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ORIGINAL PAPER

Liposomal honokiol, a promising agent for treatment of cisplatin-resistant human ovarian cancer

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Abstract
Purpose Honokiol has been receiving attention as an anti-cancer agent because of its anti-tumor effect. In the current study, we encapsulated honokiol with liposome and tested it on cisplatin-sensitive (A2780s) and -resistant (A2780cp) human ovarian cancer models.
Methods The anti-tumor activity of liposomal honokiol (Lipo-HNK) was evaluated in nude mice bearing A2780s and A2780cp s.c. tumors. Mice were treated twice weekly with i.v. administration of Lipo-HNK (10 mg/kg), control liposome (10 mg/kg), 0.9% NaCl solution or weekly with intraperitoneally administered cisplatin (5 mg/kg) for 3 weeks. Tumor volume and survival time were observed. Assessment of apoptotic cells by TUNEL assay was conducted in tumor tissue. Microvessel density within tumor tissue was determined by CD34 immunohistochemistry. For in vitro study, induction of apoptosis by Lipo-HNK was examined by PI staining fluorescence microscopy, DNA fragmentation assay and flow cytometric analysis.
Results Administration of Lipo-HNK resulted in significant inhibition (84–88% maximum inhibition relative to controls) in the growth of A2780s and A2780cp tumor xenografts and prolonged the survival of the treated mice. These anti-tumor responses were associated with marked increases in tumor apoptosis, and reductions in intratumoral microvessel density.
Conclusions The present findings suggest that Lipo-HNK may provide an effective approach to inhibit tumor growth in both cisplatin sensitive and -resistant human ovarian cancer with minimal side effects.

Keywords Liposomal honokiol (Lipo-HNK) · Apoptosis · Angiogenesis · Cisplatin-resistant ovarian cancer

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Introduction

Ovarian cancer is one of the most common tumors in female genital organs and a serious threat to the health of women because of the high mortality rate (5-year survival rate of 20–30%) (Hoskin et al. 1994). Except for some improvement in survival length with the introduction of platinum and paclitaxel therapy, the long-term survival remains poor (Ozols et al. 2004) due to eventual tumor recurrence and emergence of drug-resistant disease. Therefore, the development of more effective treatments, especially agents which lack of cross resistance with established chemotherapeutic agents would be instrumental in the ability to fight ovarian cancer.

Honokiol is an active compound isolated from the bark and seed cones of the magnolia tree (Ishitsuka et al. 2005). It has many biological activities, such as anti-tumor, antiproliferative effects and induces apoptosis of a wide range of human cancer cell lines in vitro and in vivo, for example, honokiol was highly effective against angiosarcoma in nude mice (Bai et al. 2003; Battle et al. 2005; Wang et al. 2004; Hibasami et al. 1998; Yang et al. 2002). A number of possible mechanisms of honokiol on cancer cells have been suggested: (1) suppression of NF-jB-regulated anti-apoptotic signaling (Van Antwerp et al. 1996); (2) activation of caspase-8 and triggering caspase in the TNF-a apoptotic pathway (Hsu et al. 1996); (3) promotion of c-Jun N-terminal kinase signaling (JNK) (Deng et al. 2003); (4) accumulation of ROS (Weitsman et al. 2003); (5) inhibition of p38 MAPK activation (Luschen et al. 2004); and (6) inhibition of protein synthesis (Nio et al. 1990), specifically the expression of NF-jB-regulated anti-apoptotic proteins (Li et al. 2004). These data suggested that Honokiol has potential to be developed as an anticancer agent.

However, the extreme water insolubility of honokiol hampers its delivering to the tumor at an effective concentration. In order to make it soluble, our laboratory used liposome to encapsulate the honokiol and named it as Lipo-HNK. In the previous study, we demonstrated the efficient inhibition of Lipo-HNK on SKOV3 human ovarian cystadenoma cells in vitro and in vivo (Xi Liu et al., manuscript submitted for publication). Because the mechanisms of Lipo-HNK is totally different from chemotherapeautic agents such as cisplatin, the present study was aimed to find out whether it could overcome cisplatin-resistance in ovarian cancer. The Lipo-HNK demonstrated a profound inhibiting effect on the growth of not only cisplatin-sensitive (A2780s) but also -resistant (A2780s) ovarian cancer cells in vitro and in vivo, and significantly prolonged the survival of the treated mice.

Materials and methods

Cell culture and reagents

The derivation and source of established human ovarian cancer cell lines A2780s and A2780cp have been described previously (Louie et al. 1985). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. Cisplatin and DMSO were purchased from Sigma. Cisplatin was dissolved in DMSO and diluted in NS before use, the final concentration of DMSO was 0.1%.

Preparation of liposomal honokiol

Liposomal honokiol (Lipo-HNK) was prepared in our lab and described briefly as follows: PC, cholesterol, PEG4000 and honokiol in weight ratios of 1:0.15:0.24:0.22 were mixed and dissolved in 15 ml chloroform/methanol at a ratio of 1:4 (v/v). The mixture was gently warmed to 40°C in a round-bottomed flask, and the solvent was evaporated under vacuum in a rotary evaporator until a thin lipid film was formed. The dried lipid films were left overnight and sonicated in 5% glucose solution followed by concentration and lyophilization. The preparation of empty liposome was the same way as Lipo-HNK without addition of honokiol. The final Lipo-HNK and empty liposome were small multilamellar liposomes in a size range of 150 ± 20 and 100 ± 20 nm, respectively. Lyophilized Lipo-HNK and empty liposome were dissolved in NS for vitro and vivo studies.

