RESEARCH ARTICLE

Enhanced Antitumor Effect of Curcumin Liposomes with Local Hyperthermia in the LL/2 Model

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Abstract

Curcumin previously was proven to inhibit angiogenesis and display potent antitumor activity *in vivo* and *in vitro*. In the present study, we investigated whether a combination curcumin with hyperthermia would have a synergistic antitumor effect in the LL/2 model. The results indicated that combination therapy significantly inhibited cell proliferation of MS-1 and LL/2 *in vitro*. LL/2 experiment model also demonstrated that the combination therapy inhibited tumor growth and prolonged the life span *in vivo*. Furthermore, combination therapy reduced angiogenesis and increased tumor apoptosis. Our findings suggest that the combination therapy exerted synergistic antitumor effects, providing a new perspective fpr clinical tumor therapy.

Keywords: Curcumin - hyperthermia - anti-angiogenesis - apoptosis - antitumor therapy

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Introduction

Angiogenesis, the growth of new capillary blood vessels, is important for tumor growth and expansion. (Folkman, 1992; Zetter, 1998) Without blood vessels, tumors can not grow beyond a critical size (Bergers and Benjamin, 2003). Thus, tumors must develop their own blood to supply oxygen and nutrients. Anti-angiogenesis therapy can prevent tumor cells from developing a viable blood supply and inhibit the tumor growth. It has been an important therapeutic strategy in the antitumor treatment (Gasparini et al., 2005).

Curcumin is small-molecular-weight compound that obtained from the rhizome of the plant Curcuma longa (Goel et al., 2008). Accumulating evidence suggests that curcumin can inhibit carcinogenesis in different organs and the common link between these actions is its antiangiogenic effect (Bhandarkar and Arbiser, 2007). Curcumin may be an ideal anti-angiogenesis drug. First, as a natural antioxidant, curcumin has low toxicity. Phase I and phase II studies have proved that curcumin is well tolerated for cancer patients (Cheng et al., 2001; Dhillon et al., 2008). Second, curcumin is a direct inhibitor of angiogenesis and plays an important role in the down-regulation of proangiogenic proteins, such as VEGF (Arbiser et al., 1998). Third, curcumin can inhibit several signal transduction pathways, including those involving protein kinase C and the transcription factors NF-κB and AP-1. Curcumin's antiangiogenic effect has been extensively accepted. In order to further enhance curcumin's antitumor effect, combination other forms therapy would be necessary. Past studies proved that combining antiangiogenic agents with cytotoxic therapies or different antiangiogenic agents has been shown to be potentially beneficial and improved over single antiangiogenic therapy.

Hyperthermia is one of cancer therapies, which refers to the treatment of malignant diseases by administering heat in various ways. Although hyperthermia alone does not often eradicate the established tumor, it is undoubtedly to enhance cell-killing effect of drugs (Hildebrandt et al., 2002). It has been applied in the clinical and experimental animals as an adjuvant therapy with radiotherapy or chemotherapy and local control and survival rates have been improved by adding local /regional hyperthermia to radiotherapy in patients with locally advanced cancer (Hildebrandt et al., 2002; Moyer and Delman, 2008). The antitumor mechanism of hyperthermia is most likely the result of direct cytotoxicity. Furthermore, some publications have demonstrated 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 (Roca et al., 2003). In this context, we supposed that combination curcumin with hyperthermia would have a synergistic antitumor effect.

In the present study, our results showed that the combination therapy inhibited the tumor growth. Furthermore, combination therapy reduced angiogenesis and increased tumor apoptosis. These results indicated that combination therapy is more effective than single curcumin or hyperthermia.

Materials and Methods

Cell culture

Murine Lewis lung carcinoma cell line LL/2 and endothelial cell line MS1 were provided from the

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American Type Culture Collection (ATCC). LL/2 was cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum. MS-1 were cultured in RPMI medium 1640 supplemented with 10% FBS. Cells were maintained in humidified chamber at 37°C in 5% CO_2 atmosphere.

Proliferation assay in vitro

Cell proliferation was determined by MTT assay. LL/2 and MS-1 were seeded into 96-multi well plates and cultured for 24 hours at 37°C. Then, cells were treated with different concentrations (2.5 μg/ml, 5 μg/ml, 10 μg/ml) of Lip-cur and heated to 42°C in a 42°C incubator with 5% humidified CO_2 atmosphere for 60 minutes. Cells grown in media without curcumin were used as control. After 48 hours of treatment, the cell viability was determined by MTT.

