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# Effects of amifostine on the proliferation and differentiation of megakaryocytic progenitor cells

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**Abstract**

This study investigated the effects of amifostine, a clinically usable radioprotector or chemoprotector, on the proliferation and differentiation of normal and X-irradiated cluster of differentiation 34 positive ($CD34^+$) megakaryocytic progenitor cells (colony-forming unit in megakaryocytes, CFU-Meg) from human placental and umbilical cord blood (CB) in vitro. Amifostine significantly accelerated megakaryocyte colony formation in a plasma clot culture supplemented with recombinant human thrombopoietin because of an increase in immature CFU-Meg-derived large megakaryocyte colony formation. An analysis of the cells that were harvested from the culture showed that amifostine induced a 70- and an 83-fold increase in the total cell and CFU-Meg numbers, respectively, and produced hyperploid megakaryocytes of more than 8 N ploidy. The radioprotective effect of amifostine on the clonal growth of X-irradiated $CD34^+$ CFU-Meg was observed by treatment before or after irradiation. These findings suggest that the action of amifostine extends from immature CFU-Meg to the terminal differentiation of megakaryopoiesis, and its radioprotective effect is shown in megakaryopoiesis and thrombopoiesis. 

*Keywords:* Amifostine; Hematopoietic stem cell; CD34 antigen; Colony-forming units assay; Thrombopoietin; X-ray

## 1. Introduction

Amifostine (WR-2721, Ethyol), *S*-2[3-aminopropylamino]-ethyl-phosphorothioic acid, is a phosphorylated aminothiol that has the potential to selectively protect normal tissues from damage by oxidative stress associated with cancer chemotherapy and radiotherapy (Capizzi et al., 1993; Spencer and Goa, 1995). It was originally developed as a clinically usable radioprotective agent from more than 4000 compounds by the US Army Walter Reed Institute (Washington, DC) (Kurbacher and Mallmann, 1998; Hall, 2000). A considerable amount of preclinical work suggested that amifostine, or its activated metabolite WR-1065, effectively protects normal cells against the adverse effects of irradiation and several anticancer drugs without showing tumor protection (Guest and Uetrecht, 2001). Amifostine has the potential to stimulate multipotent hematopoietic stem cells detected in normal human bone marrow cells (List et al., 1996, 1998). As the hematopoietic system is highly sensitive to various extracellular oxidative stresses, such as to radiation and cytotoxic drugs, the survival or clinical outcome of patients having these stresses depends mainly on the recovery of the hematopoiesis, especially of the hematopoietic stem and progenitor cells. A new possible function of amifostine is not only as a radioprotective agent, but also as a hematopoietic growth factor, e.g., clinical application combined with cytokines for patients having myelosuppression induced by irradiation and chemotherapy. However, evaluations are few of the action of amifostine on the proliferation and differentiation of human megakaryocytic progenitor cells (colony-forming unit in megakaryocytes, CFU-Meg) which are the progenitor cells in megakaryocyte and control platelet production, and of the radioprotective effects of amifostine in megakaryopoiesis and thrombopoiesis.

In this study, using highly purified cluster of differentiation 34 positive ($CD34^+$) cells prepared from human placental and umbilical cord blood (CB) that is the periph-

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eral blood of the fetus and neonate, we investigated the effects of amifostine on the clonal growth of normal and X-irradiated $CD34^+$ CFU-Meg in vitro.

## 2. Materials and methods

### 2.1. Reagents

Recombinant human thrombopoietin and recombinant human stem cell factor (SCF) were kindly provided by KIRIN Brewery (Tokyo, Japan). Doses of thrombopoietin and SCF per milliliter of medium were: thrombopoietin, 50 ng; SCF, 100 ng. Amifostine was purchased from Sigma (St. Louis, MO) and was dissolved in phosphate-buffered saline (PBS).