Apoptotic assessment in vitro

The MTT assay was done to investigate the resistance of A2780cp cells. For cisplatin, IC50 (48 h) of A2780s cells was 10 μM and A2780cp cells was more than 100 μM. So the A2780cp cells were at least ten times more resistant to cisplatin than the A2780s cells. For Lipo-HNK, IC50 (48 h) of A2780s cells was 36 μM and A2780cp cells was 34.7 μM (data not shown).

A2780cp cells (5×10^5) and A2780s cells (5×10^5) were grown in six-well plates and incubated for 24 h to 70% confluence. Cells were incubated with Lipo-HNK, control liposome, 0.9% NaCl solution (NS), or cisplatin (10 or 100 μM) for additional 48 h. The morphological changes of cells were observed by PI-staining fluorescence microscopy. DNA fragmentation assay was done as previously described (Wei et al. 1994). Quantitative evaluation of cellular apoptosis was performed by flow cytometric analysis using PI staining method. Image of cells was taken by using ZEISS AXIOVERT 200 microscope and Axio Cam MRm camera.

Animal groups and treatment in vivo

The following studies were approved by the Institutional Animal Care and Treatment Committee of Sichuan University. Female athymic BALB/c nude mice, 6–8 weeks old, were maintained in pathogen-free conditions and fed sterile chow. A2780s cells or A2780cp cells (2×10^6) in 0.1 ml of PBS were injected s.c. into the right flank of each mouse. Mice were assigned randomly to one of the four groups (five per group) when tumors were palpable: (a) mice treated with 100 μl Lipo-HNK (10 mg/kg), (b) mice treated with control liposome (10 mg/kg), (c) mice treated with

100 µl of 0.9% NS, (d) mice treated with cisplatin (5 mg^{-1} kg^{-1} day). Treatment of Lipo-HNK (twice/week) started when the tumor was palpable and continued for 3 weeks. To investigate cisplatin resistance of A2780cp tumors in vivo, 5 mg^{-1} kg^{-1} day of cisplatin (Qilu Pharmaceutical Co., Shandong, China) was administered i.p. once per week for 3 weeks. Tumor sizes were measured every 3 days using the formula $A \times B^2 \times 0.5236$ (A, length; B, width; all measured in millimeters).

Evaluation of possible adverse effects

Mice treated with 10 mg/kg Lipo-HNK have been investigated, in particular for potential toxicity for more than 6 months. Gross measures such as weight, fur, appetite, life span, and behavior were observed. Tissues of heart, liver, spleen, lung, kidney, bone marrow, brain, pancreas, and intestine were also fixed in 10% buffered formalin solution and embedded in paraffin. Sections of 3–5 µm were stained with hematoxylin and eosin (H&E).

Immunohistochemistry

Paraffin embedded tumor tissue was sliced into sections (5 µm) for microvessel density (MVD) quantification. Staining for MVD was done with rat anti-mouse CD34 antibody (Santa Cruz Biotechnology) using the labeled streptavidin-biotin method. Briefly, sections were baked at 56°C for 120 min, deparaffinized in xylene, rehydrated in graded alcohols, and washed in PBS. For heat-induced epitope retrieval, the sections were pretreated in a microwave oven [12 min in sodium citrate buffer (pH6.0)]. The endogenous peroxidase was inhibited by 0.3% H_2O_2 for 10 min, and the sections were incubated with 10% normal goat serum for 30 min. Primary antibody (rat anti-mouse CD34) were applied overnight in a moist chamber at 4°C. A standard avidin-biotin peroxidase technique (DAKO, Carpinteria, CA, USA) was applied. Briefly, biotinylated goat anti-rat immunoglobulin and avidin-biotin peroxidase complex were applied for 30 min each, with 30 min washes in PBS. The reaction was finally developed by DAKO Liquid DAB+ Substrate-Chromogen System. MVD was determined by examining vascular hot spots as described previously (Mukherjee et al. 2004). Image of tumor tissue and microvessels was taken by using OLYMPUS BX600 microscope and SPOT FIEX camera.

TUNEL detection of apoptotic tumor cells

Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) staining was performed with an in situ apoptotic cell detection kit according to the manufacturer's directions (Promega) as described previously (Mukherjee et al. 2002). Images of the representative sections were taken by using ZEISS AXIOVERT 200 microscope and Axio Cam MRm camera.

Statistical analysis

Comparisons of tumor volume among the different groups were performed by using one-way analysis of variance (ANOVA). Survival curves were based on the Kaplan–Meier method and statistical significance was determined by the log-rank test. $P < 0.05$ was defined as significant.

Results

Effect of Lipo-HNK on A2780s and A2780cp cancer cells in vitro

Cisplatin-sensitive (A2780s) and -resistant (A2780cp) ovarian cancer cells were seeded in six-well plates and treated with Lipo-HNK, control liposome, NS, or cisplatin, respectively. By fluorescence microscopy of PI-staining, Lipo-HNK-treated cells resulted in morphological changes characterized as apoptosis: a brightly red-fluorescent condensed nuclei (intact or fragmented), apoptotic bodies and reduction of cell volume. However, these changes seldom happened in liposome-treated or NS-treated cells (Fig. 1a). In agarose gel electrophoresis of Lipo-HNK-treated cells, there was a ladder-like pattern of DNA fragments consisting of approximately 180–200 base pairs, which was consistent with internucleosomal DNA fragmentation (Fig. 1b).