Animal experimental in vivo

Six-week-old female C57BL/6 and BALB/c mice were provided with Vital River Beijing. At the time of experiment, mice weighted about 20g. LL/2 cells were harvested and resuspended in RPMI1640 without antibiotics .The mice were injected s.c. with 1×10^6 LL/2 cells into the feet of left hind limb. About seven days after tumor cells injection, the mice randomized into 4 groups. Each group has 20 mice including 10 mice for survival analysis. Experimental group was treated with Lip-cur (10 mg/kg) by i.v. injection once a day for 14 days. One hour after Lip-cur injection, tumor-bearing legs were immersed in a water bath for 60 minutes, maintaining this temperature fluctuating less than 0.5°C.Hyperthermia was given twice a week. Tumor dimensions were measured every 3 days by calipers. Tumor volume was calculated according to the formula (Chen et al., 2008): tumor volume (mm^3) $=\pi/6\times$length (mm) $\times$width (mm) $\times$width (mm), length is the largest superficial diameter and width is the smallest superficial diameter. Mice were sacrificed when they are moribund (Wei et al., 2000).

Detection of microvessel density

The anti-angiogenesis of combination therapy was determined by immunofluorescence analysis of neovascularization in tumor tissue as described (Liu et al., 2003). Brief, frozen sections of tumors were fixed in acetone, washed with PBS, stained with rat anti-mouse CD31 polyclonal antibody (1:50; Abcam, USA), washed twice with PBS, and followed by incubation with a Rhodamine-conjugated second antibody (1:200, Abcam, USA). Vessel density was evaluated by counting the number of micro-vessel per high-power field in the sections with a fluorescence microscopy as described.

Quantitative assessment of apoptosis

Tumor sections were prepared as described previously. The apoptosis of tumor tissues was determined by Terminal deoxynucleo tidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) using an in situ cell death detection kit (DeadEnd™ Fluorometric TUNEL System, Promega, USA), following the manufacturer's instructions. The number of TUNEL-positive cells was counted under a 200× visual field magnification of the fluorescence microscope.

Toxicity evaluation

Toxicity was assessed on mice during the treatment. We observed in terms of relevant indexes such as weight loss, diarrhea, ruffled fur, feeding. Furthermore, tissues of heart, liver, spleen and kidney were examined with H&E.

Statistical analysis

Statistical analysis was performed with SPSS16.0 software system. Data was analyzed statistically by using one-way ANOVA and Student's t test. Kaplan–Meier method and the log-rank test were used to estimate the survival time. Values are shown as mean ±SD. Significance of the differences was considered at $P<0.05$

Results

Inhibition of cell proliferation of MS-1 and LL/2 in vitro

It has been reported that curcumin had antiangiogenic effect (Gururaj et al., 2002). In this research, we further investigated combination therapy reduced relative cell viability of MS-1 and LL/2 comparing with single curcumin or hyperthermia group (DMEM as control). At 48 hours of post treatment, we observed that cell viability decreased in each cell line with increasing concentration of curcumin treatment (2.5 μg/ml, 5 μg/ml, 10 μg/ml) in LL/2 and MS-1 compare to DMEM. However, the inhibition effect was enhanced by combination therapy (Figure 1). Therefore, combination therapy enhanced the inhibition of cell proliferation of MS-1 and LL/2 compared to single therapy in vitro.

Tumor growth inhibition and prolonged of life span in vivo

In order to observe the antitumor effect of combination therapy, LL/2 model was established. Figure 2A showed that single lip-cur or hyperthermia suppressed the tumor growth and the inhibition rate of tumor was respectively 40%, 24% in LL/2 model. However, the combination therapy significantly decreased tumor volume and resulted in 58% tumor regression. Figure 2B indicated that all mice received lip died at the 51th day after tumor cells injection. In contrast, combination therapy prolonged the life span .Thus, combination therapy resulted in apparent tumor regression compared to lip control ($P<0.001$), hyperthermia ($P<0.01$) and lip-cur ($p<0.05$).

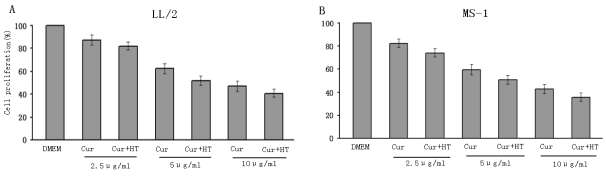

Figure 1. Inhibition of the Cell Activity of LL/2 and MS-1 in vitro. In this study, different concentration curcumin (2.5 μg/ml, 5 μg/ml, 10 μg/ml) and control (DMEM) were adopted. A, MTT assays of 4T1 (B) MTT assays of MS-1 at the 48 hours after treatment. Combination therapy made relative cell activation drop lower compared with single curcumin or hyperthermia therapy in two types of cells, especially the dilution of 5 μg/ml, 10 μg/ml Results were expressed as average ± SD (n=6, ANOVE, Student t test, $P<0.05$)