### 2.2. Collection of CB and $CD34^+$ cell purification

After obtaining informed consent from the mothers, CB was collected at the end of full-term deliveries using a sterile collection bag containing the anticoagulant citrate-phosphate dextrose according to the guidelines of the Tokyo Cord Blood Bank. Light-density mononuclear CB cells were separated by centrifugation on Ficoll-Paque (1.077 g/ml, Pharmacia,) for 30 min at 300 *g* and were washed three times with 5 mM ethylenediaminetetraacetic acid (EDTA)–PBS ( ). Light-density mononuclear cells were processed for $CD34^+$ cell enrichment according to the manufacturer's instructions, and magnetic cell sorting (Miltenyi Biotec, Germany) was used for the positive selection of $CD34^+$ cells. At the end of the procedure, the $CD34^+$ cell recovery from light-density mononuclear cells was approximately 0.5%, and the purity, measured by using a fluorescence cell analyzer (EPICS-XL, Beckman-Coulter, CA), was 90–95%.

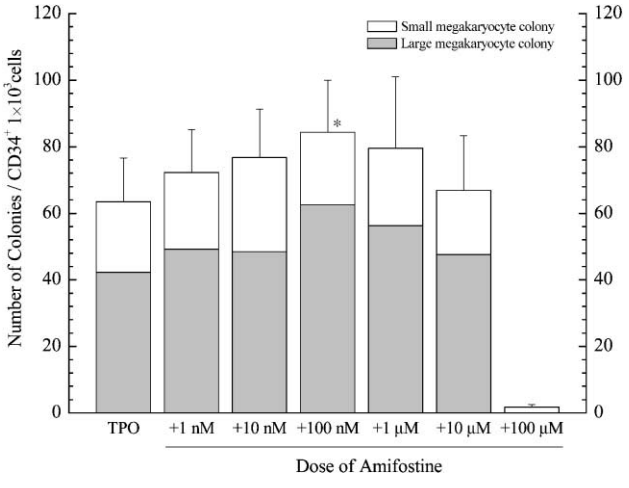


Fig. 1. Effects of amifostine concentration on in vitro clonal growth of CFU-Meg by human CB $CD34^+$ cells. Freshly prepared $CD34^+$ cells (1 $10^3$ cells/ml) were cultured in the plasma clot cultures supplemented with thrombopoietin (50 ng/ml) and various concentrations of amifostine for 12 days. Values are the mean S.D. of three separate experiments in three wells. * $P < 0.05$ by *t*-test.

Table 1
Total number of cells and CFU-Meg produced in the culture

| Treatments | Total cells ( $10^4$) (-fold) | Total CFU-Meg ( $10^3$) (-fold) |
|---|---|---|
| Day 0 | 2.4 (1.0) | 0.95 (1.0) |
| Day 12: thrombopoietin alone | 96.0 (40.0) | 29.0 (30.6) |
| Day 12: thrombopoietin and amifostine | 168.0 (70.0) | 79.1 (83.4) |

Freshly prepared CB $CD34^+$ cells were cultured in a plasma clot culture stimulated with thrombopoietin alone or thrombopoietin and amifostine for 12 days. Each aliquot of harvested cells was assayed for CFU-Meg by plasma clot culture supplemented with thrombopoietin and SCF.

### 2.3. In vitro irradiation of $CD34^+$ cells

$CD34^+$ cells were irradiated in vitro by using the method described by Kashiwakura et al. (2000). The $CD34^+$ cells were irradiated with X-rays (200 kV, 15 mA) by using 1-mm copper and 0.5-mm aluminum filters at a distance of 30 cm from the focus at a dose of 73 cGy/min.

