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FULL PAPER *Laboratory Animal Science*

Combined Effects of Treatment with Trientine, a Copper-Chelating Agent, and X-Irradiation on Tumor Growth in Transplantation Model of a Murine Fibrosarcoma

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ABSTRACT. Combined effects of treatment with trientine, a copper-chelating agent, and X-irradiation on development of fibrosarcoma using a murine transplantation model *in vivo* and on cellular survival *in vitro* were examined. Copper contents in the tumors and serum of trientine-treated mice were significantly lower than those of untreated mice. The tumor volumes of mouse fibrosarcoma QRsp-11 cells increased more slowly in the trientine-treated and the X-irradiated mice than in the control mice from 10 to 24 days postinoculation. The extent of inhibition of tumor growth by X-irradiation at 3 Gy was similar to that obtained by treatment with trientine. A combination of trientine and X-irradiation at 3 Gy showed inhibitory effects on tumor growth similar to those obtained by X-irradiation at 6 Gy. The results showed that trientine and X-irradiation interacted additively in inhibition of tumor growth. When QRsp-11 cells and mouse and bovine endothelial cells were treated with trientine after X-irradiation, the surviving fractions of the cells with combined treatments were essentially consistent with the products of the surviving fractions of trientine-treated cells and those of X-irradiated cells. When the cells were pretreated with trientine and X-irradiated, the surviving fractions of the pretreated cells were lower than those of cells without treatment.

KEY WORDS: fibrosarcoma, transplantation model, trientine, tumor growth, X-irradiation.

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A growing body of evidence indicates that angiogenesis is essential for solid tumor progression and metastasis [13]. Any solid tumors that have not acquired their own new blood supply cannot grow more than a few millimeters in size [12]. Therefore, therapies have been tested with aim of destroying tumor vasculature. Several angiogenesis mediators, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are required for angiogenesis [1, 10, 38, 43, 46]. AntiVEGF antibodies and low-molecular-weight non-protein agents that inhibit the VEGF receptor (VEGFR) can effectively inhibit tumor growth *in vivo* in animal models [7, 11, 22, 28]. However, even after prolonged administration of antiangiogenic agents, complete eradication of tumor cells is not achieved [27]. In addition, angiogenesis is essential for adipose tissue growth [36], and a VEGFR inhibitor was shown to be associated with thrombotic or hemorrhagic complications and hypertension after prolonged use [18]. Therefore, antiangiogenic agents used as monotherapy are not the anticancer panacea that they were once thought to be [27].

The copper ion is an essential element for angiogenesis [4, 5, 30, 32, 49] since Cu is a cofactor of VEGF and bFGF. It is thought that copper deficiency inhibits angiogenesis, resulting in deprivation of the supply of oxygen and nutrients for proliferation of tumor cells. Animal tumor model studies have been carried out using an anti-copper approach by feeding animals a low-Cu diet and/or using copper-chelating agents such as D-penicillamine, trientine, and tetrathiomolybdate (TM). It has been reported that anti-copper treatments inhibit development of a variety type of tumors *in vivo* in mouse, rat and rabbit models [29, 44, 45, 47, 48]. Recently, we have also reported that treatment with trientine inhibits tumor growth and induces apoptosis in tumor cells in a murine transplantation model using fibrosarcoma [15]. However, a phase II trial using low-Cu diet and D-penicillamine showed that the treatment did not improve survival of patients with neuroblastoma multiform, although the serum copper level was reduced [3]. In phase I and phase II clinical trials, it has been shown that oral administration of TM resulted in the induction of mild copper deficiency, and half of the patients showed a decrease in vascularity and increase in necrosis of tumor masses, although the disease progressed in half of the patients [6, 34]. These results suggest that monotherapy using anti-copper agents is also impractical and that a combination of anti-copper treatment and conventional tumor treatments such as radiotherapy is necessary to obtain a clinically significant benefit. However, the combined effects of anti-copper treatment and radiation on tumor growth remain unclear.

In the present study, we examined the combined effects of treatment with trientine and X-irradiation on development of fibrosarcoma using a murine trasplantation model *in vivo* and on cellular survival *in vitro*.

MATERIALS AND METHODS

Cells: C57BL/6 mouse fibrosarcoma-derived transplantable QRsp-11 cells [26] were kindly provided by Dr. F.