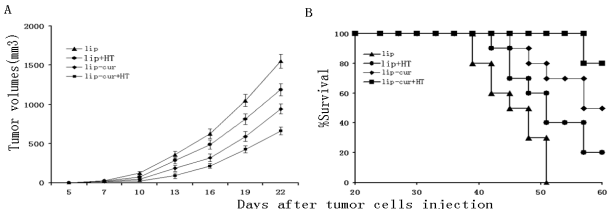

Figure 2. Tumor Growth Inhibition and Survival Advantage in vivo. LL/2 tumor-bearing mice were treated as described with lip-cur at 10 mg/kg, daily for 14 days, and/or hyperthermia twice a week or control (100 μl lipsomes) A, tumor growth curves of LL/2 Graph showed the treatment with combination of lip-cur with HT resulted in synergy to inhibit tumor growth compared with lip-cur or HT alone. Results were expressed as average ± SD (n=10, ANOVA, $P<0.01$). B, Survival curves of C57BL/6 mice-bearing LL/2 Lung cancer. The combination therapy prolonged the life span of mice (n=10, Kaplan-Meier, log-rank test, $P<0.01$)

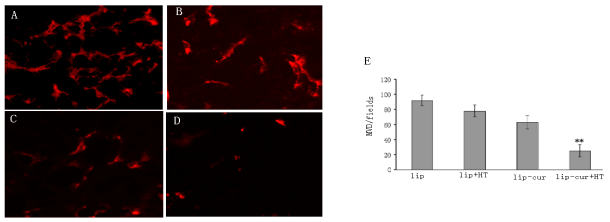

Figure 3. Inhibition of Angiogenesis Within Tumors Observed by Immunofluorescence with CD31. Frozen sections of different groups stained with CD31 were observed under fluorescence microscope. Representative tumor vasculature from lip (a), lip+HT (b), lip-cur (c), lip+HT (d) treated mice was shown. B, The vascular density was determined by counting the number of the microvessels per high-power field within hot spot area. Combination therapy displayed a significant difference compared to any other control. Data was shown as mean ± SD (n=3; ANOVA; ** $P < 0.01$)

Inhibition of tumor-induced angiogenesis

Angiogenesis within tumor tissue from each group were determined by immunofluorescence staining with anti-CD31 antibody (Figure 3). The results indicated that single lip-cur or hyperthermia therapy reduced angiogenesis compared with control. Nevertheless combination therapy was more effective on synergistic inhibition of tumor-associated angiogenesis. These results suggested that combination lip-cur with hyperthermia has a synergic effect on inhibiting tumor angiogenesis.

Induction of apoptosis in vivo

We detected the effect of lip-cur and hyperthermia on apoptosis in LL/2 by TUNEL staining. Represent fields from each group were shown (Figure 4A). Combination therapy distinctly induced tumor cell apoptosis and apoptosis rate was determined by evaluating the percentage of apoptotic cells among tumor cells. (Figure 4B). The number of apoptotic cells in 5 random fields from 3 different tumors in each group was counted.. The results indicated that combination therapy resulted in a significant increase of apoptotic cancer cells.

Observation of potential toxicity

No significant pathologic changes were found in the tissues including heart, liver, spleen, kidney of the combination treated animals in LL/2 model (Data not shown). Furthermore, there were no significant adverse effects in gross measures such as weight loss, skin ulcerations, toxic death, behavior, or feeding. Our results indicated combination therapy is safe for mice.

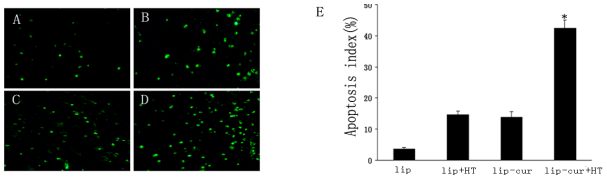

Figure 4. Evaluation of Apoptosis. The apoptosis of tumor cells was determined as material and methods. The typical sections from tumor tissue are presented: A, lip; B, .lip+HT; C, lip-cur;. D, lip-cur+HT; E, apoptotic index within tissues The combination therapy showed a significant increase of apoptotic cells in the tumor tissues compare to control (*$P<0.001$). Data expressed mean apoptotic index ± standard deviations of cancer cells

Discussion

Angiogenesis is necessary for most solid tumors growth. Tumor acquired with nutrients, oxygen and a route for metastasis through angiogenesis (Folkman, 2002). Accumulating evidence demonstrated that anti-angiogenesis therapy is effective for treatment of cancer (Granci et al., 2010). Curcumin is an important angiogenesis inhibitor, which has extensively applied in the treatment of cancer (Bhandarkar et al, 2007). Hyperthermia has been considered as a cytotoxicity enhancing and antiangiogenetic agent in tumor therapies (Pajonk et al., 2005). Combining curcumin with hyperthermia therapy may have an advantage over single curcumin therapy. According to our knowledge, it is the first time to investigate that combination therapy of curcumin with hyperthermia led to supra-additive antitumor effects.

