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Synergistic antitumor effect of CXCL10 with hyperthermia

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Abstract

Purpose IFN–inducible protein 10 (IP-10)/CXCL10 (CXC chemokine ligand 10) has been described as an antiangiogenic chemokine and displays a potent antitumor activity in vivo. In the present study, we try to investigate whether the combination therapy of hyperthermia, a physical antiangiogenic modality, with CXCL10 would completely eradicate the established solid tumors.

Methods Immunocompetent BALB/c mice bearing Meth A fibrosarcoma were established. Mice were treated with either CXCL10 at 25 µg/kg once a day for 20 days, hyperthermia was given twice (at 42°C for 1 h, on day 6 and 12 after the initiation of CXCL10), or together. Tumor volume and survival time were observed. The microvessel density was determined by CD31 immunofluorescence. Histologic analysis and assessment of apoptotic cells were also conducted in tumor tissues.

Results The results showed that CXCL10 and hyperthermia inhibited the growth of Meth A fibrosarcoma and interestingly, the combination therapy enhanced the antiangiogenic effects and completely eradicated the established solid tumors. Histological examination revealed that CXCL10 + hyperthermia led to increased induction of apoptosis, tumor necrosis, and elevated lymphocyte infiltration compared with the controls. Moreover, the tumor eradicated animals developed a protective T-cell-dependent antitumor memory response against Meth A tumor cells rechallenge.

Conclusions Our finding is that the combination therapy can achieve a synergistic antitumor efficacy, supporting the idea that the combination of two antiangiogenic agents may lead to improved clinical outcome. These findings could open new perspectives in clinical antitumor therapy.

Keywords Interferon-γ-inducible protein 10 · CXCL10 · Antitumor · Hyperthermia · Chemokine

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Introduction

Chemokines are small chemotatic cytokines which can be divided into four superfamilies based upon the positioning of the four conserved cysteine amino acid residues in mature protein (Baggiolini 1998; Luster 1998; Barrett 1997; Leonidas et al. 2001). They direct the migration of immune cells through binding to chemokine receptors that belong to the seven transmembrane domain G-protein-coupled rhodopsin superfamily and participate in many other pleiotropic functions (Loetscher et al. 1996; Murphy et al. 2000) including regulation of tumor growth. The CXC chemokine can be further divided into two classes for the presence or absence of Glu–Leu–Arg(ELR) motif (Robert et al. 1995). The ELR$^+$ CXC chemokines could stimulate angiogenesis and cause directed migration of neutrophil and endothelial cells, while the ELR$^-$ CXC chemokines are potent inhibitors of angiogenesis and lead to the chemotaxis of NK cells, lymphocytes, and monocytes (Luster 1998; Baggiolini et al. 1997).

IFN-γ-inducible protein 10 (IP-10)/CXCL10 is known to be one of the ELR$^-$ CXC chemokines and a potent inhibitor

of angiogenesis. Although, single therapy with CXCL10 displays some antitumor efficacy in vivo (Arenberg et al. 1996, 2001; Yao et al. 2002; Andrew et al. 2002), most data from previous studies suggest that the antiangiogenesis outcome generally improves with adjuvant therapy, such as radiotherapy or chemotherapy (Gang et al. 2005). We have previously shown that the combination of CXCL10 with cisplatin led to significant reductions of tumor burden but failed to cure the animals (Gang et al. 2005). Due to the multifaceted nature of the angiogenic process in malignant neoplasms, a strategy for antiangiogenic therapy is to design appropriate combination protocols that can counteract the angiogenic factors produced by the tumor and its microenvironment. Recent studies suggested that the combination of angiogenesis inhibitor with cytotoxic therapies or other antiangiogenesis agents may be a possible antitumor strategy (Gang et al. 2005; Bergers et al. 1999; Yokoyama et al. 2000; Amir et al. 2003).

Hyperthermia, one of the oldest documented tumor treatment modalities, is applied as an adjunctive therapy with various established cancer treatments such as chemotherapy, radiotherapy, and radiochemotherapy (Kampinga and Dikomey 2001; Overgaard et al. 1995; Wessalowski et al. 1998; Rau et al. 1998). The initial rationale for the use of hyperthermia is based upon a direct tumor cell-killing effect at temperatures above 41–42°C as a function of time (Dewey 1994). Although the molecular mechanisms of hyperthermia are still under investigation, recent studies indicate that the antitumor effect of hyperthermia is most likely not only the result of direct cytotoxicity but might include other mechanisms as well (Frank et al. 2005; Falk and Issels 2001).

