NOTE *Laboratory Animal Science*

Trientine, a Copper-Chelating Agent, Induced Apoptosis in Murine Fibrosarcoma Cells by Activation of the p38 MAPK Pathway

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(Received 15 May 2009/Accepted 17 July 2009)

ABSTRACT. We have reported that treatment with trientine, Cu-chelating agent, inhibits tumor growth in a murine transplantation model using fibrosarcoma and induces apoptosis in tumor cells *in vivo* and *in vitro*. When fibrosarcoma cells were treated with 10 mM trientine, the activities of p38 MAPK in treated cells were approximately 3–4 times higher than those in untreated cells. Proportions of cells in which apoptosis was induced by trientine increased in an incubation time-dependent manner from days 2 to 6. The proportions of apoptotic cells in the cells treated with trientine and SB203580, an inhibitor of p38 MAPK, were approximately 50% in those of cells treated with trientine alone. The present results showed that the p38 MAPK pathway may play an important role in induction of apoptosis in fibrosarcoma cells by trientine.

KEY WORDS: apoptosis, Cu-chelator, fibrosarcoma, p38 MAPK, trientine.

J. Vet. Med. Sci. 71(11): 1541–1544, 2009

It is now widely established that a rate-limiting step in tumor growth is the ability to recruit new blood vessels from host tissues [15]. Solid tumors that have not acquired their own new blood supply cannot grow to more than a few millimeters in size [3, 13]. Therefore, therapies have been tested with the aim of destroying tumor vasculature. Since 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, 11, 27], Cu is required for angiogenesis [18, 24, 30]. Cu stimulates proliferation of endothelial cells in culture, which plays a pivotal role in the angiogenesis process [5, 18]. It is thought that Cu 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-Cu approach by feeding animals a low-Cu diet and/or using Cu-chelating agents such as D-penicillamine, trientine, and tetrathiomolybdate. It has been reported that anti-Cu treatments inhibit development of a variety type of tumors *in vivo* in mouse, rat and rabbit models [23, 28, 29]. We have also reported that treatment with trientine inhibits tumor growth in a murine transplantation model using fibrosarcoma and that it induces apoptosis in tumor cells *in vivo* and *in vitro* [16]. Therefore, induction of apoptosis in the tumor cells by trientine may play role in inhibition of tumor growth, in addition to destroying tumor vasculature. Our study showed that trientine and X-irradiation interact additively in inhibition of tumor growth and induction of cell death *in vitro* [17]. Induction of apoptosis by trientine may reduce the radiation dose required to suppress tumor growth in radiation therapy, resulting in a decrease in occurrence of deleterious side effects of radiation, and provide clinical benefits for tumor treatments. Although it has been shown that Cu deficiency induces apoptosis in a variety of cells *in vitro* and *in vivo* [20, 21, 28, 29], the pathway that induces apoptosis by Cu deficiency remains to be elucidated. In the course of our study on the mechanisms of induction of apoptosis by Cu deficiency, we found that p38 mitogen-activated protein kinase (MAPK) plays a role in the induction of apoptosis in murine fibrosarcoma.

In the present study, we examined the effect of an inhibitor of p38 MAPK on induction of apoptosis by treatment with trientine in murine fibrosarcoma.

C57BL/6 mouse fibrosarcoma-derived transplantable QRsp-11 cells were kindly provided by Dr. F. Okada of Yamagata University [22]. QRsp-11 cells were maintained as described previously [16]. SB203580 (Wako Chemicals Co., Osaka, Japan), an inhibitor of p38 MAPK, and SP600125 (Calbiochem Co., Dormstadt, Garmany), c-jun N-terminal kinase (JNK) inhibitor II, were dissolved in dimethyl sulfoxide (DMSO). The cells were incubated in a medium containing 10 mM trientine in the absence or presence of 40 μM SB203580 or 10 μM SP600125 for 1 to 6 days. The doses of the inhibitors were selected on the basis of results of previous studies in our laboratory.