In the present study, our results showed that combination of curcumin with hyperthermia inhibited cell proliferation of LL/2, MS-1 in MTT assay at the 48 hours post treatment in vitro. Furthermore, within a certain range of concentration, the higher the concentration resulted in the lower the relative cell activity. In addition, we observed that hyperthermia enhanced inhibiting effect. We speculate that it may be associated with hyperthermia increasing duration of action of drugs (Ma et al., 2011). These results indicated that combination therapy have a higher synergistic effect in reducing relative cell activity.

In order to further investigate the antitumor effect of combination therapy in vivo, LL/2 model was established. Our results showed that combination therapy improved antitumor effect. Firstly, combination therapy inhibited tumor growth and prolonged the life span. Moreover, combination therapy increased tumor cells apoptosis and suppressed tumor-associated angiogenesis. Importantly, there are no serious adverse effect were observed , such as weight loss, behavior, feeding, or histologic changes of main organs.

However, the exact mechanism of anti-tumor efficacy induced by the combination therapy is uncertain. According to our results, we speculate that possible antitumor mechanisms of combination therapy may be

associated with three aspects. First, combination induced apoptosis. Past studies demonstrated curcumin can promote tumor cell apoptosis (Karunagaran et al., 2005). In our laboratory, we previously proved that curcumin can induce apoptosis of LL/2 tumor cells. Furthermore, Hyperthermia can induce cell apoptosis in vivo and in vitro via different pathway (Huang et al., 2000; Stankiewicz et al., 2009). In the present study, we observed that combination therapy increase the rate of apoptosis in vivo and in vitro. Second, combination therapy inhibited angiogenesis. Anti-angiogenesis effect of curcumin has been extensively accepted. Hyperthermia also inhibits angiogenesis by various ways such as suppression of VEGF (Sawaji et al., 2002), induction of gene expression (Huang et al., 2000). Our study indicated combination of curcumin with hyperthermia reduced micro-vessel density of tumor compared to control. Third, combination therapy enhanced antitumor immunity response. According to past studies, Curcumin can potentiates antitumor activity through multiple pathways, such as reversing T cell-mediated immune dysfunction (Bhattacharyya et al., 2010), preventing T cell apoptosis (Bhattacharyya et al., 2007). Recently Hyperthermia has been found to be immune regulation function through inducing of the 70-kDa heat shock protein family (Manjili et al., 2002; Calderwood et al., 2005; Zhang et al., 2008). It has been proved that HSP played a critical role in the regulation of immune, which activated the NK cell and dendritic cells (Calderwood et al., 2005; Dayanc et al., 2008). Thus, we speculate tumor regression may be partly attributed to enhancing antitumor immunity, but it needed to be further studied.

Taken together, our results indicated that the combination therapy of curcumin with hyperthermia is more effective in inhibiting tumor growth and prolonging life span than single curcumin or hyperthermia and this efficacy may be associated with promoting apoptosis, suppressing angiogenesis in vivo. The present findings provide a new strategy for the treatment of cancer.

Acknowledgements

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References

Arbiser JL, Klauber N, Rohan R, et al (1998). Curcumin is an in vivo inhibitor of angiogenesis. *Mol Med*, **4**, 376-78.

Bergers G, Benjamin LE (2003). Tumorigenesis and the angiogenic switch. *Nat Rev Cancer,* **3**, 401-10.

Bhandarkar SS, Arbiser JL (2007). Curcumin as an inhibitor of angiogenesis. *Adv Exp Med Biol*, **595**, 185-95.

Bhattacharyya S, Hossain DMS, Mohanty S, et al (2010). Curcumin reverses T cell-mediated adaptive immune dysfunctions in tumor-bearing hosts. *Cell Mol Immunol*, **7**, 306-15.

Bhattacharyya S, Mandal D, Saha B, et al (2007). Curcumin prevents tumor-induced T cell apoptosis through Stat-5a-mediated Bcl-2 induction. *J Biol Chem,* **282**, 15954-64.