Recently, Cristina et al. (2003) have documented that hyperthermia is coupled with an inhibition of angiogenesis through increasing the expression of PAI-1, suggesting that hyperthermia may improve the effectiveness of antiangiogenic drugs. Moreover, studies from other groups have shown that hyperthermia affect not only the immunological reactions of leucocytes but also the immunogeneity of certain tumor cells (Manjili et al. 2002; Multhoff 2002). These results suggest that hyperthermia may act as an enhancer of gene therapy or immunotherapy. Thus, we wonder whether the combination therapy of CXCL10 with hyperthermia would more efficiently inhibit the growth of tumors. This article shows that the combination therapy exhibited powerful antitumor effects on tumor-bearing immunocompetent animals.

Materials and methods

Cell line

Meth A fibrosarcoma (Meth A) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in RPMI medium 1640 supplied with 10% FBS, maintained in a 37°C incubator with 5% humidified CO_2 atmosphere.

Recombinant CXCL10 preparation and biological activity evaluation

The recombinant murine CXCL10 protein was generated in *Escherichia coli* and purified, renatured, and cleavaged previously (Gang et al. 2005). The biological activity was evaluated by chemotaxis as previously described with slight modification (Gang et al. 2005). Briefly, separated T lymphocytes were stimulated for 2 days in RPMI 1640 medium supplemented with 400 U/ml interleukin-2 (Sigma) and 1 µg/ml phytohemagglutinin (Murex Diagnosis, Dartford, UK). The recombinant mCXCL10 was diluted with RPMI 1640 and added to the bottom wells of the 96-well Boyden microchambers chemotaxis plate (Neuroprobe). Then 50 µl of lymphocyte cells (2.5×10^5) resuspended in RPMI 1640 medium were added to the top of the membrane (5 µm pore size). The chamber was incubated in a 37°C incubator for 4 h and transferred to 4°C for 10 min, fixed, and stained for 5 min in Wright's stain. Cells that migrated to the lower surface were counted on six randomly selected high power fields. Commercial murine IP-10 from Peprotech (Rocky Hill, NJ, USA) with biological activity was utilized as standard control.

Tumor models

All studies involving mice were performed in accordance with institutional guidelines concerning animal use and care. Sterile food and water were fed to the mice, they were maintained in sterile cages on sterile bedding and housed in rooms at a constant temperature and humidity. Meth A fibrosarcoma model was established in BALB/c female mice (purchased from the Laboratory Animal Center of Sichuan University and allowed to acclimate for 1 week) of age 6–8 weeks (Yu-Quan et al. 2000). About 5×10^5 viable tumor cells were inoculated subcutaneously in the feet of the right hind limb of each mouse (Mototsugu et al. 2001).

Experimental protocol

Mice were randomly divided into four groups when the tumors were palpable, about 5 days after tumor cell inoculation. Experiment for the observation of tumor volume and survival advantage included eight mice per group. Each mouse was given 25 µg/kg/100 µl/day for several points intratumorally (Gang et al. 2005), while the control groups were injected with 100 µl PBS solution at the same time and points. Treatment was performed once a day for

20 days. Hyperthermia was given twice (on day 6 and 12 after the initiation of CXCL10 administration), Tumor-bearing legs were immersed in a water bath at 42°C for 60 min, while the control groups were immersed in a water bath at 37°C, maintaining at this temperature to an accuracy of ±0.05°C (Song 1984). Tumor dimensions were measured with calipers every 3 days, and tumor volumes were calculated according to the formula: tumor volume (mm^3) = π/6 length (mm) width (mm) width (mm), where length is the largest superficial diameter and width is the smallest superficial diameter. The mice were sacrificed when they became moribund (Yu-Quan et al. 2000).

For the histological analysis, the mice were sacrificed by cervical dislocation on day 4 after the completion of treatment as described above. The tissues of heart, liver, spleen, lung, kidney, and excised tumors were fixed in 10% neutral buffered formalin solution.