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Okada of Yamagata University. The mouse endothelial cell line CRL-2161 and bovine brain endothelial cells (BBMC cells) were obtained from American Type Culture Collection (Manassas, VA, U.S.A.) and Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan), respectively. QRsp-11 cells were maintained in Eagle's MEM supplemented with 8% fetal calf serum (FCS), 1 mM sodium pyruvate, non-essential amino acids (Gibco), and 2 mM L-glutamine. CRL-2161 and BBMC cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FCS. The cell cultures were kept at ambient humidity and 37°C in an atmosphere containing 5% CO_2.

Mice and treatment: Specific pathogen-free male C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan) at 4 weeks of age. All research protocols were approved by the Animal Research Committee of the School of Veterinary Medicine, Rakuno Gakuen University. All mice were maintained under conditions described previously [15]. Sixty male mice were used in the present study. Triethylenetetramine dihydrochloride (trientine) (Sigma-Aldrich Co., St. Louis, Mo. U.S.A.) was administered orally to 35 mice from 5 to 10 weeks of age at a dosage of 500 mg/kg twice a week. Acute or subacute toxicity was not observed under the conditions used for administration of trientine in the present study (data not shown).

To transplant tumors, 1×10^6 QRsp-11 cells were subcutaneously injected into the flank of each mouse at 6 weeks of age. The tumor volume was calculated each day using a caliper in 2 dimensions as described by Yoshii *et al.* [46].

X-irradiation: X-irradiation was carried out utilizing a Hitachi MBR-1520R X-ray generator operating at 150 kV and 15 mA with a 0.5-mm Cu + 1.0-mm Al filter at a dose rate of 0.95 Gy/min. For *in vivo* irradiation, only the tumors of mice were irradiated by shielding the body other than tumors with lead. Mice were exposed to 3 Gy of X-rays at 10 days postinoculation (d.p.i.) of tumor cells or 3 Gy each at 10 and 12 d.p.i. (total at 6 Gy).

Clonogenic assay: Cell survival was determined using the conventional colony-forming assay. Propagated cells were collected by trypsinization and $2–50 \times 10^2$ cells were plated into 6-cm dishes. After X-irradiation, the cells were incubated for one week in the presence or absence of trientine, and then colonies were methanol-fixed and stained with May-Grunwald and Giemsa. For treatment with trientine prior to X-irradiation, the cells were incubated in the presence or absence of trientine for one week and collected. The viable cells were replated, X-irradiated, and then incubated for 1 week in the absence of trientine. Colonies containing more than 50 cells were counted as survivors.

Measurements of copper, iron and zinc: The tumor, liver, kidney and serum were obtained from four to six untreated and trientine-treated mice at 12 d. p. i. The tissues were washed repeatedly in cold saline solution. Fresh samples were frozen with liquid nitrogen and stored at –80°C until used. The mouse tissues were digested with nitric acid and perchloric acid as described previously [14]. Metal contents in the tissues were determined using an atomic absorption spectrophotometer (Perkin Elmer AAnalyst 800, Perkin Elmer Life Sciences, Shelton, CT, U.S.A.) with an air/acetylene flame or with a graphite furnace.

Statistical analysis: All data are expressed as means ± standard deviation. Differences between means were analyzed statistically by Student's *t*-test. Values of $P<0.05$ and $P<0.01$ were considered significant.

RESULTS

Combined effects of trientine and X-irradiation on tumor growth in vivo: Fibrosarcoma-derived transplantable QRsp-11 cells were subcutaneously injected into C57BL/6 mice. In the case of untreated mice, tumor volumes rapidly increased from 10 to 24 days postinoculation (d.p.i.) of tumor cells (Fig. 1). Tumor volumes increased more slowly in the trientine-treated mice than in the untreated mice from 10 to 24 d.p.i. Tumor volumes in the treated mice were significantly smaller than those in the untreated mice at 24 d.p.i. These results are essentially in good agreement with results of our recent study [15]. Tumor volumes increased more slowly in the mice X-irradiated at 3 and 6 Gy than in the unirradiated mice from 10 to 24 d.p.i. Tumor volumes in

