FULL PAPER *Laboratory Animal Science*

Trientine, a Copper-Chelating Agent, Induced Apoptosis in Murine Fibrosarcoma Cells *In Vivo* and *In Vitro*

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ABSTRACT. Anti-copper treatments have been investigated to determine whether they suppress angiogenesis and tumor development since Cu is widely accepted as being required for angiogenesis. We examined the effects of treatment with trientine, a copper-chelating agent, on tumor development in a murine xenograft model using fibrosarcoma-derived transplantable QRsp-11 cells and C57BL/6 mice and induction of apoptosis in tumor cells and endothelial cells *in vivo* and *in vitro*. The tumor volumes increased more slowly in trientine-treated mice than in untreated mice. Tumor volumes in the treated mice were significantly smaller than those in the untreated mice at 24 days postinoculation (d.p.i.) of tumor cells. A cluster of pyknotic tumor cells and morphological abnormalities in capillary endothelial cells were observed in the tumors of trientine-treated mice but not in the tumors of untreated mice. The proportions of apoptotic and necrotic cells in the tumors of treated mice were approximately 3.5-fold higher than those in the tumors of untreated mice at 14 d.p.i. When the cells were treated with trientine *in vitro*, mouse endothelial cells and bovine primary endothelial cells showed an approximately 10-fold higher sensitivity to trientine than QRsp-11 cells in terms of D_{37}. However, the proportion of apoptotic cells in endothelial cells was significantly lower than that in QRsp-11 cells after treatment with trientine. These results show that apoptosis was induced in tumor cells by treatment with trientine *in vivo* and *in vitro*.

KEY WORDS: anti-copper, apoptosis, fibrosarcoma, trientine, xenograft.

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It is now widely established that a rate-limiting step in tumor growth is the ability to recruit new blood vessels from host tissues [12]. Any solid tumors that have not acquired their own new blood supply cannot grow to more than a few millimeters in size [2, 11]. Therefore, therapies have been tested with aim of destroying tumor vasculature. The copper ion is an essential key element in several physiological and pathological events [10]. It has been demonstrated that Cu is required for angiogenesis [5, 14, 21, 22, 36]. Cu is a cofactor required for the function of several angiogenesis mediators, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) [1, 9, 27, 30, 33]. Cu stimulates proliferation of endothelial cells in culture, which plays a pivotal role in the angiogenesis process [5, 14]. In human malignancies, abnormal increases in Cu levels in serum and tumor tissues have been found in various types of tumors [4, 7, 13]. It is thought that copper deficiency inhibits angiogenesis, resulting in deprivation of 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). A low-Cu diet and treatment with D-penicillamine have been shown to inhibit the development of intracerebral tumors in the rat and rabbit [31, 32]. It has been shown that copper deficiency induced by TM suppresses tumor growth and angiogenesis in murine models of breast cancer [20]. Tumor development was suppressed and the proportion of apoptotic cells was increased in murine hepatocellular carcinoma by a low-Cu diet and treatment with trientine [34]. Trientine attenuated colorectal carcinoma development and angiogenesis in mice [35]. The results of phase I and II clinical trials using TM have been reported for metastatic and advanced kidney cancer [6, 24]. Oral administration of TM resulted in the induction of mild copper deficiency and the establishment of a stable disease in the patients. 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. 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].

It has been shown that copper deficiency induces apoptosis in a variety of cells *in vitro* and *in vivo* [15–18, 25, 34, 35]. Although apoptosis was induced *in vivo* by treatment with trientine at a late stage of tumor development [34, 35], whether apoptosis can be induced at an early stage remains unclear. Furthermore, whether apoptosis is induced in endothelial cells by anti-copper treatment *in vivo* remains unknown.

In the present study, we examined the effect of treatment with trientine on development of fibrosarcoma in a murine xenograft model and induction of apoptosis in tumor cells and endothelial cells *in vivo* and *in vitro*.