In vivo T cells depletion

T cells were depleted as previously described (Ji-yan et al. 2003) by i.p. injection with a mixture of 500 μg of anti-CD4 (clone GK1.5, rat IgG) monoclonal antibody (mAb) and 500 μg of anti-CD8 (clone 2.43, rat IgG) monoclonal antibody (mAb), 1 day before the tumor cells challenge and then twice per week for 3 weeks. Control mice were given 1,000 μg of rat IgG i.p. according to the same schedule. These hybridomas were obtained from the American Type Culture Collection (Manassas, VA, USA). As determined by flow cytometry, the depletion of CD4^{+} and CD8^{+} T cells was consistently >98%.

Histological analysis

Tumors from each group were embedded in paraffin. Sections 3–5 μm were stained with hematoxylin and eosin (H&E), according to the standard procedures (Gang et al. 2005). Histological assessment was performed using the computer-aided image analysis system Quantimet 600 and Qwin software (Leica, Benshaim, Germany). The mean diameter of necrosis was determined and recorded at various points in a blind manner. The percentage of the tumor necrosis area to total tumor area was evaluated by the image analysis system. Leukocytes were quantitated as previously described (Gang et al. 2005).

The combination-induced antiangiogenesis was determined by immunofluorescent analysis of neovascularization in tumors as described (Ji-yan et al. 2003). Briefly, frozen sections were fixed in acetone, incubated with PBS, and stained with rabbit antimouse CD31 (platelet/endothelial cell adhesion molecule 1) polyclonal antibody (1:50;

Labvision, California, USA), washed, followed by incubation with a Cy3-conjugated second antibody (Sigma). Vessel density was determined by counting the number of microvessels per high-power field in the sections with a fluorescence microscopy, as described by Ji-yan et al. (2003).

Quantitative assessment of apoptosis

Apoptosis was determined in two ways. Tumor sections were prepared as described previously. Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining was done using an in situ cell death detection kit (DeadEnd™ Fluorometric TUNEL System, Promega, Madison, USA) following the manufacturer's protocol. It is based on the enzymatic addition of digoxigenin-nucleotide to the nicked DNA by the recombinant terminal deoxynucleotidyl transferase (rTdT) (Ben-sasson et al. 1995). Hoechst staining was performed as described previously (Xiaoling et al. 2006). Briefly, sections were deparaffinized in xylene and rehydrated through the graded ethanol into PBS. Subsequently, the sections were stained with Hoechst 33258 staining solution according to the manufacturer's introductions (Beyotime, Jiangsu, China). In the tissue sections, four equal-sized fields were randomly chosen and analyzed. The apoptotic index was calculated as a ratio of the apoptotic cell number to the total tumor cell number in each high-powered field.

Evaluation of potential adverse effects

Potential toxicities were studied as previously described (Ji-yan et al. 2003). The mice treated with recombinant CXCL10 or hyperthermia without carcinomatosis were investigated, in particular for potential toxicity for >6 months. Gross measures such as weight loss, ruffling of fur, life span, behavior, and feeding were investigated. Tissues of heart, liver, spleen, lung, kidney, etc., were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Sections of 3–5 μm were stained with hematoxylin and eosin (H&E), according to the standard procedures (Gang et al. 2005).

Statistical analysis

ANOVA and unpaired Student t test were used to analyze the data (Bernhard et al. 2000). Survival curves were constructed according to the Kaplan–Meier method (Kaplan and Meier 1958). Statistical significance was determined by the log-rank test (Peto and Peto 1972). Differences between means or ranks as appropriate were considered significant when yielding a $P < 0.05$.

Results

Biological activity of CXCL10

The *E. coli* expressing fusion murine CXCL10 was purified, renatured, cleaved, and freezed at −80°C (Gang et al. 2005). Before use, the biological activity was identified by chemotactic assay. Recombinant CXCL10 in our experiment had similar capacity of chemotaxis compared to commercially purchased IP-10 in vitro (Gang et al. 2005), which indicated that our product was valid for further animal experiment.