The numbers of apoptotic cells were quantitatively estimated by observing sub-G1 (apoptotic) cells using flow cytometric analysis of PI staining method. From the result of flow cytometry, we were convinced that cisplatin was effective in A2780s cells and ineffective in A2780cp cells, because the apoptotic cells of A2780cp cells only accounted for 20% when treated with 100 µM cisplatin, while the percentage of sub-G1 cells was 50.8% in A2780s cells when treated with 10 µM cisplatin. There were a large amount of apoptotic cells in Lipo-HNK-treated cells compared with liposome-treated or NS-treated cells in both tumor models. In A2780s tumor model, the apoptotic cells accounted for 61.3% in Lipo-HNK-treated group versus 13.5% in liposome-treated group and 3.5% in NS-treated group. In A2780cp tumor model, the apoptotic cells accounted for 55.1% in Lipo-HNK-treated group versus 14.2% in liposome-treated group and 5.3% in NS-treated group (Fig. 2).

Results obtained from flow cytometry strongly correlated with DNA fragmentation in agarose gel electrophoresis and morphological changes in fluorescence microscopy of PI-staining.

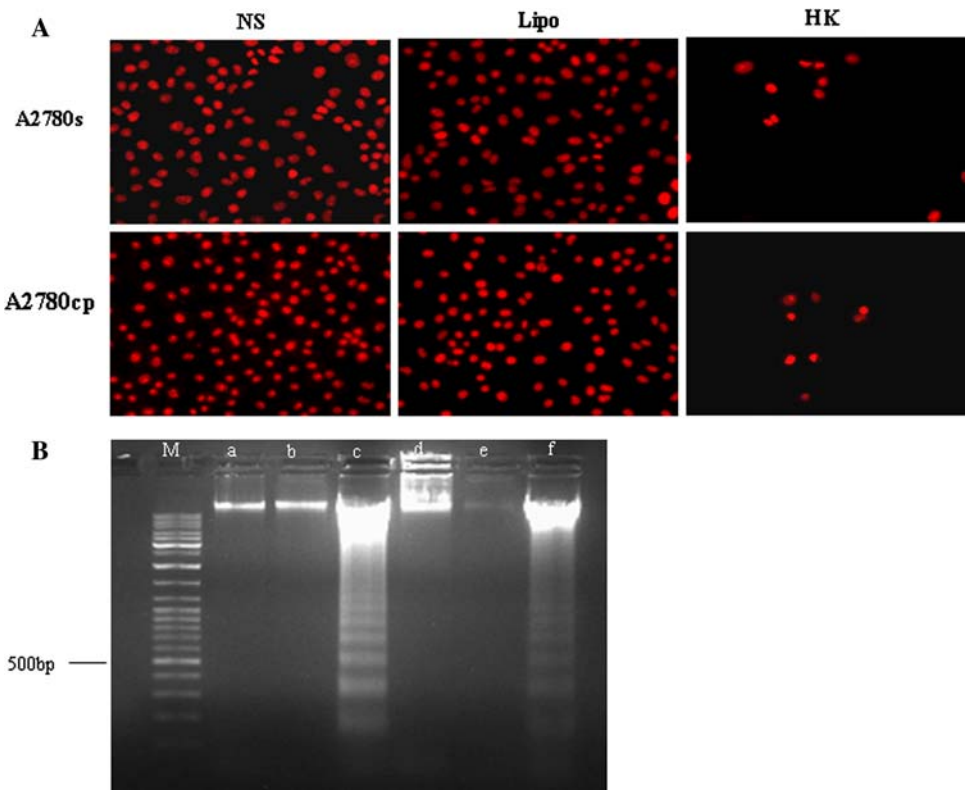

Fig. 1 PI stained fluorescence microscopy of A2780s and A2780cp cells and DNA fragmentation of Honokiol-treated cells. **a** Cells were treated with NS, liposome, or Lipo-HNK for 48 h. Lipo-HNK-treated cells resulted in morphological changes characterized as apoptosis: a brightly *red*-fluorescent condensed nuclei (intact or fragmented), reduction of cell volume, and apoptotic bodies. Original magnification, ×200. **b** DNA fragmentation of Lipo-HNK-treated cells (72 h after transfection): A2780s cells were treated with NS (*lane a*), liposome (*lane b*) or Lipo-HNK (*lane c*); A2780cp cells were treated with NS (*lane d*), liposome (*lane e*) or Lipo-HNK (*lane f*)