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MATERIALS AND METHODS

Cells: C57BL/6 mouse fibrosarcoma-derived transplantable QRsp-11 cells [18] were kindly provided by Dr. F. Okada of Yamagata University. 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. The mice were housed at a temperature 25 ± 2°C and exposed to a daily cycle of 14 hr of light and 10 hr of darkness. A solid diet (MF-Food, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided *ad libitum*. Forty male mice were used in the present study. Triethylenetetramine dihydrochloride (trientine) (Sigma-Aldrich Co., St. Louis, Mo. U.S.A.) was administered orally to 20 mice from 5 to 10 weeks of age at a dosage of 500 mg/kg twice a week. Acute and subacute toxicity was not observed under the conditions used for administration of trientine in the present study (data not shown).

To create the xenograft model, 5×10^5 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.* [33].

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 one week of incubation in the presence of trientine at concentrations from 0.025 to 10 mM, colonies were methanol-fixed and stained with May-Grunwald and Giemsa. Colonies containing more than 50 cells were counted as survivors.

Flow cytometory: Apoptotic cells were estimated using an Annexin V-FITC Apoptosis Detection Kit (BioVision, Inc., Mountain View, CA, U.S.A.) for tumors and a MEBSTAIN Apoptosis Kit Direct (Medical and Biological Laboratories Co., Ltd., Nagoya, Aichi, Japan) for *in vitro* cultured cells according to the manufacturers' instructions. Briefly, single-cell suspensions were prepared for tumors by pressing the tumors through wire mesh screens and then two passages through a 25-gauge needle. The cells were washed several times with PBS containing 0.2% bovine serum albumin (BSA) and stained with Annexin V-FITC and propidium iodide. Fluorescence was measured with a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA, U.S.A.) using a 530-nm filter by excitation at 480 nm. The percentages of apoptotic and necrotic cells were determined using the Multicycle software (Phoenix Flow Systems, San Diego, CA, U.S.A.). In the case of cultured cells, trientine-treated cells (1×10^6) were collected by trypsinization and washed several times with PBS containing 0.2% BSA. The cells were fixed with 0.1 M NaH_2PO_4 containing 4% paraformaldehyde at 4°C for 30 min, washed 2 times with PBS containing 0.2% BSA, and then pelleted by centrifugation at 500 × g. The cells were fixed in 5 m*l* of cold 70% ethanol for 30 min at room temperature and stored at –20°C. Just prior to flow cytometric analysis, individual samples were labeled with fluorescein-dUTP. Flow cytometric analysis was carried out as described above for tumors.

Histopathology: Tumor tissues approximately 0.5 cm × 0.5 cm in size were isolated, fixed in Carnoy's solution for 30 min, dehydrated, and then embedded in paraffin. Five μm-thick sections were then stained with hematoxylin and eosin (HE) for histopathological examinations.

Statistical analysis: All data were 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

Effects of trientine on tumor development and induction of apoptosis in vivo: When fibrosarcoma-derived transplantable QRsp-11 cells were subcutaneously injected into C57BL/6 mice, no significant difference was found between the tumor volumes of untreated and trientine-treated mice until 12 days postinoculation (d.p.i.) of tumor cells (Fig. 1). In the case of untreated mice, tumor volumes rapidly increased from 15 to 24 d.p.i. Tumor volumes increased more slowly in the trientine-treated mice than in the untreated mice from 15 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.

