SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 4: 107-112, 2012

Liposomal curcumin inhibits Lewis lung cancer growth primarily through inhibition of angiogenesis

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Received October 21, 2011; Accepted March 23, 2012

DOI: 10.3892/ol.2012.686

Abstract. Curcumin has been proven to effectively inhibit tumor growth by both targeting tumor cells and angiogenesis; however, poor water solubility limits further clinical application. In the present study, we prepared water-soluble liposomal curcumin to investigate its anti-tumor effects and the underlying mechanism. The MTT assay was used to test the anti-proliferative activities for the MS1 murine endothelial and LL/2 Lewis lung cancer cell lines. Apoptosis and cell cycle arrest induced by liposomal curcumin were analysed by flow cytometry. Anti-angiogenic agents and the resulting anti-tumor effects were investigated in a murine lung cancer model. Zebrafish were used to investigate the anti-angiogenic effect of liposomal curcumin in the development of embryos. *In vitro*, liposomal curcumin inhibited the proliferation of MS1 cells and induced cell cycle arrest and apoptosis. Notably, LL/2 cells showed less sensitivity to the liposomal curcumin *in vitro*. *In vivo*, the systemic administration of liposomal curcumin resulted in significant inhibition of tumor growth. CD31 immunohistochemical analysis and alginate encapsulation assay revealed that angiogenesis was decreased by liposomal curcumin treatment. Angiogenesis was also suppressed in the development of zebrafish. Liposomal curcumin showed potent inhibitory activity against murine endothelial cells but not lung cancer cells. Liposomal curcumin treatment is capable of significantly inhibiting tumor growth *in vivo*, a process that may depend primarily on its anti-angiogenic effects. Our study also indicates that liposomal curcumin may be developed not only for cancer therapy, but also for the treatment of other angiogenesis-related diseases.

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Key words: liposomal curcumin, angiogenesis, anti-angiogenic agents, lung cancer

Introduction

Curcumin has been proven to be a promising anti-cancer drug by induction of apoptosis and apoptosis-independent death, and inhibition of proliferation and angiogenesis (1-4). Phase I and II studies of this compound have shown that curcumin is well tolerated and is effective for cancer patients; however, its benefits may be attenuated due to its low bioavailability through oral administration for non-gastrointestinal cancers (5,6). Therefore, novel strategies are required to overcome these limitations, which are mostly due to the low water solubility and low stability of curcumin against gastrointestinal fluids (7). Investigators have recognized that liposomes have the advantage of improving water insolubility and enhancing delivery efficacy of drugs (8). At present, various methods have been reported for the preparation of liposomes (9). Curcumin acts as an anticancer drug through multiple mechanisms; however, the activity of curcumin may be changed in a liposomal form. Furthermore, the water solubility of liposomal curcumin provides a new strategy for intravenous administration. It is speculated that, systemically, intravenous administration may exhibit marked inhibitory effects due to circumvention of the first-pass effect.

Angiogenesis, the process by which capillaries sprout from pre-existing vasculature, is a hallmark of the majority of solid tumors (10). Targeting tumor vascular endothelial cells has proven to be an effective therapeutic strategy in anti-tumor treatment (11). Therefore, anti-angiogenic agents have gained increasing importance in cancer research. Mounting evidence indicates that curcumin inhibits carcinogenesis in various organs and that the common link between these actions is its anti-angiogenic effect (4). Although the anti-cancer and anti-angiogenic effects of curcumin have been evaluated comprehensively, the anti-angiogenic effect of the liposomal form of these compounds has not been extensively studied, particularly when the liposomal curcumin is administered intravenously.

Previously, we developed water-soluble liposomal curcumin with the ethanol injection method. In the current study, we examined the anti-angiogenic and anti-cancer effects of liposomal curcumin on Lewis lung cancer *in vitro* and *in vivo*. Our study indicated that the liposomal curcumin primarily inhibits

tumor growth due to its anti-angiogenic activity. Our results also indicate that liposomal curcumin may be used in tumor treatment for further clinical application.