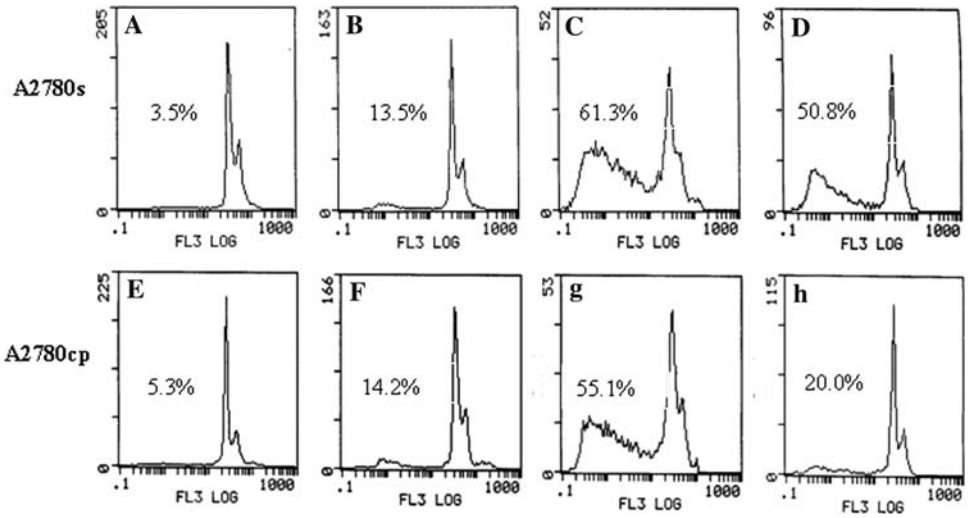

Fig. 2 DNA fluorescence histograms of PI-stained A2780s and A2780cp cells. A2780s cells were treated with NS (**a**), liposome (**b**), Lipo-HNK (**c**) or 10 μM cisplatin (**d**) for 48 h; A2780cp cells were treated with NS (**e**), liposome (**f**), Lipo-HNK (**g**) or 100 μM cisplatin (**h**) for 48 h

Lipo-HNK inhibits both A2780s and A2780cp ovarian cancer growth in vivo

A2780s or A2780cp ovarian cancer cells were injected into the right flank of each mouse. Mice were treated twice weekly via the tail vein with 10 mg/kg Lipo-HNK, 10 mg/kg control liposome, 0.9% NS or weekly with i.p. administration of cisplatin (5 mg/kg) for 3 weeks. Tumor volumes were measured every 3 days. In A2780s tumor model, tumor volumes of groups treated with Lipo-HNK or cisplatin were significantly smaller in comparison to the control groups treated with NS or control liposome. Mean tumor volume (±SD) in Lipo-HNK-treated mice was 222 ± 71 versus $1{,}823 \pm 606\ mm^3$ in liposome-treated mice and $3{,}921 \pm 235\ mm^3$ in NS-treated mice, $P < 0.01$. Although cisplatin-treated tumor is smaller (tumor volume $112 \pm 157\ mm^3$), there was no significant difference in tumor volume between Lipo-HNK-treated and cisplatin-treated mice, $P > 0.05$ (Fig. 3a); Complete tumor regression happened in one of the five mice administrated with Lipo-HNK. In A2780cp tumor model, Lipo-HNK significantly inhibited tumor growth of the treated mice in comparison to the controls. Mean tumor volume (±SD) in Lipo-HNK-treated mice was 408 ± 165 versus $2{,}575 \pm 701\ mm^3$ in

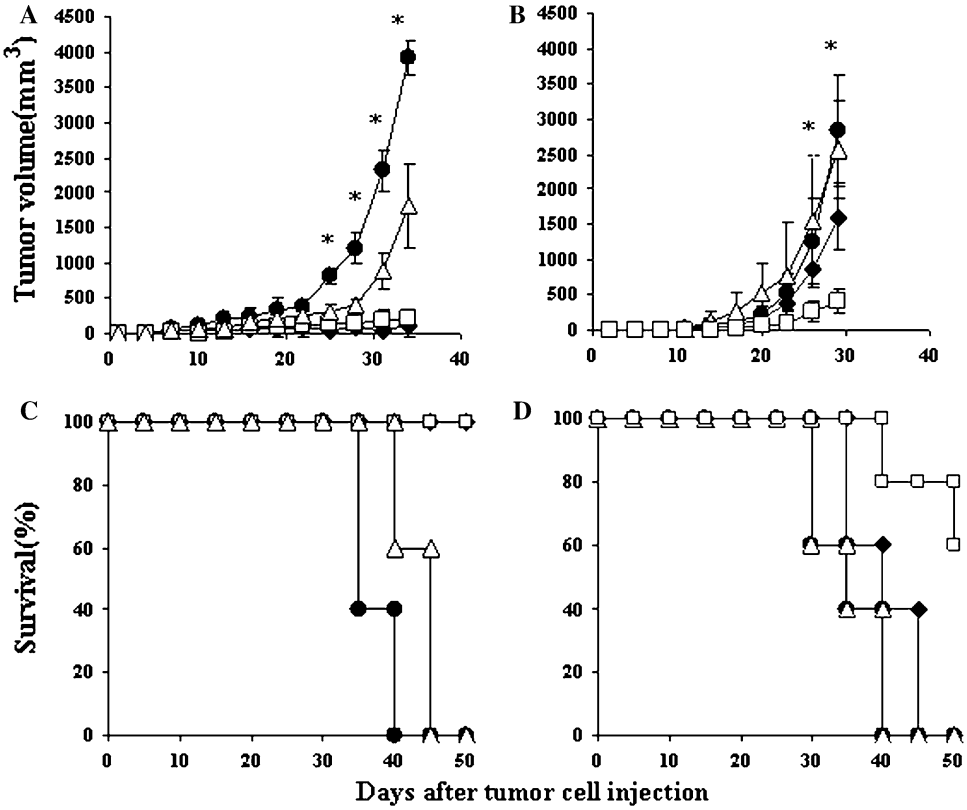

Fig. 3 Tumor suppression and survival advantage in mice. A2780s cells (**a**, **c**) or A2780cp cells (**b**, **d**) of 2×10^6 were inoculated s.c. into nude mice. Mice (five per group) were treated with i.v. administration of Lipo-HNK (10 mg/kg) (*open square*), liposome (*open rectangle*), or NS (*filled circle*) twice per week or with i.p. administration of cisplatin (5 mg/kg) (*open rectangle*) once per week for 3 weeks, when tumors were palpable. *Asterisks* indicate a significant difference in tumor volume ($P < 0.01$) between Lipo-HNK-treated and control groups. Points, average tumor volume; *bars* ±SD. A significant increase in survival in Lipo-HNK-treated mice was also found in both tumor models, compared with the control groups ($P < 0.01$, by log-rank test)

liposome-treated mice, $2{,}828 \pm 796\ mm^3$ in NS-treated mice and $1{,}607 \pm 466\ mm^3$ in cisplatin-treated mice, $P < 0.05$ (Fig. 3b); complete tumor regression happened in three of the five mice treated with Lipo-HNK.