Tumor growth inhibition

Tumor volume and life span of mice assay showed that the single CXCL10 treatment resulted in reduction of tumor growth and led to tumor stasis. Remarkably, the combination treatment exihibited a superior antitumor efficacy and resulted in complete tumor eradication (Fig. 1a). No regrowth of tumors was observed 3 months post-termination of the combination treatment. The control animals that received PBS survived 33 days on an average, in contrast, the combination of CXCL10 with hyperthermia resulted in a significant increase in the life span (Fig. 1b) and the mice were maintained even after 90 days of tumor cell injection. Thus, combination therapy with CXCL10 + hyperthermia resulted in apparent tumor inhibition versus PBS controls ($P < 0.01$) and hyperthermia or CXCL10 alone ($P < 0.01$).

Induction of T-cell memory in mice receiving combined treatment

To determine whether the mice receiving combination therapy develop a T-cell-dependent antitumor memory, those eight surviving mice, after combination therapy, were rechallenged with Meth A tumor cells (1×10^6) by subcutaneous inoculation in the right flank on day 90 after terminating the treatment. Four of the mice were T-cell depleted and the other four were mock depleted. After rechallenge, all the mock depleted mice demonstrated a memory response and remained tumor free. In contrast, none of the T cells depleted mice was able to resist the rechallenge (Fig. 2). Four naive mice were implanted with the same number of Meth A tumor cells in the same place and they served as the positive controls.

Immunolabeling of CD31

Combination therapy resulted in an apparent inhibition of tumor angiogenesis. Angiogenesis within tumor tissue was measured by counting the number of microvessels by immunolabeling of CD31 in tissue sections. Angiogenesis was inhibited by CXCL10 or hyperthermia. Nevertheless, the combination therapy resulted in synergistic inhibition of tumor angiogenesis when compared with the controls (Fig. 3).

Histologic analysis

In the present study, CXCL10 treated tumors showed a significant increase in necrosis compared to controls. Hyperthermia also affected the necrosis of tumor. Analysis of the extent of tumor necrosis revealed that the combination therapy was clearly more effective (Fig. 4a–d). We also evaluated leukocyte infiltration by histology within the tumor tissue. Clustering-like leukocyte infiltration was apparently found in the margin of both CXCL10 alone and CXCL10 in
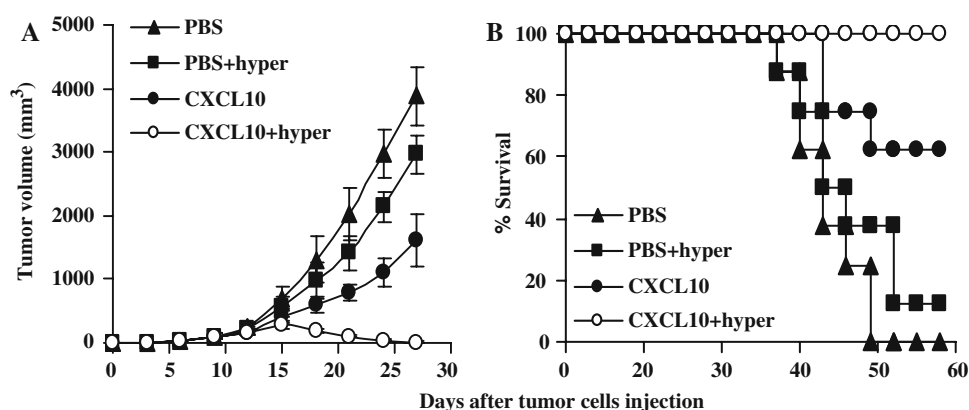