Materials and methods

Cell lines and animals. Murine Lewis lung carcinoma cell line LL/2 and endothelial cell line MS1 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in DMEM supplemented with 10% (vol/vol) fetal bovine serum and were maintained in a humidified chamber at 37˚C in 5% CO_2 atmosphere. C57BL/6 and zebrafish (FLK-1:EGFP) were purchased from the West China Experimental Animal Center. The study protocol was reviewed and approved by the institutional animal care and treatment committee of Sichuan University, Chengdu, China.

Cell proliferation assay. The MTT assay was performed to determine the effect of curcumin on MS1 and LL/2 viability. Briefly, cells were plated in a 96-well plate at a density of 3000 cells per well and were exposed to liposomal curcumin at different concentrations for 48 h. Cells grown in media without curcumin were used as a control. Following treatment, the media were carefully removed. Then, 20 μl MTT (5 mg/ml) was added to each well and incubated with the cells for 3 h. Dimethyl sulfoxide (150 μl) was added to each well and the plates were read at 570 nm in an ELISA reader.

Flow cytometry. The percentage of apoptotic cells and the cell cycle distribution of curcumin-treated cells were analyzed by flow cytometry. Briefly, cells ($1x10^5$/well) were plated in 6-well plates. Following incubation overnight, the cells were treated with various concentrations of liposomal curcumin (0-40 μg/ml) for 48 h, trypsinized and washed with PBS, and centrifuged. Supernatants were removed and the cells were resuspended in 1 ml of hypotonic fluorochrome solution containing 50 mg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100 and immediately subjected to flow cytometry (ESP Elite, Beckman Coulter Fullerton, CA, USA).

Tumor growth inhibition experiment in vivo. Six-week-old female C57BL/6 mice were acclimatized for one week and fed with animal chow and water *ad libitum*. The mice were injected subcutaneously in the right leg with $5x10^5$ Lewis lung carcinoma cells with a total volume of 50 μl. Seven days later, when the tumors were palpable, the mice were randomized into two groups (n=6 per group). The experimental group was treated with liposomal curcumin (10 mg/kg) by intravenous injection once a day for two weeks. The control mice were administered normal saline (NS). Tumor dimensions were measured every three days with calipers. Tumor volume was calculated according to the formula: volume = $width^2$ x length x 0.52.

Detection of microvessel density. Frozen sections of the tumor tissue from the mice were used to determine vessel density with an anti-CD31 antibody, as described in a previous study in detail (12). The following antibodies and reagents were used: monoclonal rat anti-mouse CD31 antibody (1:400, Santa Cruz Biotechnology, Santa Cruz, CA, USA),

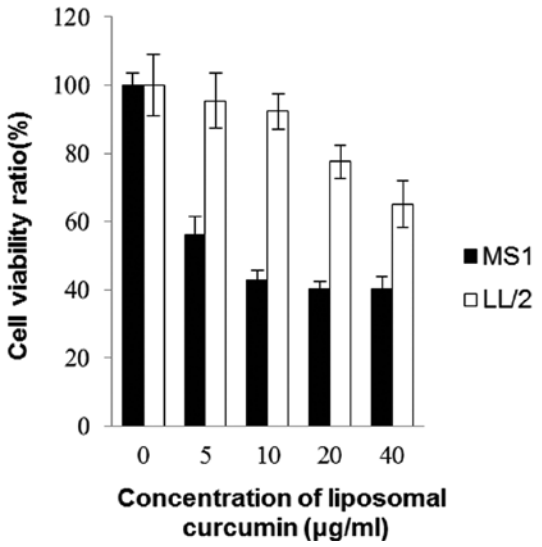

Figure 1. The effects of curcumin on cell viability were examined using the MTT assay. The values for each concentration tested are the average (mean ± SD) from six replicate wells. Curcumin had higher cytotoxic activity against MS1 compared with LL/2 ($P<0.05$).

biotinylated polyclonal goat anti-rat antibody (1:200, Vector Laboratories, Peterborough, UK), ABC kit (Boster Biological Engineering Co., Wuhan, China) and DAB visualization system (ZSJQ Biotechnology, Beijing, China). Sections were counterstained with hematoxylin and mounted with glass coverslips. Images were captured using an Olympus fluorescence microscope at an original magnification of x200. Microvessel density (MVD) was assessed within hot spots.