Lipo-HNK prolonged the survival of tumor-bearing mice

The beneficial effects of Lipo-HNK on cisplatin-sensitive and -resistant ovarian cancer models were also reflected in the survival time. Survival of the tumor-bearing mice treated with Lipo-HNK in both tumor models was significantly prolonged compared with the control therapies, $P < 0.01$.There was no significant difference in survival time between Lipo-HNK-treated mice and cispatin-treated mice in A2780s tumor model. Mice with complete tumor regression gained long time survival in Lipo-HNK-treated groups of both tumor models (Fig. 3c, d).

Toxicity observation

Animals treated with 10 mg/kg Lipo-HNK were particularly investigated for potential toxicity for more than 6 months. No changes in gross measures, such as weigh loss, feeding, ruffling of fur, behavior, and so on, were indicated. Furthermore, H&E histological staining of the heart, liver, spleen, lung, kidney, pancreas, intestine, brain and bone marrow did not reveal any significant differences between Lipo-HNK-treated and the control mice of both tumor models.

Increased intratumoral apoptosis in Lipo-HNK treated mice

To further explore the role of Lipo-HNK therapy in tumor in vivo, we applied TUNEL assay to detect apoptosis of tumor cells within tumor tissue. Cell nuclei stained with dark green, as viewed by fluorescence microcopy (magnification, 200×), indicated apoptosis, and were recorded as TUNEL-positive nuclei. As shown in Fig. 4, significant increases of TUNEL-positive nuclei were found in Lipo-HNK-treated group compared with the two controls ($P < 0.05$): In A2780s tumors, mean apoptotic index ± SD of cancer cells treated with Lipo-HNK was 28.67 ± 2.5 versus $6.8 \pm 1.1\%$ in liposome-treated group and $4.07 \pm 0.5\%$ in NS-treated group, respectively ($P < 0.05$). In A2780cp tumors, the figure was $19.3 \pm 1.5\%$ in Lipo-HNK-treated group versus $6.2 \pm 0.7\%$ in liposome-treated group and $3.2 \pm 0.7\%$ in NS-treated group, respectively ($P < 0.05$).

Lipo-HNK inhibits intratumoral angiogenesis

Angiogenesis within tumor tissues was evaluated by counting the number of microvessels in paraffin embedded sections stained with an antibody reactive to CD34. The most

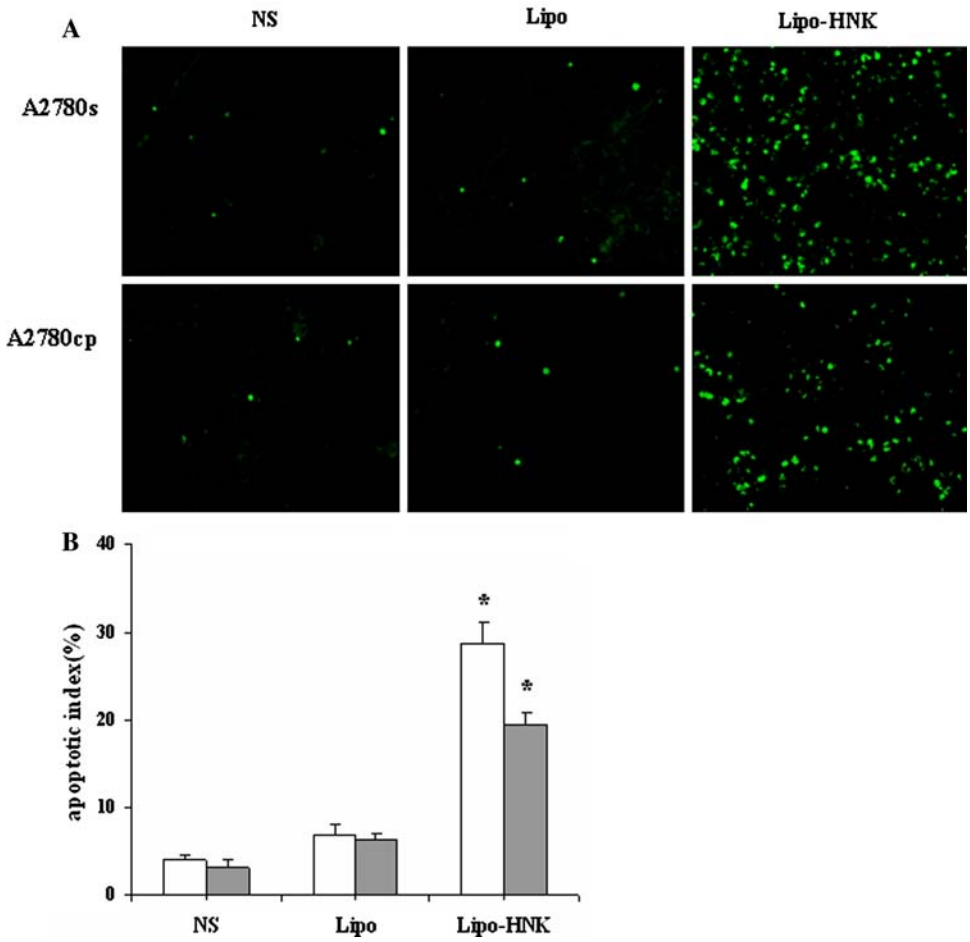

Fig. 4 Terminal deoxynucleotidyl transferase-mediated nick-end labeling staining of tumor tissues. **a** Representative sections were taken from A2780s and A2780cp tumor tissue of NS-treated, liposome-treated and Lipo-HNK-treated mice. Original magnification, ×200. **b** In A2780s tumors (*open square*)and A2780cp tumors (*filled square*), percent apoptosis in the treatment group markedly increased in comparison with controls. * $P < 0.05$