Fig. 1 Tumor growth inhibition and survival advantage in mice. Mice (eight mice per group) were intratumorally injected with mCXCL10 at 25 µg/kg/day for 20 days, and/or administration of hyperthermia cycle twice (on day 6 and 12 after initiation of CXCL10) or appropriate controls (0.1 ml PBS) at the same time points. **a** Suppressed growth of tumor in mice. Graph shows the treatment with CXCL10 or hyperthermia alone inhibiting the growth of tumor, while combination therapy with CXCL10 + hyperthermia results in the eradication of the established Meth A fibrosarcoma. Results were expressed as average tumor volume ± standard deviations. **b** Survival curves of BALB/c mice bearing Meth A fibrosarcoma. The combination of CXCL10 with hyperthermia resulted in a significant increase in life span
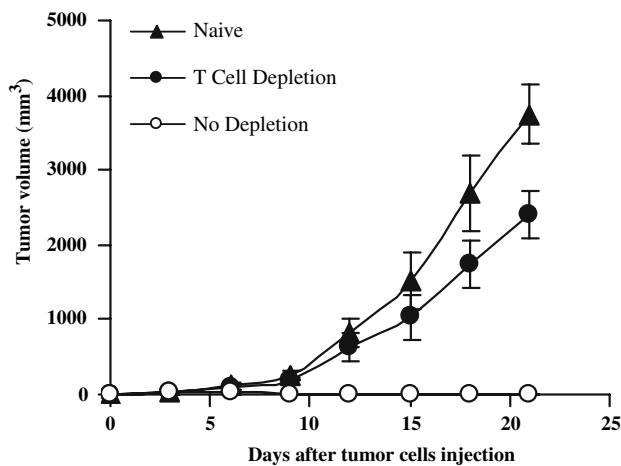
Fig. 2 Combination-treated mice develop T-cell-dependent antitumor memory. Mice remaining tumor free after combination therapy were rechallenged with s.c. injection of 1×10^6 Meth A tumor cells on day 90, post-termination of the combination treatment. Naive mice implanted with s.c. Meth A fibrosarcoma served as a positive control. Graph shows that the tumor growth in T-cell depleted mice is progressive, while no tumor regrowth can be observed in the mock depleted mice

conjunction with hyperthermia groups. Representative sections of tumor tissue from CXCL10 and CXCL10 + hyperthermia treated groups were depicted (Fig. 4e–f). Apparently elevated lymphocyte infiltration could be found in the tumor tissue from CXCL10 or CXCL10 + hyperthermia treated groups.

Therapeutic effect on apoptosis

To better understand the relationship between antitumor effect and apoptosis of tumor cells, tumors resected 4 days after the completion of treatment were subjected to TUNEL assays and Hoechst 33258 staining. The apoptosis rate of tumor cells was affected by hyperthermia or CXCL10 alone treatments, whereas the combination therapy resulted in a significant increase of apoptotic cancer cells (Fig. 5a–d, f–j). Data were shown as the mean apoptotic index ± standard deviations of cancer cells as percent normalized to apoptotic index of cancer cells (Fig. 5e, i).

Observation of potential toxicity

CXCL10 + hyperthermia treated animals without a tumor burden were particularly investigated for potential toxicity of CXCL10 and hyperthermia for >6 months. No pathologic changes were found in the tissues studied, including liver, lung, kidney, etc., (all organs) of the combination treated animals. No adverse consequences were noted in gross end-points such as weight loss, ruffling of fur, life span, behavior, or feeding.
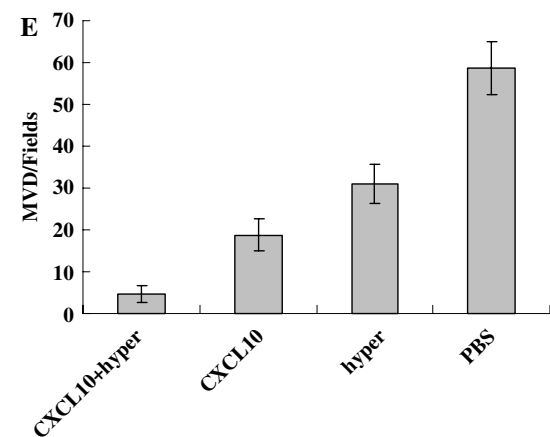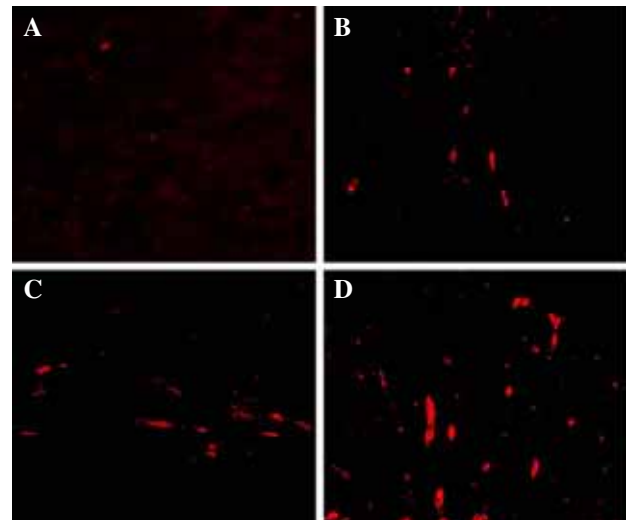