Activities of p38 MAPK and JNK were measured using a p38 MAP Kinase Assay Kit and an SAPK/JNK Assay Kit (Cell Signaling Thechnology™ Co., Beberty, U.S.A.), respectively, according to the manufacturer's protocol. Briefly, cells were washed with PBS and lysed by sonication in cell lysis buffer. A modified Lowry assay was used to quantify relative protein levels in the samples. Equal amounts of proteins were used for each assay. An immobilized phospho-p38 MAP kinase (Thr180/Tyr182) monoclonal antibody bead and c-Jun fusion protein bead were used for immunoprecipitation of p38 MAPK and JNK, respectively. After incubation overnight at 4°C, the beads

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were precipitated by centrifugation at 14,000 × g for 30 sec at 4°C. The pellets were washed with lysis buffer and suspended in 50 μl kinase buffer supplemented with 200 μM ATP and 2 μg ATF-2 fusion protein (only for p38 MAPK assay), and then 25 μl SDS sample buffer (187.5 mM Tris-HCl, 6% SDS, 30% glycerol, 150 mM DTT, 0.03% BPB) was added. Proteins were resolved by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore Co., Bedford, U.S.A.) using an iBlot™ Dry Blotting System (Invitrogen Co., Carlsbad, U.S.A.). The membrane was incubated in blocking buffer (5% w/v nonfat dry milk in TBS/T) at room temperature for 2 hr and then with the primary antibodies at 4°C overnight. Proteins were detected by a Phototope®-HRP Western detection kit (Cell Signaling Thechnology™ Co.). The densities of the bands were determined using LumiVision PRO (Aisin Co., Kariya, Japan).

Proportions of apoptotic cells were estimated using an APOPercentage™ Apoptosis Assay kit (Biocolor Co., Brendale, Australia) according to the manufacturer's protocol. Briefly, after the cells had been incubated with 10 mM trientine with or without 40 μM SB203580 for 1 to 6 days, APOPercentage dye was added to the cell suspensions and the cell suspensions were incubated for 30 min at 37°C. The cells were washed twice with PBS and photographed under a light microscopy. The percentage of apoptotic cells was determined by counting the 200 cells in randomly selected fields of 5 independent regions.

All data were expressed as means ± standard deviation. Differences between means were analyzed statistically by two-factor repeated measure ANOVA. Values of $P<0.05$ were considered significant.

When fibrosarcoma-derived transplantable QRsp-11 cells were incubated with or without 10 mM trientine for 1 to 4 days, the activities of p38 MAPK in trientine-treated cells were approximately 3–4 (3.0 ± 0.5–3.9 ± 1.0) times higher than those in untreated cells (Fig. 1a). On the other hand, no significant differences were found between JNK activities in untreated and treated cells with trientine (Fig. 1b).

The QRsp-11 cells were incubated with trientine in the absence or presence of SB203580, an inhibitor of p38 MAPK, and the proportions of apoptotic cells were estimated. Apoptotic cells were stained in red in the present assay. As shown in Fig. 2a, treatment of cells with trientine induced apoptosis. Proportions of cells in which apoptosis was induced by trientine increased in an incubation time-dependent manner from days 2 to 6 (Fig. 2b). The proportions of apoptotic cells were around 40% of total cells on days 5 to 6. These results are essentially in good agreement with results of our previous study using flow cytometoric analysis [16]. To determine whether induction of apoptosis by trientine is associated with activation of p38 MAPK activity, cells were incubated with trientine and SB203580. The proportions of apoptotic cells were significantly lower in trientine and SB203580-treated cells than in cells treated with trientine alone (Fig. 2a and b). The proportions of apoptotic cells in trientine and SB203580-treated cells were approximately 50% of those in cells treated with trientine alone from days 2 to 5. There were no significant differences in proportion of apoptotic cells between cells treated with trientine alone and treated with trientine and SP600125, JNK inhibitor II (data not shown). The present results showed that treatment of fibrosarcoma with trientine activated p38 MAPK and that the p38 MAPK pathway played an important role in induction of apopotosis by trientine.