Since it is thought that tumor size affects histopathology, samples were isolated from tumors of almost the same size (5 mm × 5 mm) for untreated and treated mice at 14 d.p.i. A cluster of pyknotic cells was found in the tumors of the trientine-treated mice but not in the tumors of the untreated mice (Figs. 2A-D). Morphological abnormalities were found in capillary endothelial cells of treated mouse tumors but not in untreated mouse tumors (Figs. 2E and F). No pyknotic capillary endothelial cells were found in either untreated or treated mouse tumors. To determine whether the pyknotic cells represented apoptotic cells, the proportions of apoptotic and necrotic cells were analyzed using a flow cytometer. Since it is thought that tumor size affects the incidence of apoptosis and necrosis, cells were prepared from tumors of almost the same size (5 mm × 5 mm) for untreated and treated mice at 14 d.p.i. The proportions of apoptotic and necrotic cells in the tumors of the treated mice

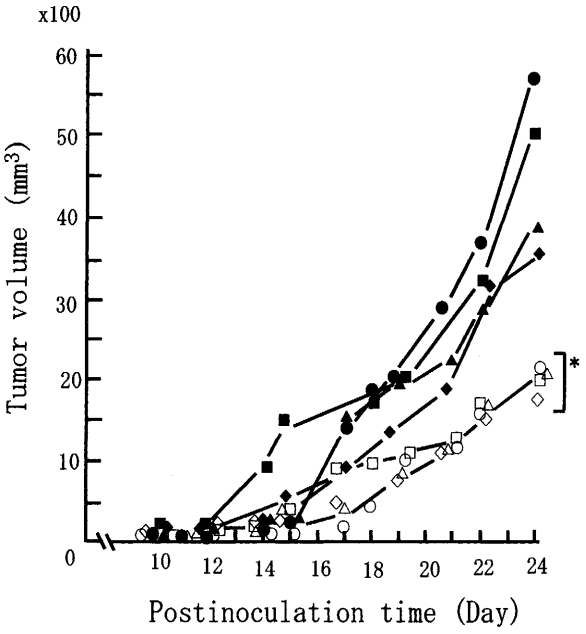

Fig. 1. Effects of trientine on tumor development. QRsp-11 cells (5×10^5) were injected subcutaneously into 4 untreated (closed symbols) and 4 trientine-treated mice (open symbols). The tumor volumes were determined using calipers at the indicated time points. * Represents a significant difference compared to the untreated group ($p<0.05$).

were approximately 3.5-fold higher than those in the tumors of the untreated mice (Table 1).

Effects of trientine on cell survival and induction of apoptosis in vitro: When cells were treated *in vitro* with trientine, the surviving fraction decreased in a dose-dependent manner at concentrations from 0.025 to 10 mM (Fig. 3). Mouse endothelial cells (CRL-2161) and bovine primary endothelial cells (BBMC) showed a higher sensitivity to trientine than QRsp-11 cells. When the concentration of trientine required to reduce cell survival to 37% (D_{37}) was used as an index to compare cellular sensitivities, the D_{37} values of CRL-2161 and BBMC cells were approximately 0.1 mM and the D_{37} value of QRsp-11 cells was approximately 1 mM. Thus, endothelial cells were approximately 10-fold more sensitive to trientine than QRsp-11 cells. To determine whether treatment of cells with trientine induced apoptosis *in vitro*, cells were incubated with trientine and collected after 1 to 7 days of incubation. To compare induction of apoptosis in similar surviving fractions of each cell type, QRsp-11 cells were treated with trientine at 10 mM and CRL-2161 and BBMC cells were treated with trientine at 1 mM. The surviving fractions were 7.3% for QRsp-11, 16.7% for CRL-2161, and 9.3% for BBMC cells. In the case of QRsp-11 cells, apoptotic cells appeared after 3 days of incubation and increased in an incubation time-dependent manner from days 3 to 6. The proportions of apoptotic cells were around 60% of total cells on days 6 and 7 (Fig. 4). In contrast, no apoptotic cells were found in trientine-treated BBMC cells until 7 days of incubation. The proportion of apoptotic cells in CRL-2161 cells was slightly higher than the proportions in QRsp-11 and BBMC cells on day 0 and increased from days 1 to 4 but not from days 4 to 7. The proportions of apoptotic cells in CRL-2161 cells were approximately 25% from days 4 to 7 and were significantly lower than those in QRsp-11 cells.