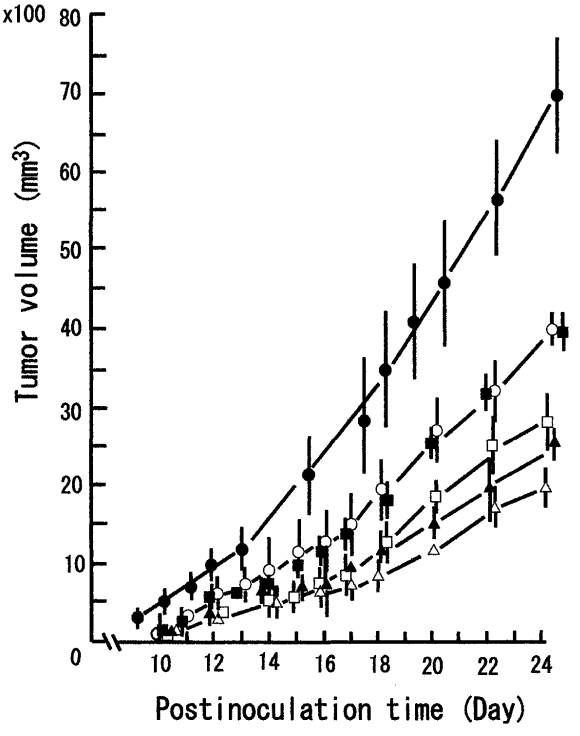

Fig. 1. Combined effects of trientine and X-irradiation on tumor development. QRsp-11 cells (1×10^6) were injected subcutaneously into untreated (closed symbols) and trientine-treated (open symbols) mice. The mice were exposed to X-rays at 0 (○, ●), 3 (□, ■) and 6 Gy (△, ▲). Tumor volumes were determined using calipers at the indicated time points. Mice were exposed to 3 Gy of X-rays at 10 d.p.i. or 3 Gy each at 10 and 12 d.p.i. (total 6 Gy). Points represent averages obtained from 5–8 separate experiments (± standard deviation).

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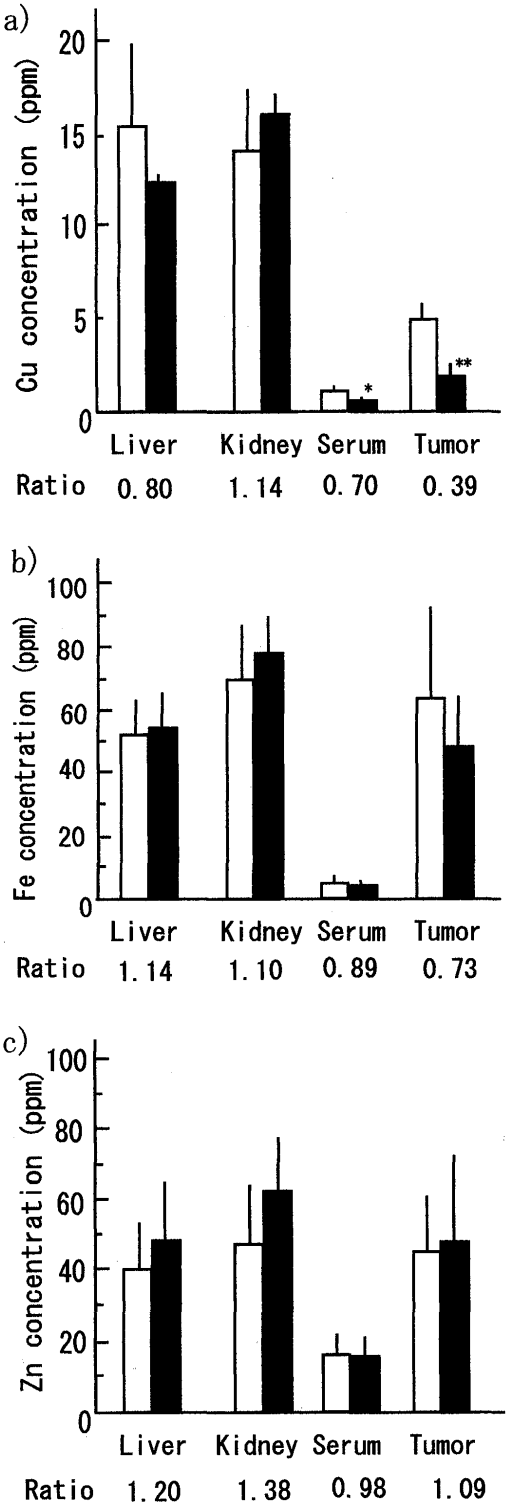