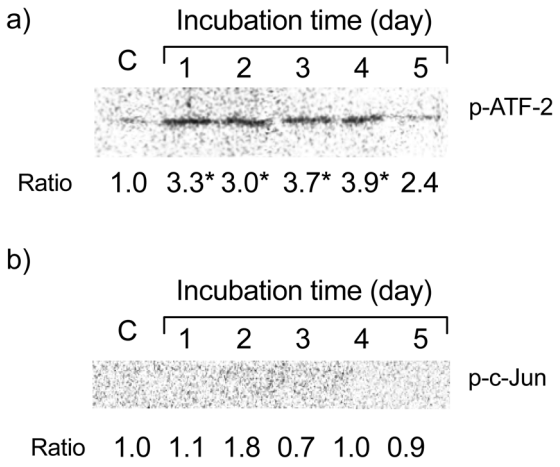

Fig. 1. Effects of trientine on activities of mitogen-activated protein kinase. QRsp-11 cells were treated with 10 mM trientine for 1 to 5 days and activities of p38 MAPK (a) and JNK (b) were measured. Ratio represents band intensities of the cells treated with trientine for each day to those of untreated control cells. C represents control. Averages of ratios were obtained from 3 separate experiments. * Represents a significant difference compared to control activities ($p<0.05$).

Therapies that aim to destroy tumor vasculature by anti-Cu treatments have been tested [4, 6] since Cu has been shown to be required for angiogenesis [18, 24, 30]. Trientine is an effective medicinal Cu-chelating agent for patients with human Wilson disease, which is characterized by hepatic Cu accumulation [26]. Previously, we showed that the tumor volumes of fibrosarcoma increased more slowly in trientine-treated mice than in untreated mice and that treatment with trientine induced apoptosis in the tumor cells *in vivo* and *in vitro* [16]. Therefore, it was suggested that the slower increase in tumor volume in treated mice might have been due, at least in part, to induction of apoptosis in the tumor cells by trientine.

MAPK cascades are required for many cellular functions, including cell growth, proliferation, differentiation, inflammation, apoptosis and malignant transformation [2, 7]. One of these cascades, p38 MAPK, is activated by exposures of cells to many types of stresses, including UV, chemotherapeutic agents and oncogenes. Activation of p38 MAPK in response to several anticancer agents is necessary and, in some cases, sufficient, to induce apoptosis in a variety of

