensitivity is lowered\(^4\). We found that serum taken from an AMM-injected animal had direct effects on the GM-CFC (Fig. 5), indicating that there is less possibility of the third being true. The fact that SAR had an effect after dialysis (Table 1) may be against the second possibility because each metabolite of AMM would be dialyzable. However, we still cannot exclude the second possibility, assuming that active metabolites form complexes with large molecular substances in serum. Further studies are needed to determine which possibility is correct.

We used an in vitro colony formation method here. The method is available for purifying and identifying the factor(s) responsible for radioprotection by AMM. The factor(s) in the serum
are now in the process of being characterized.

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