### 2.4. Plasma clot cultures

CFU-Meg was assayed by using the plasma clot technique with platelet-poor human plasma. The culture medium contained a 1 $10^3$ cells/ml concentration of $CD34^+$ cells and 15–20% human platelet-poor AB plasma and growth factor(s) in Iscove's modified Dulbecco's medium (Gibco) with additives of 100 U/ml penicillin (Gibco), 100 g/ml streptomycin (Gibco), 1 mM sodium pyruvate (Gibco), 1% minimum essential medium (MEM) vitamin (Gibco), 1% MEM non-essential amino acids (Gibco), 1 $10^{-5}$ M thioglycerol (Sigma), 2 g/ml L-asparagine (Wako, Tokyo, Japan), 74 g/ml $CaCl_2$ (Wako), and 0.2% bovine serum albumin (Boehringer Mannheim, Germany). The medium (0.3 ml) was plated in 24-well culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) and was incubated at 37 C in a humidified atmosphere of 5% $CO_2$ for 11–12 days.

### 2.5. Immunofluorescence staining to identify megakaryocyte colonies

Each well was fixed twice with a 2:1 mixture of acetone and methanol for 15 min. The plates were dried in an airflow overnight and then they were kept at 20 C until staining. For the staining, the plates were removed from the freezer and were returned to room temperature. Then, PBS containing 0.5% bovine serum albumin (PBS-B) was added to soften the clot. After discarding the solution, fluorescein isothiocyanate (FITC)-CD41 (gpIIbIIIa, Pharmingen, San Diego, CA) monoclonal antibody diluted 1:100 in PBS-B was added, and the plates were incubated for 1 h at room temperature and were washed once with PBS-B. The nuclei

were counterstained with propidium iodine (PI, 0.3 ng/ml, Sigma). The colonies were washed again and were counted by using a fluorescence microscope (Olympus, Tokyo, Japan) at 100 magnification. Megakaryocyte colonies were classified into two types: large colonies of more than 50 cells (immature CFU-Meg), and small colonies of 3–50 cells (mature CFU-Meg) (Siena et al., 1993; Hagiwara et al., 1998). The total number of CFU-Meg (total CFU-Meg) was calculated by summing immature CFU-Meg and mature CFU-Meg.

### 2.6. Immunological marker analysis

The expression of cell surface antigens was analyzed by direct immunofluorescence flow cytometry by using triple-staining combinations of monoclonal antibodies including phycoerythrin (PE)-Cy5 (PC5)-CD45, FITC-CD34, and phycoerythrin (PE)-CD41. Briefly, the cells were incubated with saturated concentrations of the relevant monoclonal antibodies for 20 min at room temperature, were washed, and were analyzed by using a flow cytometer. For each ex-