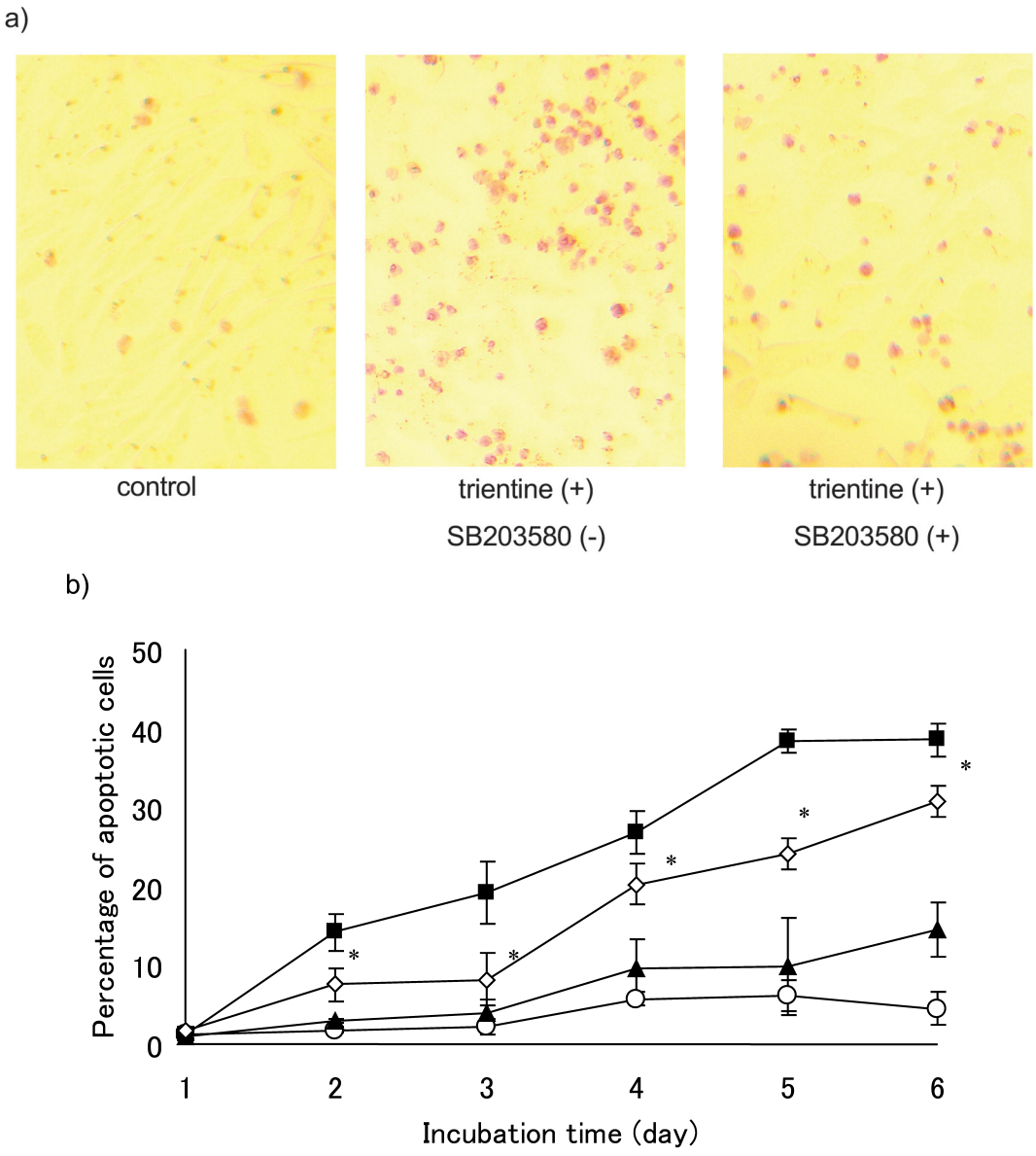

Fig. 2. Effects of SB203580 on trientine-induced apopotosis. a) QRsp-11 cells were incubated for 5 days without trientine (control) or with 10 mM trientine in the absence or presence of 40 μM SB203580 and stained with APOPercentage dye. b) Proportions of apoptotic cells incubated without trientine and SB203580 (–○–), with 40 μM SB203580 (–▲–), and with 10 mM trientine in the absence (–■–) or presence (–◇–) of 40 μM SB203580. Points represent averages obtained from 5 separate experiments (± standard deviation). * Represents a significant difference between the cells treated with trientine and SB203580, and cells with trientine alone (p<0.05).

cancer cell lines [8–10, 25]. This is the first report of trientine inducing apoptosis by activation of p38 MAPK, although it has been shown that Cu deficiency induces apoptosis in a variety of cells *in vitro* and *in vivo* [20, 21, 28, 29]. Furthermore, p38 MAPK has been reported to positively regulate several tumor suppressor pathways, such as replicative senescence and contact inhibition [12, 14, 19].

The present study suggested that treatment with trientine provides a clinical benefit by induction of apoptosis in tumor cells by activation of p38 MAPK, which may result in inhibition of tumor growth

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