highly vascularized area of each tumor was identified on low power and five high-powered fields were counted in this area of greatest vessel density. Lipo-HNK-treated tumors resulted in the apparent inhibition of angiogenesis in both A2780s and A2780cp tumors compared with liposome-treated and NS-treated groups, $P < 0.05$ (Fig. 5).

Discussion

Despite the common clinical use of cisplatin for the treatment of ovarian cancer, the resistance of tumor cells to cisplatin remains a major therapeutic problem. It has been reported that drug resistance to currently used chemotherapeutics is thought to be partly mediated by the ability to circumvent apoptosis (Malaguarnera 2004; Ferreira et al. 2002; Vasey 2003). If chemotherapeutic-induced DNA damage accumulates beyond a threshold, programmed cell death will be initiated (Ferreira et al. 2002; Vasey 2003; Shepherd 2000). Previous studies have stated that the acquired drug resistance of ovarian cancer cells is associated with alterations in apoptosis (Perez 1998); inhibition of apoptosis is taken to be a major contributing factor to cisplatin-resistance in various ovarian cancer cell lines (Henkels and Turchi 1999; Li et al. 2001; Liu et al. 2002). In this regard, new treatment approaches with different mechanisms of apoptosis induction are all urgently needed to address the limited effectiveness of current treatment modalities for ovary cancer.

Honokiol, a component of traditional tea extracts, is known for its low toxicity and favorable oral absorption. It has multiple pharmacologic actions including anti-oxidative, anti-angiogenesis, and anti-tumor effects and other less well-defined effects on behavior (Ishitsuka et al. 2005). Because NF-jB and ROS activation correlate with the initiation of carcinogenesis, the anti-oxidative effects of honokiol could also justify its use to prevent cancer development (Valko et al. 2006). Bai et al. (2003) demonstrated that 100 mg/kg honokiol administered via the intraperitoneal route in nude mice revealed significant inhibition of tumor growth in vivo without any systemic toxicities. Other studies have also demonstrated the apoptosis inducing ability of honokiol in vitro and in vivo of tumor cells (Bai et al. 2003; Battle et al. 2005; Wang et al. 2004; Hibasami et al. 1998; Yang et al. 2002).

However, the extreme water insolubility of honokiol hampers it's delivering to the tumor at an effective concen-