Alginate encapsulation for tumor cell assay. Alginate bead-containing tumor cell assays were described in detail in a previous study (13). Briefly, cultured LL/2 cells were resuspended with 1.5% (m/v) sodium alginate (Sigma-Aldrich, St. Louis, MO, USA). The tumor cell alginate solution was then dropped into a swirling bath of 0.25 M $CaCl_2$ in order to form droplets containing approximately $1x10^5$ tumor cells per bead. After being anesthetized, the C57BL/6 mice were implanted subcutaneously with four beads through an incision on the back and the incisions were sutured with surgical clamps. Treatment with liposomal curcumin (10 mg/kg) was performed once per day following bead implantation, with normal saline (NS) as a control. At 14 days, the mice were injected intravenously with 100 μl FITC-dextran solution (Sigma Chemical) (100 mg/kg) and were sacrificed 20 min later. Images of the alginate implants were captured using a SPOT FIEX camera. Alginate beads were transferred to tubes containing 2 ml of saline. The tubes were mixed in a vortex for 20 sec and centrifuged (3 min; 1000 x g). Finally, the fluorescence of the supernatant was measured to quantify blood vessel formation.

Zebrafish embryo development assay. FLK-1 promoter EGFP transgenic zebrafish (FLK-1:EGFP) were used. Fertilized eggs were incubated for 8 h, after which liposomal curcumin was added to the water at a concentration of 5 μg/ml. The same water without curcumin was used as a control. Following incubation for 72 h, the larvae were placed on glass slides

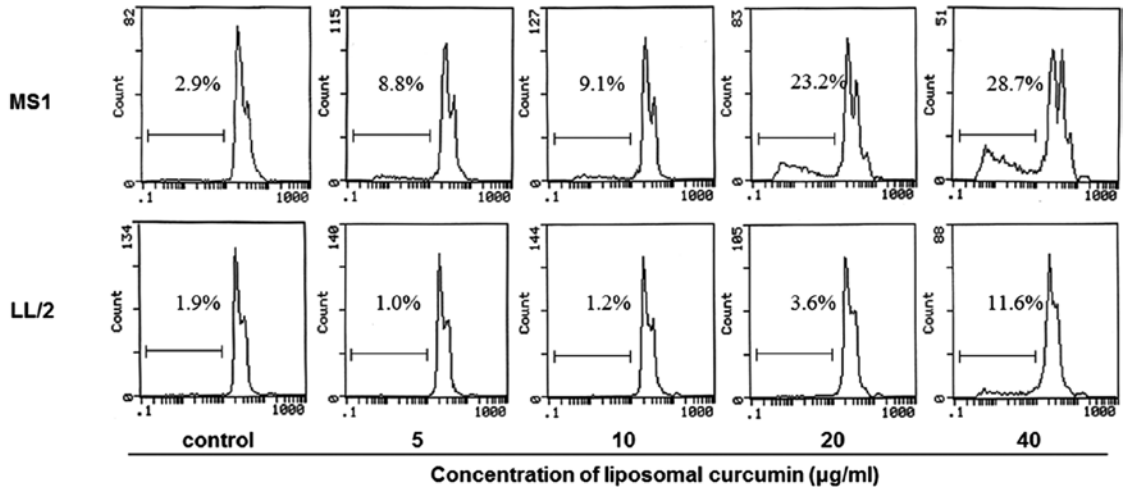

Figure 2. Effects of liposomal curcumin on cell apoptosis. Following treatment with liposomal curcumin at various concentrations for 48 h, apoptotic MS1 and LL/2 cells were assessed by flow cytometry.

and examined using a Zeiss microscope. Fluorescence signals were detected and images were captured.