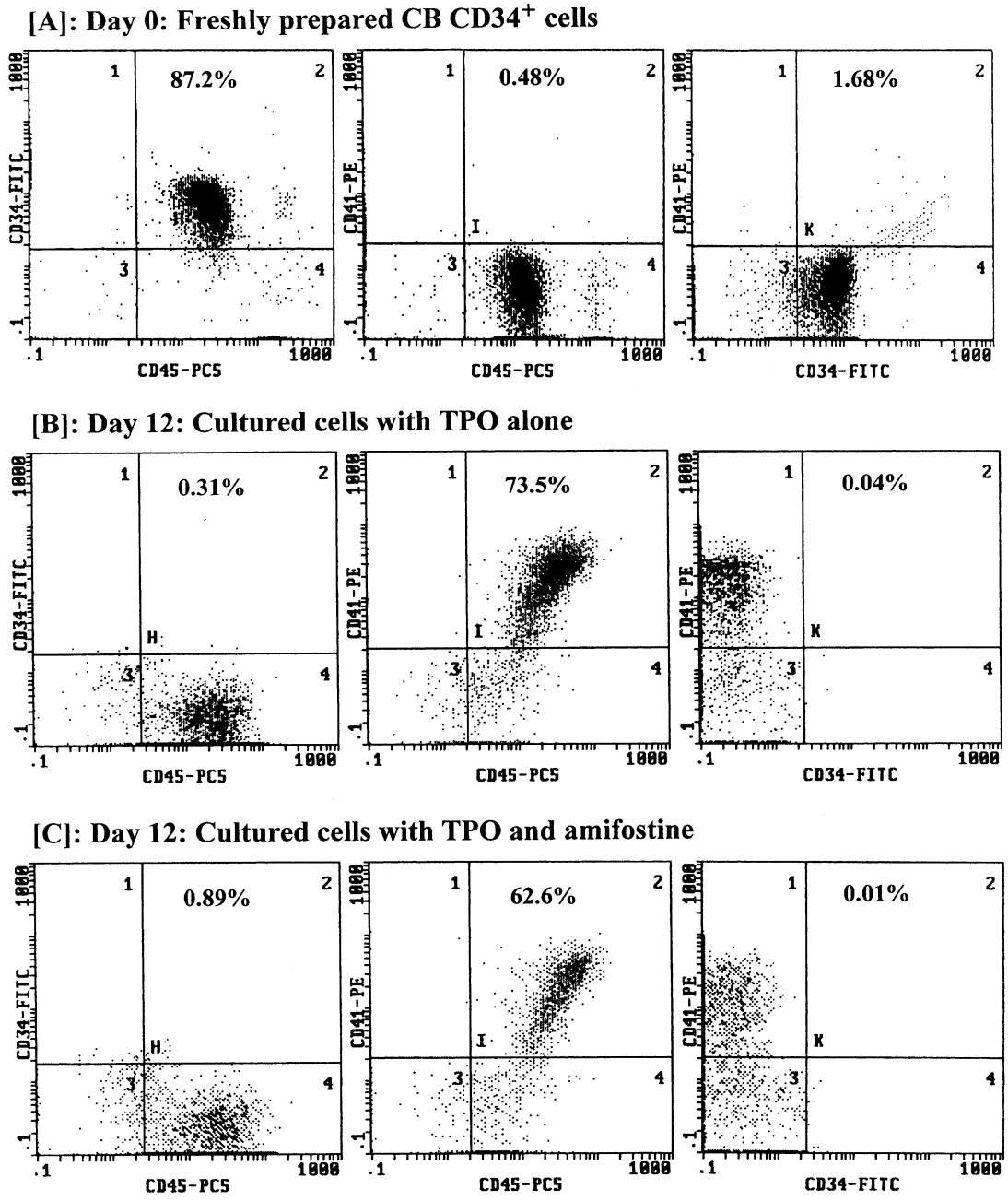


Fig. 2. Representative flow cytograms of cells harvested from a culture stimulated with thrombopoietin alone or thrombopoietin and 100 nM amifostine. The cells were treated with anti-human FITC-CD34, PE-CD41 and PC5-CD45 monoclonal antibody. The expression of each surface antigen was analyzed using a flow cytometer.

Table 2
DNA ploidy distributions of megakaryocytes produced in the culture

| Treatments | DNA ploidy (%) | | | |
|---|---|---|---|---|
| | 2 N | 4 N | 8 N | 16 N |
| Freshly prepared CD34$^+$ cells | 95.4 | 3.9 | 0.7 | 0 |
| Day 12: thrombopoietin alone | 62.6 | 22.0 | 4.0 | 0.5 |
| Day 12: thrombopoietin and amifostine | 46.3 | 32.2 | 10.1 | 1.6 |

The cells harvested from the culture were treated with FITC-CD41 monoclonal antibody and plopidium iodide, then each sample was analyzed for DNA ploidy distribution using a flow cytometer. Values are derived from flow cytometer analysis, as shown in Fig. 3.

periment, a negative control experiment was done using isotype-matched irrelevant control monoclonal antibodies.