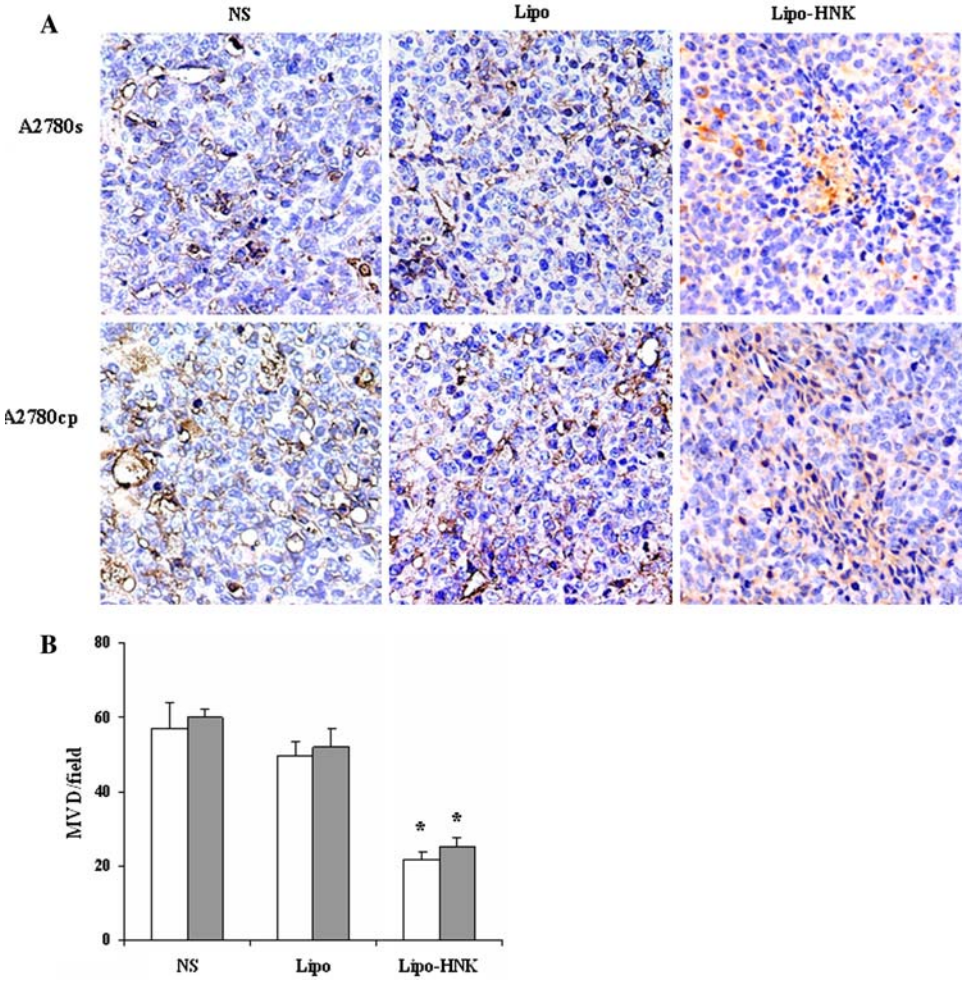

Fig. 5 Inhibition of intratumoral angiogenesis assayed by CD34 staining of microvessels. Tumor angiogenesis was assessed by immunohistochemical staining with anti-CD34 antibody (*brown*) on paraffin-embedded sections. Representative sections were taken from A2780s and A2780cp tumor tissue of NS-treated, liposome-treated and honokiol-treated mice. Original magnification, ×400. **b** In A2780s tumors (*white bar*) and A2780cp tumors (*gray bar*), the number of microvessels was significantly smaller in Lipo-HNK-treated group compared with two controls. * $P < 0.05$

tration. To solve this problem, a suitable drug delivery system with the aim of making the agent soluble and enhancing its accumulation at the site of tumor is needed. The best selection is liposome. Liposomes have been investigated extensively as carriers for anticancer drugs in attempts to direct active agents to tumors in vivo (Senior 1987; Ranade 1989) or to protect sensitive tissues from toxicity (Rahman et al. 1978). They can provide slow release of an encapsulated drug, resulting in sustained exposure to tumor cells and enhanced efficacy (Webb et al. 1995). They accumulate preferentially at tumor sites as a result of their ability to extravasate through "pores" or "defects" in the capillary endothelium, while these "pores" appear to be a consequence of the rapid angiogenesis occurring in tumors and are generally not present in normal tissues or organs (Yuan et al. 1995). Further more, when combined with a hydrophilic coat such as PEG, liposome provides long retention in circulation (Gabizon 2001). In our previous study, the Lipo-HNK has exhibited profound anti-tumor and anti-angiogenesis ability against SKOV3 ovarian cancer cells in vitro and in vivo. These results of the previous work may suggest that the anti-tumor effect of liposome-HNK is proportional to tumor delivery and that, once within the tumor, the entrapped drug retains its full antineoplastic activity.

In the current study, we tested the Lipo-HNK on cisplatin sensitive (A2780s) and -resistant (A2780cp) human ovarian cancer models. The A2780cp cells are at least ten times more resistant to cisplatin than the A2780s cells in vitro, and cisplatin could not suppress the A2780cp tumor growth under the conditions where the drug is effective against the A2780s tumor. Lipo-HNK efficiently inhibited the growth of both A2780s and A2780cp cancer xenografts, and prolonged the survival of the treated mice. Further more, complete tumor regression happened in one of the five mice administered with Lipo-HNK in A2780s tumor model, and there was no significant difference in tumor volume between Lipo-HNK-treated and cisplatin-treated mice. For mice bearing A2780cp tumor, three of the five mice in Lipo-HNK-treated group demonstrated complete tumor regression. Under the conditions of our study, with the drug administered in this dose and schedule, the histopathology of animal tissues revealed no systemic toxicity. These results strongly suggest that Lipo-HNK may overcome

cisplatin resistance at least partly in some tumors. Although we did not examine the synergism of Lipo-HNK with cisplatin, it should be confirmed by in vivo experiments in the future with other cell lines.

We had also made several observations concerning the effect of Lipo-HNK on A2780s and A2780cp cells in vitro, including PI staining fluorescence microscopy, DNA fragmentation assay, and flow cytometric analysis of Lipo-HNK-treated cancer cells. Our findings were consistent with the apoptosis-inducing ability of honokiol reported before (Kopecky et al. 2001). Having witnessed the profound anti-tumor activity of Lipo-HNK in vitro, we did TUNEL staining of Lipo-HNK-treated tumor tissue to study the anti-tumor mechanism in vivo. Our finding was also consistent with the apoptosis-inducing ability of honokiol mentioned above.

In addition, the anti-angiogenic activity of honokiol could have general importance in eradicating cancer growth locally. Angiogenesis plays a vital role in tumor growth and metastasis. One measure of angiogenic activity, MVD, is high in ovarian cancer tissue. Treatment with Lipo-HNK resulted in the apparent reductions in intratumoral MVD of both A2780s and A2780cp tumors compared with liposome-treated or NS-treated mice.

In conclusion, anti-tumor and anti-angiogenesis ability integrated with perfect drug delivery system have made the Lipo-HNK efficacious against both cisplatin sensitive and -resistant human ovarian cancer. Our findings collectively suggest that Lipo-HNK is a promising agent that can be considered as a new treatment approach for human ovarian cancer, particularly for patients with cisplatin-resistance.