Fig. 2. Metal contents in the tissues. Copper (a), iron (b) and zinc (c) contents in the liver, kidney, serum and tumor from untreated mice (□) and trientine-treated mice (■) at 12.d.p.i. * Represents a significant difference between the untreated group and trientine-treated group (*: $p<0.05$ and ** $p<0.01$). Ratio represents metal contents of tissues from treated mice to those from untreated mice. Averages obtained from 4–6 separate experiments are shown (± standard deviation).

the irradiated mice were significantly smaller than those in the unirradiated mice at 24 d.p.i. No significant differences were found between the tumor growth curves in the 3-Gy-irradiated mice and the trientin-treated mice. Tumor volumes increased more slowly in the trientine-treated and X-irradiated mice than in the mice that received X-irradiation alone or treatment with trientine alone from 10 to 24 d.p.i. The tumor growth curve in the mice that received combined treatment with trientine and 3 Gy irradiation was similar to that in the 6-Gy- irradiated mice.

Metal contents: To examine the effects of administration with trientine on metal contents in the tissues of mice, we obtained tumors, livers, kidneys and serum from untreated and trientine-treated mice at 12 d.p.i. and then measured metal contents. Copper contents in the tumor and serum of treated mice were significantly lower than those of untreated mice (Fig. 2a). The ratios of copper contents in the tumor and serum of treated mice to those of untreated mice were 0.39 and 0.7, respectively. No significant differences were found between copper contents in the liver and the kidney of untreated and treated mice.

Copper is a catalytic cofactor for ceruloplasmin (Cp) [33]. The ferroxidase activity of Cp mediates the oxidation of ferrous ions to the ferric state. It has been thought that absorption of copper interacted with absorption of zinc in the intestine [37]. Therefore, we measured iron and zinc contents in the tissues of untreated and treated mice. No significant differences were found between iron contents or zinc contents in the tumor, liver, kidney and serum of untreated and treated mice (Fig. 2b and c).

Combined effects of trientine and irradiation on cellular survival in vitro: Since it has been reported that sensitivity of endothelial cells to radiation affects radiosensitivity of the tissues [20, 31], combined effects of trientine and irradiation on survival of endothelial cells and tumor cells were examined. Mouse endothelial cells (CRL-2161) and bovine primary endothelial cells (BBMC) showed a higher sensitivity to trientine than did QRsp-11 cells [15]. BBMC cells has a higher radiosensitivity than did CRL-2161 and Qrsp-11 cells (Fig. 3 and data not shown). To compare the combined effects of trientine and X-irradiation on cellular survivals in similar surviving fractions of each cell type, QRsp-11 cells were treated with trientine at 1 mM and CRL-2161 and BBMC cells were treated with trientine at 0.1 mM. The surviving fractions were 0.25 for QRsp-11, 0.34 for CRL-2161, and 0.29 for BBMC cells (Fig. 3). When the cells were treated with trientine after X-irradiation, the surviving fractions of the cells with combined treatments were lower than those of the cells with trientine treatment alone and with X-irradiation alone. The surviving fractions of the cells with combined treatments were essentially consistent with the products of the surviving fractions of the trientine-treated cells and those of X-irradiated cells. Furthermore, the surviving fractions of 3-Gy-irradiated and trientine-treated cells were similar to those of 6-Gy-irradiated cells. When the cells were treated with trientine for 1 week, and viable cells were replated and then X-irradiated, the surviving frac-