### 2.7. Measurement of megakaryocyte DNA ploidy

Megakaryocyte DNA ploidy was measured by using the method of Hagiwara et al. (1998) with modifications. Freshly isolated CD34$^+$ cells or cultured cells harvested from plasma clot culture were centrifuged for 5 min at 250 *g*, and the cell pellets were resuspended in PBS-B containing 5 mM EDTA and were incubated with FITC-CD41 monoclonal antibody for 20 min at room temperature. Then, the cells were washed with PBS and the cell pellets were resuspended in modified CATCH medium (Miyazaki et al., 1992) containing 3.5% bovine serum albumin and 0.5% Tween 20 (Wako). After 1 h of incubation at 4 C, the cells were fixed for 5 min by adding an equal volume of the same medium supplemented with 1% paraformaldehyde (Wako) to the cell suspension. After washing with PBS, the cells were resuspended in 50 g/ml of propidium iodide (PI) dissolved in a solution containing 0.7% citric acid and 0.6% NaCl, and the mixture was incubated for 1 h at 4 C. After incubation, the cells were further incubated with 50 g/ml of RNase (Sigma) for 30 min at room temperature, and were then passed through a 35- m nylon mesh. Freshly prepared CD34$^+$ cells were used as the standard of the 2 N DNA content, and the mean channel of the 2 N peak was measured by using a flow cytometer.

### 2.8. Statistical analysis

The significance of differences between the control and experimental groups was calculated using the Student's *t*-test. The data of the multiple groups were analyzed using the one-way layout analysis of variance and Fisher's least significant difference. The $D_0$ value, the radiation dose that reduces survival to e $^{-1}$ (i.e., 0.37) of its previous value on the exponential portion of the survival curves, and the extrapolation number *n*, the point on the survival scale to which the straight part of the curve back-extrapolates, were calculated by using a single-hit multi-target equation programmed in a computer.

## 3. Results

### 3.1. Dose–response relationship of amifostine on the clonal growth of CB CD34$^+$ CFU-Meg

The effect of the concentration of amifostine on the in vitro clonogenic potentials of CFU-Meg from freshly purified nonirradiated CB CD34$^+$ cells was assessed by plasma clot culture in the presence of thrombopoietin. In the absence of exogenous thrombopoietin, no colonies were observed in the culture with or without the presence of amifostine (data not shown). Thrombopoietin alone supported approximately 63 colonies/1 $10^3$ cells in the plasma clot culture (Fig. 1), and the proportion of large megakaryocyte colonies, immature CFU-Meg, was 66.8% of the total number of colonies. Addition of 100 nM amifostine resulted in an approximately 33% increase ($p < 0.05$) in the total number of colonies, due to an increase in large megakaryocyte colonies. All other experiments in this study were done with 100 nM amifostine.

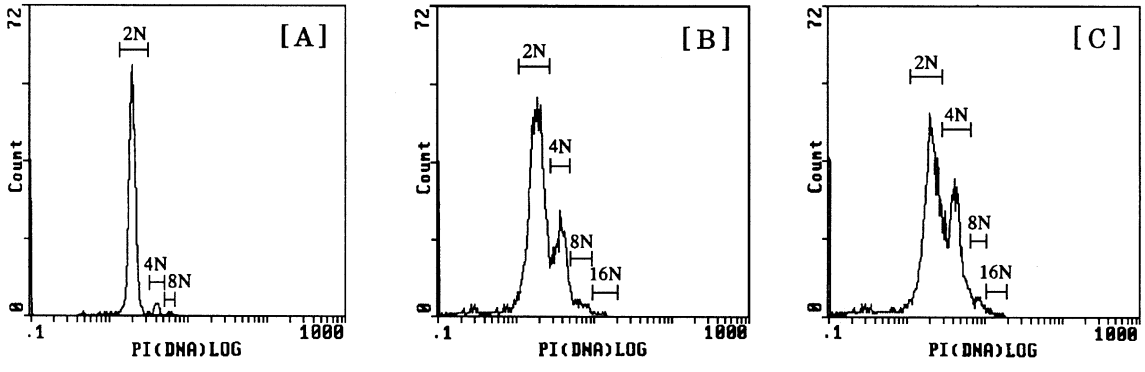


Fig. 3. DNA ploidy distributions of megakaryocytes produced in a culture. The cells harvested from the culture were treated with anti-human FITC-CD41 monoclonal antibody and plopidium iodide. DNA ploidy distributions of CD41 positive cells were analyzed using a flow cytometer. Panel [A]: Freshly prepared CB CD34$^+$ cells. Panel [B]: Cultured cells with thrombopoietin alone. Panel [C]: Cultured cells with thrombopoietin plus amifostine.