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References

Bai X, Cerimele F, Ushio-Fukai M et al (2003) Honokiol, a small molecular weight natural product, inhibits angiogenesis in vitro and tumor growth in vivo. J Biol Chem 278:35501–35507

Battle TE, Arbiser J, Frank DA (2005) The natural product honokiol induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells. Blood 106:690–697

Deng Y, Ren X, Yang L, Lin Y, Wu X (2003) A JNK-dependent pathway is required for TNFalpha-induced apoptosis. Cell 115:61–70

Ferreira CG, Epping M, Kruyt FA, Giaccone G (2002) Apoptosis: target of cancer therapy. Clin Cancer Res 8:2024–2034

Gabizon AA (2001) Pegylated liposomal doxorubicin:metamorphosis of an old drug into a new form of chemotherapy. Cancer Invest 19:424–436

Henkels KM, Turchi JJ (1999) Cisplatin-induced apoptosis proceeds by caspase-3-dependent and -independent pathways in cisplatin-resistant and -sensitive human ovarian cancer cell lines. Cancer Res 59:3077–3083

Hibasami H, Achiwa Y, Katsuzaki H et al (1998) Honokiol induces apoptosis in human lymphoid leukemia Molt 4B cells. Int J Mol Med 2:671–673

Hoskin WJ, Mcguire WP, Brandy MF et al (1994) The effect of diameter of largest residual disease on survival after primary cytoreductive surgery in patients with suboptimal residual epithelial ovarian carcinoma. Am J Obstet Gynecol 174:974–979

Hsu H, Shu HB, Pan MG, Goeddel DV (1996) TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84:299–308

Ishitsuka K, Hideshima T, Hamasaki M et al (2005) Honokiol overcomes conventional drug resistance in human multiple myeloma by induction of caspase-dependent and -independent apoptosis. Blood 106:1794–1800

Kopecky SA, Willingham MC, Lyles DS (2001) Matrix protein and another viral component contribute to induction of apoptosis in cells infected with vesicular stomatitis virus. J Virol 75:12169–12181

Li J, Feng Q, Kim JM et al (2001) Human ovarian cancer and cisplatin resistance: possible role of inhibitor of apoptosis proteins. Endocrinology 142:370–380

Li L, Thomas RM, Suzuki H, De Brabander JK, Wang X, Harran PG (2004) A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. Science 305:1471–1474

Liu JR, Opipari AW, Tan L et al (2002) Dysfunctional apoptosomal activation in ovarian cancer: implications for chemoresistance. Cancer Res 62:924–931

Louie KG, Behrens BC, Kinsella TJ et al (1985) Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. Cancer Res 45:2110–2115

Luschen S, Scherer G, Ussat S, Ungefroren H, Adam-Klages S (2004) Inhibition of p38 mitogen-activated protein kinase reduces TNF-induced activation of NF-kappaB, elicits caspase activity, and enhances cytotoxicity. Exp Cell Res 293:196–206

Malaguarnera L (2004) Implications of apoptosis regulators in tumorigenesis. Cancer Metastasis Rev 23:367–387

Mukherjee P, Abate LE, Seyfried TN (2004) Antiangiogenic and proapoptotic effects of dietary restriction on experimental mouse and human brain tumors. Clin Cancer Res 10:5622–5629

Mukherjee P, El-Abbadi MM, Kasperzyk JL, Ranes MK, Seyfried TN (2002) Dietary restriction reduces angiogenesis and growth in an orthotopic mouse brain tumor model. Br J Cancer 86:1615–1621

Nio Y, Zighelboim J, Berek J, Bonavida B (1990) Cycloheximide-induced modulation of TNF-mediated cytotoxicity in sensitive and resistant ovarian tumor cells. Cancer Chemother Pharmacol 26:1–8

Ozols RF, Bookman MA, Connolly DC et al (2004) Focus on epithelial ovarian cancer. Cancer Cell 5:19–24

Perez RP (1998) Cellular and molecular determinants of cisplatin resistance. Eur J Cancer 34:1535–1542

Rahman YE, Hanson WR, Bharucha J, Ainsworth EJ, Jaroslow BN (1978) Mechanisms of reduction of antitumor drug toxicity of liposome encapsulation. Ann NY Acad Sci 308:325–341

Ranade VV (1989) Drug delivery systems. 1. Site-specific drug delivery using liposomes as carriers. J Cancer Pharmacol 29:685–694

Senior JH (1987) Fate and behavior of liposomes in vivo: a review of controlling factors. CRC Crit Rev Ther Drug Carrier Syst 3:123–193

Shepherd JE (2000) Current strategies for prevention, detection, and treatment of ovarian cancer. J Am Pharm Assoc (Wash) 40:392–401

Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006) Free radicals, metals and antioxidants in oxidative stressinduced cancer. Chem Biol Interact 160:1–40

Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM (1996) Suppression of TNF-alpha-induced apoptosis by NFkappaB. Science 274:787–789

Vasey PA (2003) Resistance to chemotherapy in advanced ovarian cancer: mechanisms and current strategies. Br J Cancer 89(Suppl 3):S23–S28

Wang T, Chen F, Chen Z et al (2004) Honokiol induces apoptosis through p53-independent pathway in human colorectal cell line RKO. World J Gastroenterol 10:2205–2208

Webb MS, Harasym TO, Masin D, Bally MB, Mayer LD (1995) Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetics and therapeutic properties of vincristine in murine and human tumor models. Br J Cancer 72:896–904

Wei Y-Q, Zhao X, Kariya Y, Fukata H, Teshigawara K, Uchida A (1994) Induction of apoptosis by quercetin: involvement of heat shock protein. Cancer Res 54:4952–4957

Weitsman GE, Ravid A, Liberman UA, Koren R (2003) Vitamin D enhances caspase-dependent and -independent TNFalphainduced breast cancer cell death: the role of reactive oxygen species and mitochondria. Int J Cancer 106:178–186

Yang SE, Hsieh MT, Tsai TH, Hsu SL (2002) Down-modulation of Bcl-XL, release of cytochrome c and sequential activation of caspases during honokiol-induced apoptosis in human squamous lung cancer CH27 cells. Biochem Pharmacol 63:1641–1651

Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, Jain RK (1995) Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. Cancer Res 55:3752–3756