Results

Higher sensitivity to liposomal curcumin in murine endothelial cell line MS1. An MTT assay was conducted on murine Lewis lung carcinoma cell line LL/2 and endothelial cell line MS1. Fig. 1 shows the effects of liposomal curcumin on cell viability following 48 h of drug exposure. The cell viability decreased in each cell line with the increasing concentration of curcumin treatment. The sensitivity to curcumin differed markedly between MS1 and LL/2 cells. Curcumin had higher cytotoxic activity towards MS1, but lower cytotoxic activity towards LL/2 ($p<0.05$). It was clear that mouse endothelial cells were more sensitive to curcumin in comparison to Lewis lung cancer cells. In addition, flow cytometry was performed to investigate whether liposomal curcumin induced MS1 and LL/2 cell apoptosis. The quantitative assessment of sub-G1 cells by flow cytometry was used to estimate the number of apoptotic cells. Liposomal curcumin was found to increase the number of sub-G1 cells compared with the control groups (Fig. 2). Notably, no marked pro-apoptotic effect of liposomal curcumin was observed against LL/2 cells.

Changes of cell cycle phase distribution mediated by liposomal curcumin. To evaluate the cell cycle phase distribution of MS1 and LL/2 cells with curcumin treatment, the DNA content was measured using flow cytometry. FACS analysis of MS1 cells revealed that exposure to liposomal curcumin from 5 to 40 μg/ml for 48 h caused an increase of the G2/M-phase population from 15.8 to 37.8%, compared to control cells with 16.2% G2/M phase cells (Fig. 3A). This increase was accompanied by a significant decrease in the percentage of S-phase cells, whereas the fraction of G1-phase cells was mainly unchanged (Fig. 3C). This result demonstrates that curcumin induces growth inhibitory effects on MS1 at least in part via G2/M phase arrest. Results of the FACS analysis of propidium iodide-stained LL/2 cells showed that liposomal curcumin also reduced S phase and increased G2/M percentages in LL/2 compared to the control