Calderwood SK, Theriault JR, Gong J (2005). Message in a bottle: role of the 70-kDa heat shock protein family in anti-tumor immunity. *Eur J Immunol,* **35**, 2518-27.

Chen P, Yang L, Yang H, et al (2008). Synergistic antitumor effect of CXCL10 with hyperthermia. *J Cancer Res Clin*, **134**, 679-87.

Cheng AL, Hsu CH, Lin JK, et al (2001). Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res*, **21**, 2895-990.

Dayanc BE, Beachy SH, Ostberg JR, et al (2008). Dissecting the role of hyperthermia in natural killer cell mediated anti-tumor responses. *Int J Hyperther*, **24**, 41-56.

Dhillon N, Aggarwal BB, Newman RA, et al (2008). Phase II trial of curcumin in patients with advanced pancreatic cancer. *Clin Cancer Res*, **14**, 4491-9.

Folkman J (1992). The role of angiogenesis in tumor growth. *Semin Cancer Biol*, **3**, 65-71

Folkman J (2002). Role of angiogenesis in tumor growth and metastasis. *Semin Oncol*, **29**, 15-8.

Gasparini G, Longo R, Toi M, et al (2005). Angiogenic inhibitors: a new therapeutic strategy in oncology. *Nat Clin Pract Oncol,* **2**, 562-77.

Goel A, Kunnumakkara AB, Aggarwal BB (2008). Curcumin as " Curecumin": From kitchen to clinic. *Biochem Pharmacol*, **75**, 787-809.

Granci V, Dupertuis YM, Pichard C (2010). Angiogenesis as a potential target of pharmaconutrients in cancer therapy. *Curr Opin Clin Nutr,* **13**, 417-22.

Gururaj AE, Belakavadi M, Venkatesh DA, et al (2002). Molecular mechanisms of anti-angiogenic effect of curcumin. *Biochem Biophys Res Commun,* **297**, 934-42.

Hildebrandt B, Wust P, Ahlers O, et al (2002). The cellular and molecular basis of hyperthermia. *Crit Rev Oncol*, **43**, 33-56.

Huang Q, Hu JK, Lohr F, et al (2000). Heat-induced gene expression as a novel targeted cancer gene therapy strategy. *Cancer Res*, **60**, 3435-39.

Karunagaran D, Rashmi R, Kumar T (2005). Induction of apoptosis by curcumin and its implications for cancer therapy. *Curr Cancer Drug Tar*, **5**, 117-29.

Liu JY, Wei YQ, Yang L, et al (2003). Immunotherapy of tumors with vaccine based on quail homologous vascular endothelial growth factor receptor-2. *Blood*, **102**, 1815-23.

Ma J, Chen CS, Blute T, Waxman DJ (2011). Antiangiogenesis enhances intratumoral drug retention. *Cancer Res*, **71**, 2675-85.

Manjili M, Wang XY, Park J, et al (2002). Cancer immunotherapy: stress proteins and hyperthermia. *Int J Hyperther,* 18, 506-20.

Moyer HR, Delman KA (2008). The role of hyperthermia in optimizing tumor response to regional therapy. *Int J Hyperther,* **24**, 251-61.

Pajonk F, van Ophoven A, McBride WH (2005). Hyperthermia-induced proteasome inhibition and loss of androgen receptor expression in human prostate cancer cells. *Cancer Res*, **65**, 4836-43.

Roca C, Primo L, Valdembri D, et al (2003). Hyperthermia inhibits angiogenesis by a plasminogen activator inhibitor 1-dependent mechanism. *Cancer Res*, **63**, 1500-07.

Sawaji Y, Sato T, Takeuchi A, et al (2002). Anti-angiogenic action of hyperthermia by suppressing gene expression and production of tumour-derived vascular endothelial growth factor in vivo and in vitro. *Brit J Cancer*, **86**, 1597-603.

Stankiewicz A, Livingstone A, Mohseni N, et al (2009). Regulation of heat-induced apoptosis by Mcl-1 degradation and its inhibition by Hsp70. *Cell Death Differ,* **16**, 638-47.

Wei YQ, Wang QR, Zhao X, et al (2000). Immunotherapy of tumors with xenogeneic endothelial cells as a vaccine. *Nat Med,* **6**, 1160-66.

Zetter P, Bruce R (1998). Angiogenesis and tumor metastasis. *Annu Rev Med*, **49**, 407-24.

Zhang HG, Mehta K, Cohen P, et al (2008). Hyperthermia on immune regulation: a temperature's story. *Cancer Lett*, **271**, 191-204.