### 3.2. Flow cytometric measurement of cell surface antigens and DNA ploidy in the generated cells of the plasma clot culture

To evaluate the action of amifostine on the proliferation and differentiation of CB hematopoietic stem and progenitor cells, generated cells were harvested from the plasma clot culture, and the number of surface antigens on the cells and DNA ploidy were measured by using a flow cytometer. In the control culture, the total number of cells and CFU-Meg increased 40- and 30-fold, respectively, from the number of the initial input (Table 1). Adding amifostine produced a significant increase in total cell and CFU-Meg number compared with the control (70- and 83-fold, respectively). The expressions of $CD45^+CD34^+$, $CD45^+CD41^+$, and $CD34^+CD41^+$ in the freshly prepared $CD34^+$ cells before culture were 87.2%, 0.48%, and 1.68%, respectively (Fig. 2). Each value in the cells prepared from the control culture stimulated with thrombopoietin alone was 0.31%, 73.5%, and 0.04%, respectively (Fig. 2). Similarly, the cells from the culture with thrombopoietin plus amifostine showed almost the same values (0.89%, 62.6%, and 0%, respectively) as the control (Fig. 2). These results indicated that the $CD45^+CD34^+$ cells, relatively immature cells in hematopoiesis, rapidly decreased in vitro, and that megakaryocytes became the majority in the generated cells. Estimations of the DNA ploidy of generated megakaryocytes showed that 62.6% of megakaryocytes were 2 N ploidy, and hyperploid megakaryocytes of more than 8 N ploidy were 4.5% of the total megakaryocytes detected in the control culture (Table 2; Fig. 3). By adding amifostine, megakaryocytes with 2 N ploidy decreased to 46.3% and hyperploidy megakaryocytes increased to 11.7% of the total megakaryocytes. This result indicated that amifostine can promote maturation of megakaryocytes from $CD34^+$ CFU-Meg.

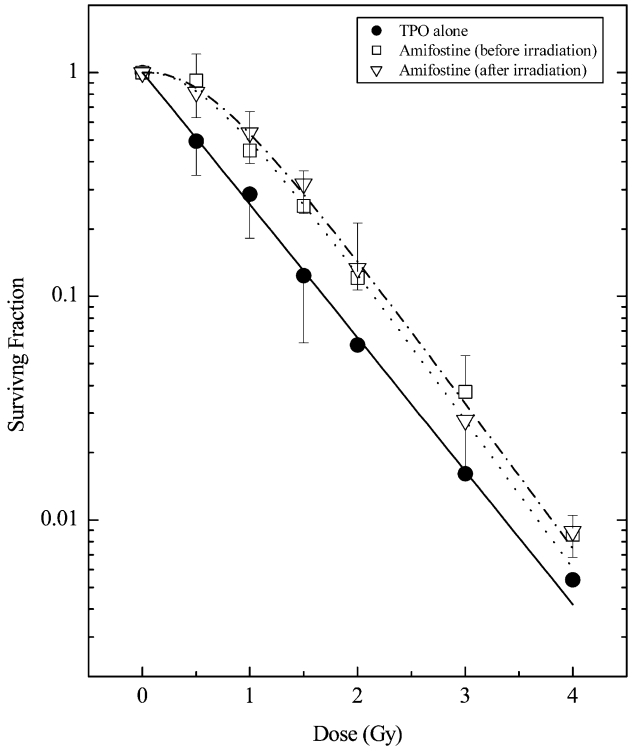


Fig. 4. Effects of amifostine on the radiation survival of CFU-Meg. Human CB $CD34^+$ cells were irradiated with 0–4 Gy of X-rays. Control culture was made with X-irradiated cells and thrombopoietin alone (●). Cells were treated with amifostine (100 nM) for 1 h before irradiation and were plated with the plasma clot culture in the presence of thrombopoietin ( ). X-irradiated cells were plated with the culture in the presence of thrombopoietin and amifostine (100 nM) ( ). The surviving fraction values are the mean S.D. of three separate experiments in three wells.