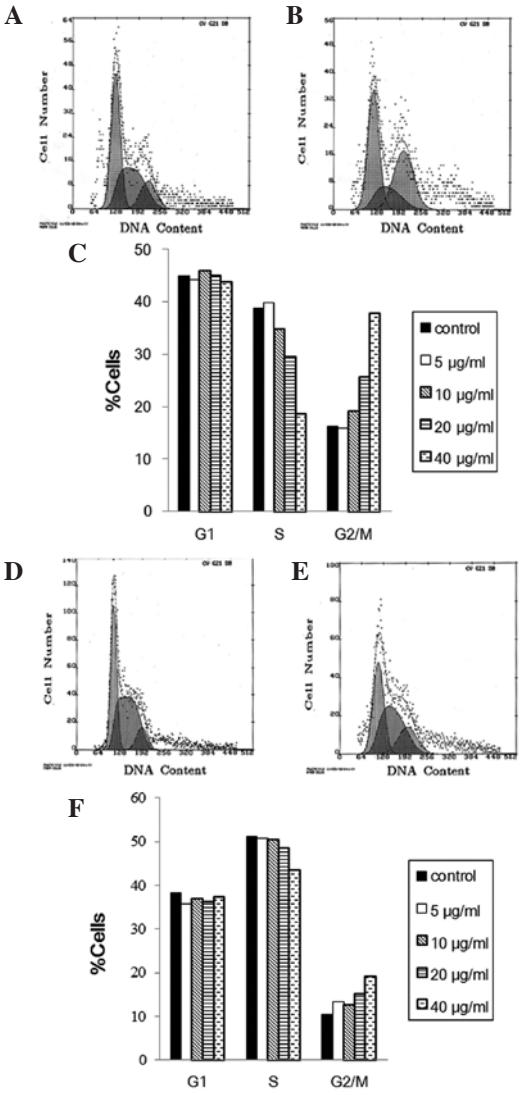

Figure 3. Cell cycle distribution of MS1 and LL/2 cells. Cell cycle distribution of MS1 and LL/2 cells following treatment with various concentrations of curcumin for 48 h. (A) Untreated control MS1 cells; (B) MS1 cells treated with 40 μg/ml curcumin; (C) MS1 cell cycle distribution (percentage of total) of various concentration treatment groups; (D) untreated control LL/2 cells; (E) MS1 cells treated with 40 μg/ml curcumin; (F) LL/2 cell cycle distribution (percentage of total) of different concentration treatment groups.

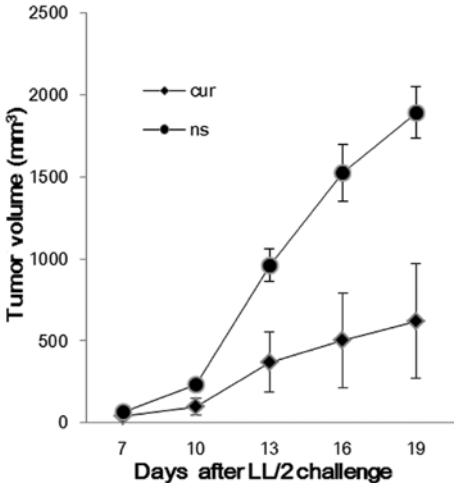

Figure 4. Liposomal curcumin-mediated inhibition of tumor growth in the LL/2 model. Mice were treated intravenously with NS or curcumin (10 mg/kg) every day. Tumor sizes on each mouse were measured every three days. Treatment with liposomal curcumin resulted in the marked inhibition of tumor growth (P<0.05).

cells (Fig. 3D). However, the change of cell cycle distribution with the increasing concentration of liposomal curcumin treatment was relatively insignificant in the LL/2 cell line (Fig. 3F).

Tumor growth inhibition in vivo. The established LL/2 tumor model was used to observe the effect of liposomal curcumin on the tumor burden of mice. The treatment regimens were carried out as described in Materials and methods. Compared with the control group, the liposomal curcumin-treated group was found to significantly inhibit tumor growth (Fig. 4). The tumor volume of the control and treated groups was (1892.26±158.03 mm^3) vs. (618.64±350.26 mm^3) on day 19.

Inhibition of tumor-induced angiogenesis. Tumor sections from each group were stained with anti-CD31 antibody (Fig. 5). Liposomal curcumin treatment resulted in the significant inhibition of angiogenesis in tumors (Fig. 5B) compared with the controls (Fig. 5A). Angiogenesis within tumor tissue was estimated by counting the number of microvessels on the section stained with an antibody reactive to CD31. Tumors from the liposomal curcumin group exhibited lower vessel density than those of the NS group (Fig. 5C). In addition, the inhibition of angiogenesis *in vivo* was observed through alginate encapsulation assay. Alginate implant angiogenesis was quantitated by measuring the uptake of FITC-dextran into beads. Vascularization of the alginate beads was reduced, and FITC-dextran uptake was decreased in liposomal curcumin-treated mice compared to the controls (Fig. 6).

Liposomal curcumin-mediated anti-angiogenesis in development of the zebrafish embryo. Our data demonstrate that liposomal curcumin effectively inhibited endothelial cell growth *in vitro* and *in vivo*. The endothelial cell line MS1 is a pancreatic islet endothelial cell line derived from C57BL/6