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, 24, 28, 35]. Trientine is an effective medicinal copper-chelating agent for patients with human Wilson disease, which is characterized by hepatic copper accumulation [29]. In the present study, we showed that the tumor volumes of mouse fibrosarcoma QRsp-11 cells increased more slowly in trientine-treated mice than in untreated mice from 15 to 24 d.p.i. Thus, tumor growth was suppressed at later stages by trientine. A cluster of pyknotic cells was found in the tumors of trientine-treated mice but not in the tumors of untreated mice at 14 d.p.i. Flow cytometric analysis showed that the pyknotic cells were apoptotic cells. Although it is thought that inhibition of angiogenesis leads to deficiency of blood supply and results in an increase in anoxic tumor cells and induction of necrosis in large solid tumors, the present study showed that treatment with trientine induced apoptosis even when no significant differences were observed between the tumor volumes of the untreated and trientine-treated mice. Furthermore, the proportions of apoptotic cells were about 3-fold higher than those of necrotic cells in both the tumors of trientine-treated and untreated mice. Therefore, the slower increase in tumor volume in the treated mice at the late stages of tumor development might have been due, at least in part, to induction of apoptosis in the tumor cells at an earlier stage by trientine, although the proportion of necrotic cells was also increased by the treatment. On the other hand, the morphology of the capillary endothelial cells was abnormal; however, no pyknotic endothelial cells were observed.

Although mouse endothelial cells and bovine primary endothelial cells showed higher sensitivity to copper-deficiency than QRsp-11 cells in our clonogenic assay, endothelial cells treated with trientine *in vitro* showed greater resistance to trientine in induction of apoptosis than QRsp-11 cells. These results suggest that copper deficiency inhibits cellular proliferation of endothelial cells but does not induce apoptosis efficiently, although the possibility that apoptosis of endothelial cells may be induced by treatment with trientine at high concentrations cannot be ruled out. In contrast, QRsp-11 cells were resistant to trientine in our clonogenic assay but were sensitive to trientine in induction of apoptosis. These *in vitro* results are in good agreement with our *in vivo* observations.

Copper is a catalytic cofactor for Cu,Zn superoxide dismutase (Cu,Zn SOD) and ceruloplasmin (Cp) [23]. Cu,Zn SOD catalyzes dismutation of superoxide anion. The fer-