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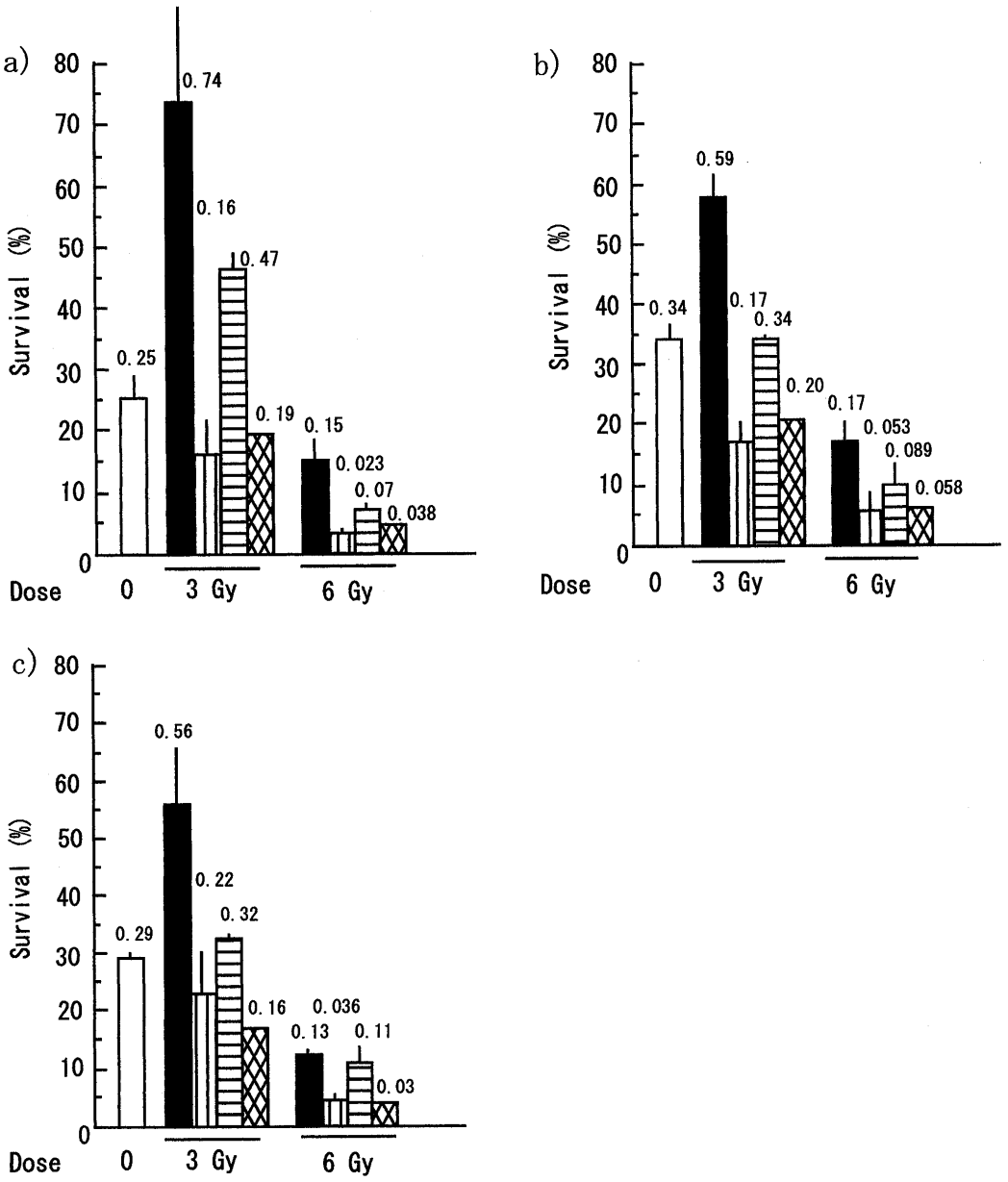

Fig. 3. Combined effects of trientine and X-irradiation on cellular survival in a clonogenic assay. QRsp-11 (a), CRL-2161 (b) and BBMC (c) cells were treated with trientine at 1 mM, 0.1 mM and 0.1 mM, respectively. The cells were subjected to treatment with trientine alone (□), treatment with X-irradiation alone (■), treatment with trientine after X-irradiation (▥) and pretreatment with trientine and X-irradiation (▤). Products of the surviving fractions of trientine-treated cells and those of X-irradiated cells (▩). Numbers in the figures are surviving fractions of the cells. Averages obtained from 4–5 separate experiments are shown (± standard deviation).

tions of the X-irradiated cells after pretreatment with trientine were lower than those of the X-irradiated cells without trientine treatment (Fig. 3).