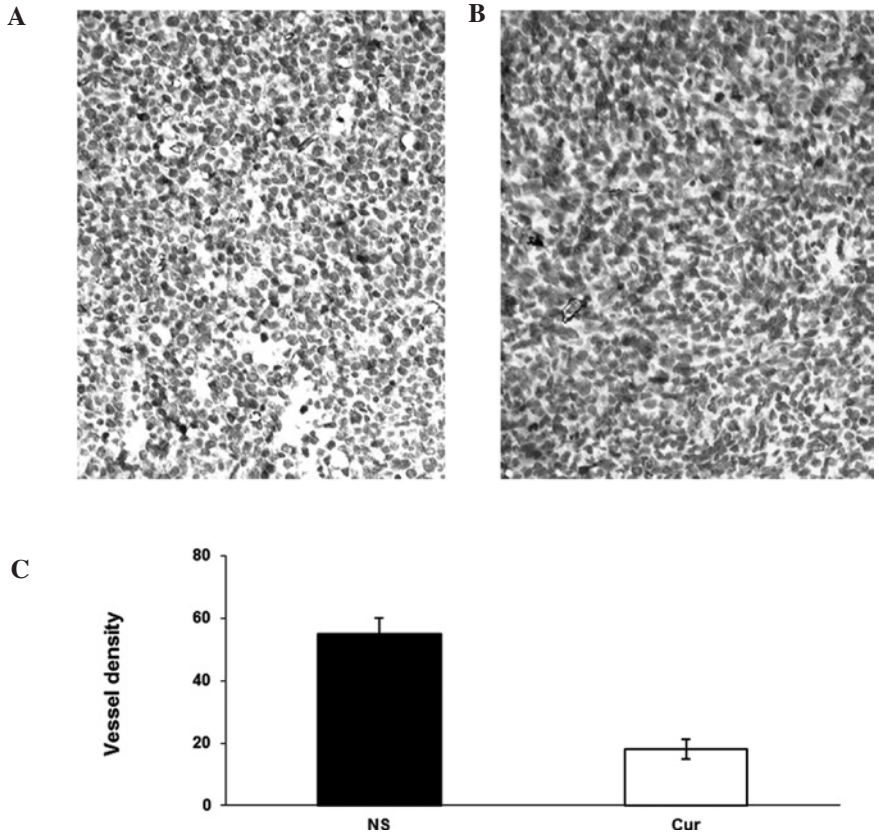

Figure 5. Inhibition of angiogenesis within the tumor estimated by CD31 immunohistochemical analysis. (A) Representative sections from the normal saline (NS) group. (B) Representative sections from the liposomal curcumin (Cur) group. (C) Vessel density was determined by counting the number of the microvessels per high-power field within a hot-spot area. Tumors of the liposomal curcumin group showed a smaller microvessel count than the NS group (P<0.05).

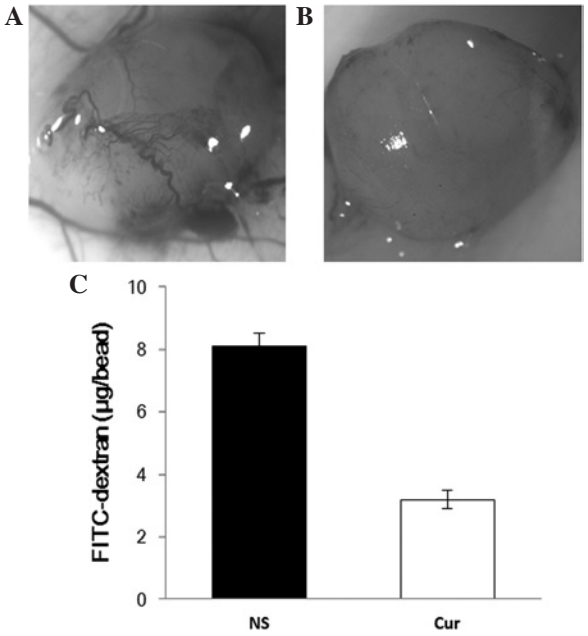

Figure 6. Inhibition of angiogenesis in tumors was estimated by encapsulation assay. Alginate beads containing $1x10^5$ LL/2 cells were implanted subcutaneously into C57BL/6 mice, which were treated with liposomal curcumin (Cur) once a day. Fourteen days later, beads were surgically removed, and FITC-dextran was quantified as described in Materials and methods. (A) Representative alginate beads from the normal saline (NS) group. (B) Representative alginate beads from liposomal curcumin group. (C) FITC-dextran uptake of beads from each group. The curcumin group showed a significant decrease in vascularization compared with the control group ($P<0.05$).