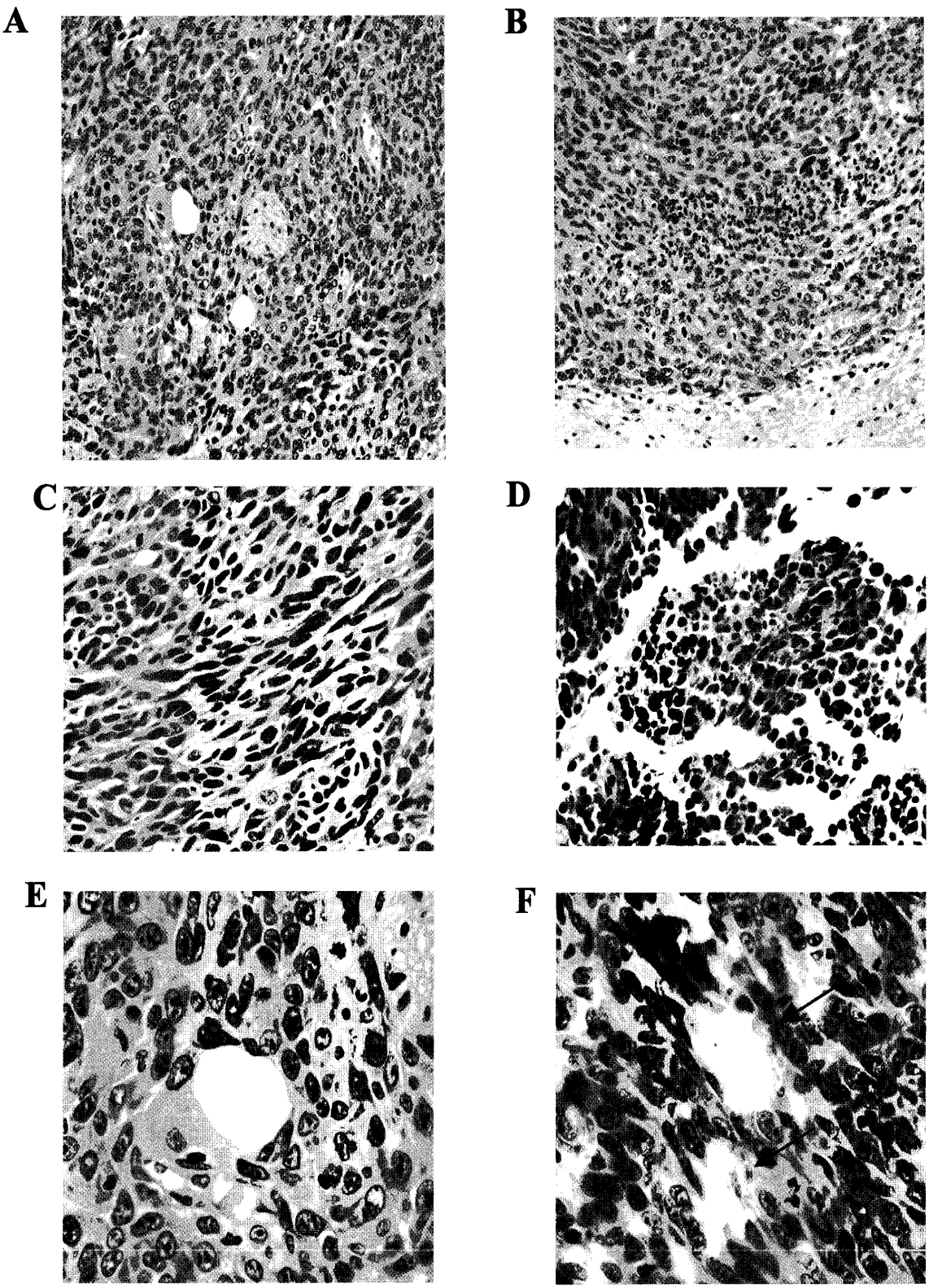

Fig. 2. Tumor tissues of mice at 14 d.p.i. (HE). A, C, and E: tumor tissues from untreated mice. B, D, and F: tumor tissues from trientine-treated mice. A cluster of pyknotic cells is shown in B and D. Arrows indicate typical morphological abnormalities of capillary endothelial cells in F.

Table 1. Induction of apoptosis *in vivo* by treatment with trientine

	Proportion of cells (%)	
	Untreated	Treated with trientine
Apoptotic cells	3.5 ± 2.0	11.96 ± 3.65
Necrotic cells	1.10 ± 0.44	3.94 ± 2.18
Normal cells	93.1 ± 1.64	83.1 ± 7.95

Tumor cells were isolated from untreated and trientine-treated mice at 14 d.p.i. Proportions of apoptotic and necrotic cells were determined using a flow cytometer, Annexin V-FITC, and propidium iodide. Each value represents the average obtained from 5–6 separate experiments (± SD).