Fig. 3 Inhibition of angiogenesis within tumors estimated by immunofluorescence with CD31. The frozen sections of tumor tissues were obtained from mice treated with **a** CXCL10 + hyperthermia, **b** CXCL10, **c** hyperthermia, and **d** PBS. Vessel density was determined by counting the number of the microvessels per high-power field in the CD31 stained sections. **e** Combination treatment group displayed a significantly decreased microvessel when compared to the control groups ($P < 0.05$)

Discussion

Angiogenesis, the formation of new capillaries from preexisting vessels, is essential for growth and malignancy of tumors (Folkman 1995). The expansion of solid tumors is strictly dependent on the angiogenesis and solid tumors cannot grow beyond 2–3 mm in diameter without vascularization (Hanahan and Folkman 1996). Thus, antiangiogenesis is now considered as one of the most promising new therapeutic approaches which has gained strong impetus in antitumor therapy (Yang et al. 2003). Combining antiangiogenic agents with cytotoxic therapies or different antiangiogenic agents has been shown to be potentially beneficial and improved over single antiangiogenic therapy (Gang et al. 2005; Amir et al. 2003; Teicher et al. 1992; Helena
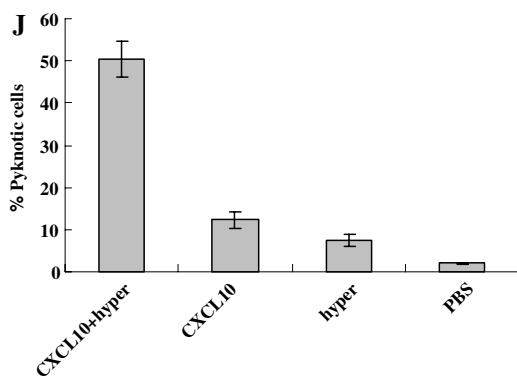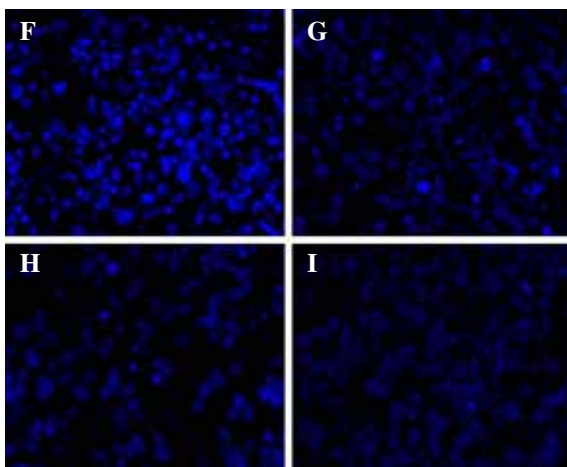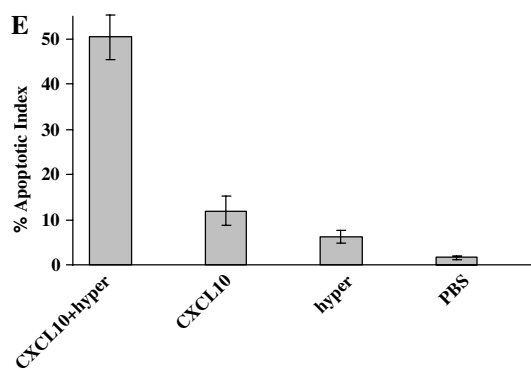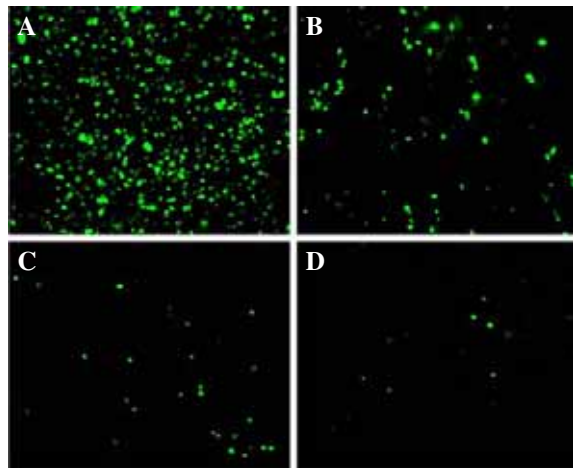