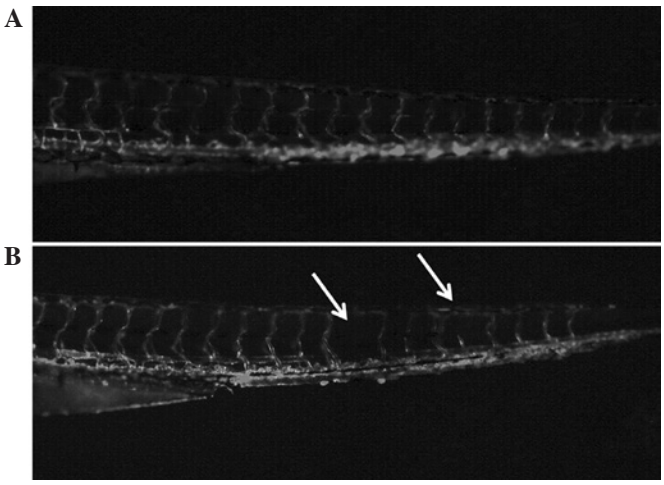

Figure 7. Effects of liposomal curcumin on the development of zebrafish. Transgenic FLK-1:EGFP zebrafish larvae were visualized under a Zeiss microscope. (B) Larvae hatching out of the liposomal curcumin-treated fertilized eggs, as indicated by the white arrows, showed a reduction or disappearance of certain vessels compared to the (A) controls.

mice and may exhibit physiological and pathological roles in the current C57BL/6 mouse model. Therefore, our data also revealed that the inhibition of angiogenesis mediated by liposomal curcumin is non-specific to tumor angiogenesis. To verify this, the anti-angiogenic effects of liposomal curcumin on the development of the zebrafish embryo were investigated. Larvae hatched from fertilized eggs treated with liposomal curcumin exhibited developmental defects. In the control fish, vascularization was normal. The angiogenetic defects caused by liposomal curcumin were evident compared to the control group (Fig. 7), indicating that inhibition of angiogenesis mediated by liposomal curcumin is non-specific and thus may show toxicity in physiological processes, such as wound healing.

Discussion

Curcumin inhibits tumor growth by targeting tumor cells and endothelial cells. The current study evaluated the anti-tumor and anti-angiogenesis activity of liposomal curcumin in a Lewis lung cancer model. Since a critical step in angiogenesis involves the proliferation of endothelial cells, we also determined the effect of liposomal curcumin on the viability of murine endothelial cells both *in vitro* and *in vivo*. Our data showed that the inhibitory effects of liposomal curcumin on angiogenesis were more effective. Inhibition of the NF-κB pathway mediated by curcumin is known to play a key role in its pharmacological activity (14,15). Accumulating evidence has shown that both physiological and pathological angiogenesis rely on the activity of the NF-κB pathway (16). However, NF-κB signaling has not been a preferred drug target in current clinical cancer therapy since the activity of NF-κB signaling is not necessary for tumor growth.