Table 3
In vitro radiation response characteristics of CB $CD34^+$ CFU-Meg

| Treatments | $D_0$ (cGy) | | $n$ | |
|---|---|---|---|---|
| Control | 72.8 | 12.5 | 1.02 | 0.28 |
| Amifostine (before irradiation) | 70.7 | 14.6 | 2.78 | 0.52[a] |
| Amifostine (after irradiation) | 66.8 | 15.6 | 3.00 | 0.46[a] |

X-irradiated $CD34^+$ cells with 0–4 Gy were plated in plasma clot culture with thrombopoietin. Amifostine treatment was before or after irradiation. Values are the mean S.D. of three separate experiments in three wells.

[a] $P<0.001$ when each group was compared by the one-way layout analysis of variance.

### 3.3. Effects of amifostine on the clonal growth of X-irradiated CB $CD34^+$ CFU-Meg

CB $CD34^+$ cells were irradiated with X-rays from 0 to 4 Gy and were plated in the plasma clot culture supplemented with thrombopoietin alone. Amifostine (100 nM) was treated with $CD34^+$ cells before irradiation for 1 h or immediately after irradiation. Fig. 4 shows the radiation survival curves of total CFU-Meg, and Table 3 summarizes the $D_0$ and $n$ values of these results. Thrombopoietin alone showed an exponential survival curve ($D_0 = 72.8$ cGy, $n = 1.02$), but, in contrast, amifostine treatment showed marked shoulders in the survival curves ($D_0 = 70.7$ and 66.8 cGy, $n = 2.78$ and 3.00, respectively). However, both parameters of radiation sensitivities from different amifostine treatments showed no differences.

## 4. Discussion

In this study, amifostine appeared to produce growth factor-like properties promoting growth of megakaryocytic progenitor cells in vitro. Amifostine particularly increased the formation of immature CFU-Meg-derived large megakaryocyte colonies in the presence of thrombopoietin alone, but amifostine alone did not support the growth of CFU-Meg in our culture condition (Fig. 1). Amifostine showed a 70- and an 83-fold increase in total cell numbers and CFU-Meg, respectively, after 12 days of culture (Table 1), suggesting that amifostine synergistically acts with thrombopoietin to promote the proliferation of immature hematopoietic progenitor cells in vitro. The results of flow

cytometric analysis of the cells harvested from the culture showed that amifostine accelerates the generation of hyperploid megakaryocytes. These findings suggest that the target cells of amifostine are immature progenitor cells in the differentiation pathway of hematopoiesis, and that the action of amifostine extends to the maturation of megakaryopoiesis. A preincubation exposure to amifostine increases clonal growth of relatively immature hematopoietic progenitor cells in cultures initiated with optimal concentrations of interleukin-1, interleukin-3, or SCF (List et al., 1998). Amifostine acts early in the regulation of hematopoiesis, consistent with the results of this study.