Fig. 4 Histochemical analysis of tumors. Tumor species from each group were embedded in paraffin, sections of 3–5 μm were stained with hematoxylin and eosin (H&E). **a** There was a little tumor tissue necrosis in the tumor tissues from control mice. Tumors with distinct necrosis were found in Meth A neoplasm tissue (×200) of **b** hyperthermia, **c** CXCL10, and **d** hyperthermia + CXCL10 treated groups. Little leukocytes infiltration was found in the control groups, while lymphocyte infiltration was enhanced in the margin or central region of CXCL10 or CXCL10 + hyperthermia treated mice. Representative sections of tumor tissues from **e** CXCL10 and **f** hyperthermia + CXCL10 treated groups (×400)
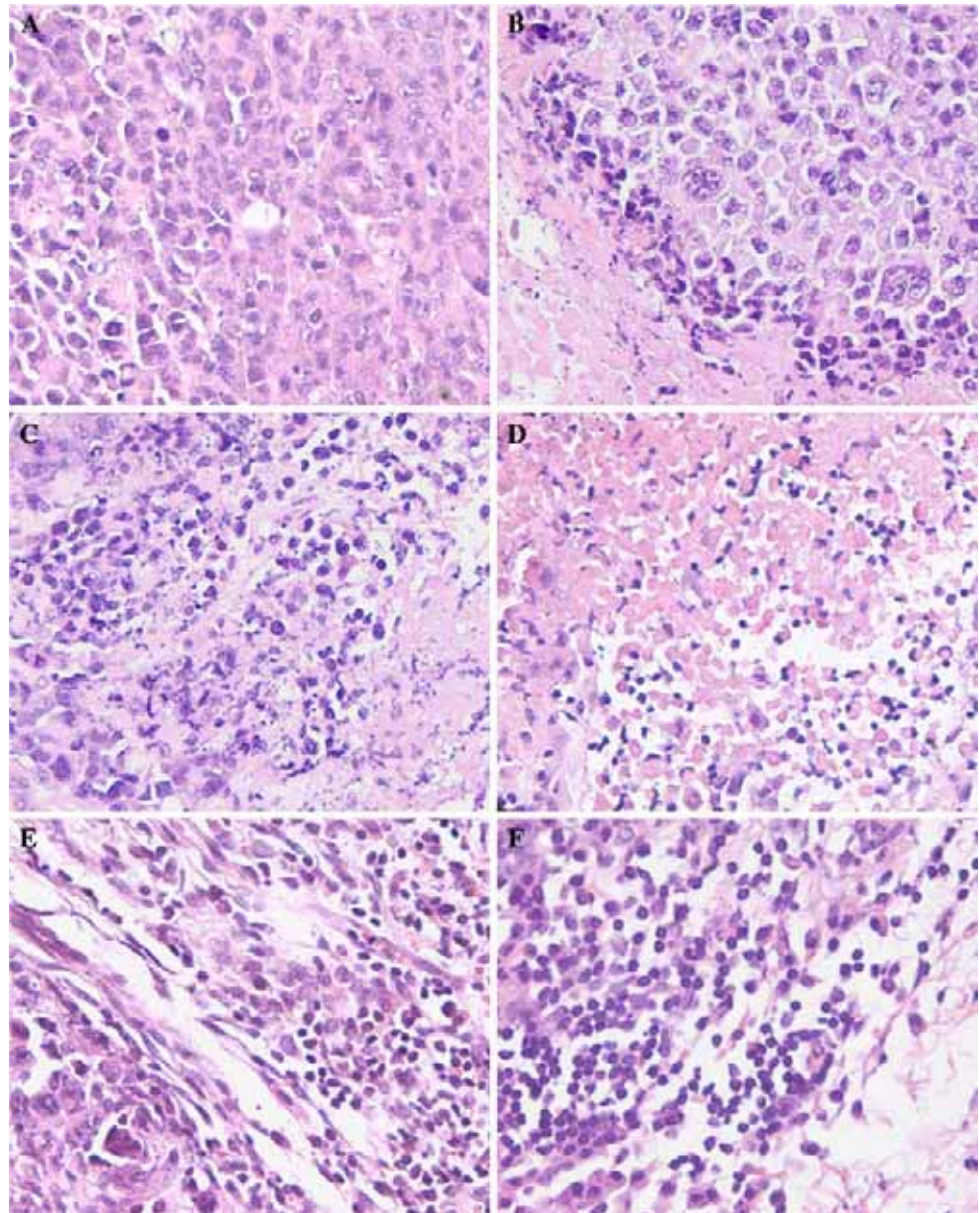
et al.1998). Recently, it has been suggested that hyperthermia acts as a cytotoxicity enhancing and angiogenesis inhibiting agent in tumor treatments (Frank et al. 2005; Falk and Issels 2001; Cristina 2003). Previous studies have shown that the antitumor efficacy of CXCL10 in vivo (Baggiolini 1998; Luster 1998; Barrett 1997; Leonidas et al. 2001; Loetscher et al. 1996; Murphy et al. 2000; Robert et al. 1995; Baggiolini et al. 1997; Arenberg et al. 1996, 2001; Yao et al. 2002; Andrew et al. 2002; Gang et al. 2005), the present study was designed to test whether the combination therapy of CXCL10 and hyperthermia would improve antitumor effects. To our knowledge, this was also the first time to demonstrate that the combination therapy of CXCL10 and hyperthermia led to supra-additive antitumor effects.