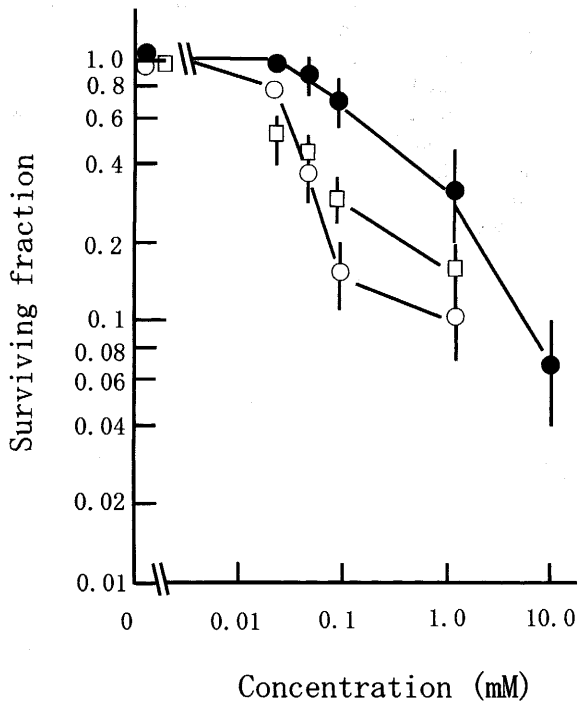

Fig. 3. Effects of trientine on cellular survival in a clonogenic assay. CRL-2161 (□), BBMC (○), and QRsp-11 (●) cells were treated with trientine at various concentrations. Points represent averages obtained from 5 separate experiments (± standard deviation). Standard deviations were within the symbols at some points.

roxidase activity of Cp mediates the oxidation of ferrous ions to the ferric state, thereby preventing ferrous ion-dependent formation of hydroxyl radicals *via* the Fenton reaction. Furthermore, copper is a cofactor of cytochrome c oxidase (Cytox). Impairment of Cytox may lead to the production of partially reduced oxygen species [8]. Therefore, copper deficiency may increase the production of reactive oxygen species (ROS) and reduce the antioxidant ability of cells [8, 26]. It is known that ROS induces apoptosis in a variety of cells. Therefore, apoptosis may be induced by treatment with copper-chelating agents. A study aimed at determining which signal transduction pathway is involved in induction of apoptosis is now in progress. However, the reason for why endothelial cells showed greater resistance

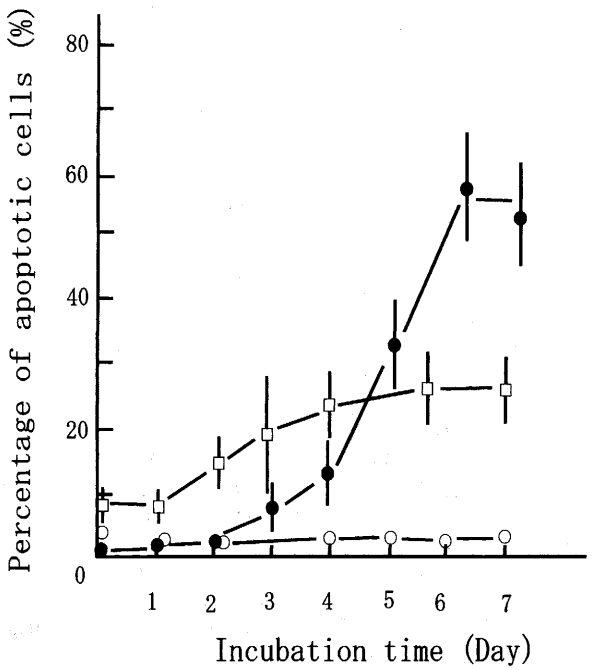

Fig. 4. Effects of trientine on induction of apoptosis *in vitro*. CRL-2161 (□), BBMC (○) and QRsp-11 (●) cells were treated with trientine at 1 mM, 1 mM and 10 mM, respectively. Points represent the average obtained from 4-5 separate experiments (± SD). Standard deviations were within the symbols at some time points.

to trientine in induction of apoptosis remains unknown, although cellular proliferation was effectively inhibited.

The present study showed that treatment with trientine, a copper-specific chelating agent, inhibits the growth of fibrosarcoma in a mouse xenograft model and that trientine induces apoptosis in tumor cells *in vivo* and *in vitro*. Although the results of phase I and II clinical trials have been reported, a clinically significant benefit has not been shown [3, 6, 24]. The sensitivity of tumor cells to anti-copper agents in induction of apoptosis may affect the efficiency of the treatment.

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