DISCUSSION

Therapies that aim to destroy tumor vasculature by anti-copper treatments have been tested since Cu has been shown to be required for angiogenesis, [3, 6, 34, 39, 48]. However, it has been reported that the results of phase I and phase II clinical trials have not shown a clinically significant benefit [3, 6, 34]. Therefore, it is thought that a combination of anti-copper treatment and conventional tumor treatments such as radiotherapy is necessary to obtain an effective response to tumor treatment. Trientine is an effective medicinal copper-chelating agent for patients with human Wilson disease, which is characterized by hepatic copper accumulation [42]. Copper contents in the tumor and the serum of trientine-treated mice were significantly lower than those of untreated mice. However, no significant dif-

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ferences were found between copper contents in the liver and kidney of untreated and treated mice. Since it has been thought that Cu binds proteins such as metallothionein in the liver and kidney and trientine mainly binds free Cu but not protein-associated Cu [16], hepatic and renal copper contents may not be decreased by treatment with trientine. On the contrary, a decrease in serum copper content may result in a decrease in transport of copper into the tumor. Although it has been reported that copper interacts with other metal metabolism [33, 37], no significant differences were found between iron contents or zinc contents in the tumor, liver, kidney and serum of untreated and treated mice.

In the present study, we showed that the tumor volumes of mouse fibrosarcoma QRsp-11 cells increased more slowly in the trientine-treated mice or in the X-irradiated mice than in the control mice from 10 to 24 d.p.i. Thus, tumor growth was suppressed by trientine and X-irradiation. The extent of inhibition of tumor growth by X-irradiation at 3 Gy was similar to that obtained by treatment with trientine. A combination of trientine and X-irradiation at 3 Gy showed inhibitory effects on tumor growth similar to those obtained by X-irradiation at 6 Gy on tumor growth. These results showed that trientine and X-irradiation interacted additively in inhibition of tumor growth under the conditions used in the present study. It has been shown that copper deficiency induces apoptosis in a variety of cells *in vitro* and *in vivo* [1, 17, 21, 25, 48]. We have shown that treatment with trientine induced apoptosis in tumor cells at an early stage of tumor development [15]. Thus, induction of apoptosis by trientine and cell killing by X-irradiation may occur independently in the tumor. It is thought that inhibition of angiogenesis leads to a deficiency of blood supply and results in an increase in the proportion of hypoxic cells in large solid tumors. Although it is well known that hypoxic cells are more radioresistant than are oxygenated cells, the present study suggests that treatment with trientine does not affect the radiosensitivity of cells *in vivo*.

Although it has been reported that sensitivity of endothelial cells to radiation affects radiosensitivity of the tissues [20, 31], combined effects of radiation and antiangiogenic agents are still controversial. *In vitro* studies showed that the combination produced increase in cytotoxicity on endothelial cells [22]. Anginex, antiangiogenic peptide, showed an endothelial cell-specific radiosensitizing activity [8]. On the contrary, radiation induces vascular cytokines, such as VEGF [19], which functions as a powerful antiapoptotic factor for endothelial cells in new blood vessels [2, 40]. Thus, radiation-induced VEGF might result in tumor radioresistance through vascular radioprotection [23, 24]. In the present study, when mouse endothelial cells, bovine primary endothelial cells and QRsp-11 cells were treated with trientine after X-irradiation, the surviving fractions of the cells with combined treatments were essentially consistent with the products of the surviving fractions of the trientine-treated cells and those of X-irradiated cells. These results suggest that trientine and X-irradiation act on induction of cell death independently *in vitro*. These *in vitro* results are in good agreement with our *in vivo* observations. On the other hand, when the cells were pretreated with trientine and then X-irradiated, the surviving fractions of the X-irradiated cells after treatment with trientine were lower than those of the X-irradiated cells without trientine treatment. These results showed that pretreatment with trientine sensitized the cells to X-irradiation. Copper is a catalytic cofactor for Cu,Zn superoxide dismutase (Cu,Zn SOD), which catalyzes dismutation of superoxide anion, and cytochrome c oxidase (Cytox). Impairment of Cytox may lead to the production of partially reduced oxygen species [9]. X-irradiation produces reactive oxygen species (ROS) such as superoxide anion in cells. Copper deficiency may increase the production of ROS and reduce the antioxidant ability of cells [9, 21, 35]. Therefore, pretreatment of cells with trientine may result in an increase in radiosensitivity. A study on combined effects of trientine and X-irradiation on angiogenesis in tumor tissues is now in progress.

The present study showed that trientine and X-irradiation interacted additively in inhibition of tumor growth of fibrosarcoma in a mouse transplantation model and induction of cell death *in vitro*. A combination of treatment with trientine and X-irradiation may reduce the radiation dose required to suppress tumor growth, resulting in a decrease in occurrence of deleterious side effects of radiation, and provide clinical benefits for tumor treatments.

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