To study the antitumor efficacy of the combination therapy, a Meth A fibrosarcoma model was established. The Meth A tumor-bearing animals received daily intratumoral injections of CXCL10 protein in combination with hyperthermia. This resulted in complete tumor regression and impalpable tumors 10 days after the completion of treatment. No regrowth of tumors was observed 3 months after completion of treatment. Animals receiving the combined therapy had developed a T-cell-dependent antitumor memory. These results suggest that the combination of CXCL10 with hyperthermia exhibits a higher degree of synergistic activity.

The mechanism by which the combination therapy can enhance the antitumor efficacy in vivo may be attributed to the increased induction of the apoptosis according to our

previous study (Gang et al. 2005). This suggestion is supported by the findings that there are more apparent apoptotic cells in the combination treated tumors (Fig. 5). Previous reports indicate that both CXCL10 and hyperthermia act as angiogenic inhibitors (Arenberg et al. 1996; Yao et al. 2002; Gang et al. 2005; Cristina et al. 2003). Our findings are that CXCL10 and hyperthermia reduce the microvessel density of tumor, which coincides with previous studies. Nevertheless, the combination treatment did remarkably decrease microvessel density and increase treatment response compared to single therapy, which would impair nourishment to tumor cells during their growth and then lead to the apoptosis and necrosis (Figs. 3, 4, 5).

Recently, hyperthermia has also been shown to have important stimulatory effects on the immune system (Manjili et al. 2002; Li and Dewhirst 2002). Among the various immunologically relevant changes in tumour cell physiology occuring after heat shock treatment, hyperthermia induced extracellular localized HSPs have been found to play key roles in the induction of immune response (Multhoff 2002; Srivastava 2002; Calderwood et al. 2005; Vabulas et al. 2002). It has been shown that NK cells could be activated by membrane-bound HSP70 through a target recognition structure binding to transiently plastic adherent NK cells (Multhoff 2002). Some other groups had demonstrated that hyperthermia induced HSP/peptide complexes of tumor can activate the dendritic cells through binding of toll-like receptors (TLRs) on their surface and induce DCs to cross-present antigens to CD8+ T cells (Li and Dewhirst 2002; Srivastava 2002; Calderwood et al. 2005; Vabulas et al. 2002). Therefore, the synergistic activity of combination therapy may in part result from hyperthermia, enhancing the antigen presentation on tumor cells. This may, in turn, explain the result that the combination treated mice develop a T-cell-dependent antitumor memory.

Taken together, our data in the present study suggest that the combination therapy of CXCL10 and hyperthermia exhibits supra-additive antitumor efficacy in vivo, and this efficacy may result from increasing apoptosis and enhanced antigen presentation on tumor cells. The present findings also provide a new strategy for the treatment of some cancers that are resistant to chemotherapy and radiotherapy and may be of importance for further exploration in the treatment of cancer.

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