Amifostine is a prodrug that is dephosphorylated by the action of membrane-bound alkaline phosphatase to the active metabolite WR-1065, 2-(3-aminopropyl) aminoethanethiol (Purdie et al., 1983; Kurbacher and Mallmann, 1998). This metabolite is believed to be responsible for reducing the toxic effects of radiation in normal oral tissues and reducing cumulative renal toxicity of cisplatin (Kurbacher and Mallmann, 1998; Hall, 2000). It is assumed that this is also true for protection against chemotherapeutic agents, due to the more rapid uptake of amifostine in normal tissues compared to hypoxic tumor tissue (Smoluk et al., 1988). Other intracellular thiol compounds, including glutathione (GSH), metallothionein, and sodium thiosulfate, also protect against hematopoietic toxicity of anticancer drugs without interfering in the antitumor efficacy (Treskes and van der Vijgh, 1993). Amifostine/WR-1065 binds to DNA and interferes in thymidylate kinase and topoisomerase activities, inhibits endonucleases, and affects transcription factor regulation, possibly as a result of increased intracellular thiol concentration (List et al., 1996, 1998). -Phenyl N-*tert*-butylnitrone (PBN), a spin trap reagent, accelerates the proliferation of murine hematopoietic progenitor cells in bone marrow in vitro (Kashiwakura et al., 1997). PBN also stimulates the level of intracellular GSH, suggesting a relationship between the intracellular redox state and the proliferation and differentiation of hematopoietic stem and progenitor cells. Thus, amifostine may affect the intracellular redox state of hematopoietic stem and progenitor cells, leading to an increase in megakaryopoiesis and thrombopoiesis when combined with thrombopoietin. In this study, radioprotection by amifostine of the clonal growth of X-irradiated $CD34^+$ CFU-Meg was observed by treatments before and after irradiation of amifostine (Table 3). A marked shoulder appeared in the survival curve from both treatments (Fig. 4).

Combinations of cytokines, including thrombopoietin, SCF, and interleukin-3, are effective for the radiation survival of CB CFU-Meg compared with thrombopoietin alone, or thrombopoietin combined with Flt3/Flk2-ligand or interleukin-11 or both (Kashiwakura et al., 2000). The radioprotective potential of amifostine to irradiated CFU-Meg seems to be similar to SCF and interleukin-3, and the radioprotective mechanisms of the synergism by thrombopoietin and SCF or interleukin-3 are uncertain. Thiol metabolites activated by amifostine, WR-1065, protects cells from cytotoxic damage by binding to highly reactive nucleophiles that would normally cross-link and damage DNA (Treskes and van der Vijgh, 1993). Additionally, p53 expression might be upregulated by either amifostine or WR-1065, to give more efficient DNA repair (North et al., 2000). Thiols modulate early-response genes and increase the binding of transcription factors that result in protection against oxidative stress (List et al., 1996, 1998). Also, thiol molecules increase the activities of transcriptional factors, such as NF- B and AP-1 (Arrigo, 1999). Amifostine inhibits apoptosis in murine hematopoietic stem and progenitor cells by activating NF- B/Rel transcription factors (Romano et al., 1999). These functions by amifostine may lead to its radioprotection of CB $CD34^+$ CFU-Meg.

Problems exist with CB transplantation, such as small volumes of samples and severe thrombocytopenia after transplantation. Possible approaches to solve these problems are to expand hematopoietic stem and progenitor cells and to transplant differentiated progenitor and matured cells obtained by ex vivo expansion. Transfusion of differentiated hematopoietic progenitor cells shortens the thrombocytopenia period (Bertolini et al., 1997; Gehling et al., 1997; Bachier et al., 1999; Paquette et al., 2000). Cytokine administration accelerates hematopoiesis in victims receiving irradiation. The synergistic activity of growth factor combinations is important to recover hematopoietic stem and progenitor cells after exposure to irradiation. However, as we should pay attention to the costs and patents in the clinical application of cytokines for myelosupression and ex vivo expansion, simple and economical clinical regimens are required. Although more detailed study is needed of whether amifostine has therapeutic potential as a hematopoietic regulatory factor, it can act as a simulator to proliferate and differentiate CFU-Meg. A study of the maturation of megakaryocytes when combined with thrombopoietin is needed. Amifostine may promote megakaryopoiesis and thrombopoiesis recovery in patients with little tolerance of radiation or cytotoxic drugs, and it promotes ex vivo expansion.

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