The significant finding of the current study is that murine endothelial cells (MS1) were more sensitive than murine lung tumor cells (LL/2). The cytotoxic activity of curcumin against these two cell lines was evaluated by MTT assay. Following treatment with curcumin (5 to 40 μg/ml) for 48 h, the viability of endothelial cells was markedly inhibited and lung cancer cells by comparison were only slightly inhibited. Moreover, the difference is clearer in low concentrations of curcumin (<20 μg/ml). We further investigated the possible mechanisms of this result. Curcumin inhibits cell proliferation through diverse mechanisms. However, as yet, the exact mechanism is not clear since various mechanisms act on different cells. Two possible mechanisms may have been involved in generating the results of this study. The first is induction of apoptosis: Our study showed that liposomal curcumin induces more cells to apoptosis in MS1 murine endothelial cells than in LL/2 murine lung tumor cells. It is also noteworthy that it had almost no effect on LL/2 at a concentration of 20 μg/ml. The possible mechanisms underlying the induction of apoptosis by liposomal curcumin may differ in the two cell lines; a more mechanistic study is therefore required in this area. The second possible mechanism is cell cycle arrest: We report in this study that liposomal curcumin treatment leads to G2/M arrest in MS1 and LL/2 cell lines. However, the effect on MS1 is more obvious than that on LL/2. In recent studies, other authors have reported that certain tumor cells are resistant to curcumin (17,18), which may correspond with the different genetics and biologies of individual tumors. The lack of effect of curcumin on LL/2 shown in our current study is consistent with their reports. Therefore, the inhibition of tumor angiogenesis is thought to play a more significant role in its anti-tumor effects. In addition, it is known that endothelial cells are genetically stable, and therefore less likely to rapidly develop drug resistance. Taken together, these results indicate that liposomal curcumin targeting angiogenesis that supports

tumor growth rather than the tumors themselves is a promising therapeutic approach for certain cancer types.

Various angiogenetic inhibitors have been developed to target vascular endothelial cells and block tumor growth. The majority of these inhibitors have limited potential since they are extremely toxic or highly expensive, and are therefore beyond the reach of most patients. Curcumin, a plant-derived compound, has been used safely as a food additive for centuries without reports of significant toxicity. In addition, curcumin is affordable and has been found to suppress angiogenesis through multiple mechanisms (18). Most of the studies used free curcumin, which is poorly water-soluble and has low bioavailability, and is therefore limited in its clinical efficacy. We prepared liposomal curcumin solution via the ethanol injection method. This solution is well dispersed and shows an average size of 125.7 nm, determined using a nano-particle size analyzer (data not shown). Liposome encapsulation of curcumin renders this agent amenable to intravenous administration.

A particularly encouraging aspect of this study is the observation that liposomal curcumin significantly suppressed LL/2 tumor growth *in vivo*, although it was almost ineffective on LL/2 cells *in vitro*. This result indicates that liposomal curcumin can be used for cancer therapy in curcumin-sensitive and -resistant tumor cells. The approach of anti-angiogenesis for the curcumin treatment of cancer appears promising. Our results also indicate that curcumin has the ability to block angiogenesis *in vivo*. This result may be associated with the inhibition of proliferation and the induction of apoptosis and cell cycle arrest in endothelial cells *in vitro*. Liposomal curcumin has been reported to exhibit anti-cancer activity on colorectal cancer (19), prostate cancer (20), head and neck squamous cell carcinoma (21), and pancreatic (22) and cervical cancer (23). In this study, we revealed that liposomal curcumin inhibits tumor growth in the Lewis lung cancer mouse model primarily by targeting tumor angiogenesis.

The effect of liposomal curcumin on physiological angiogenesis was also investigated in this study. We used zebrafish as vertebrate model organisms to investigate the anti-angiogenic effect of curcumin in the development of the embryo. Advantages of using zebrafish as model organisms include their fecundity, optical clarity, and genetic similarity to mammals. Research using rats as animal models revealed that orally administered curcumin had no toxic effects on fertility or pregnancy (24). Our findings have shown that, unlike in rats, liposomal curcumin had embryotoxic and anti-angiogenic effects on the development of zebrafish embryos. Therefore, our study indicates that liposomal curcumin-mediated anti-angiogenic effects are not tumor-specific but broad-spectrum and may be used to treat angiogenesis-related diseases. However, further investigation into the toxicity of liposomal curcumin is required.

Acknowledgements

This study was financially supported by the Chinese National Natural Science Foundation.

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