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Oriental Pharmacy and Experimental Medicine 2005 **5(4)**, 283-293

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DMNQ S-52, a new shikonin derivative, inhibits lymph node metastasis *via* inhibition of MMPs production

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SUMMARY

Our previous study showed that a novel synthetic shikonin derivative, 6-(1-hydroxyimino-4-methylpentyl)5,8-dimethyoxy 1,4-naphthoquinone S-52 (DMNQ S-52) induced apoptosis. In the present study, we investigated its anti-metastatic activities as compared with shikonin because DMNQ S-52 was synthesized for overcoming weak points of shikonin such as high toxicity, low solubility and deleterious effects. DMNQ S-52 showed the weaker cytotoxicity (IC_{50}; 12.3 ± 1.6 μM) against Lewis lung carcinoma (LLC) cells than that of shikonin (IC_{50}; 4.2 ± 1.1 μM). DMNQ S-52, at non-toxic concentrations (less than 10 μM), significantly inhibited the invasion and migration of LLC cells. DMNQ S-52 also significantly inhibited the production of MMP-9, MT1-MMP and uPAR. Moreover, daily i.p. administration of DMNQ S-52 at dose of 5 mg/kg in mice resulted in a potent inhibition of the primary tumor size of LLC in the lung as well as the metastasis of lymph nodes. These findings suggest that the DMNQ S-52 has therapeutic potential to inhibit metastasis *via* inhibition of MMP family and uPA/plasminogen system.

Key words: DMNQ S-52; Lymph node metastasis; Lewis lung carcinoma; MMP; uPA

INTRODUCTION

Lung cancer is now the most common fatal malignant disease in the world and the presence of lymph node metastasis is an important prognostic factor in primary lung cancer (Han *et al.*, 2003). Despite development of prognosis and treatment of cancer, five-year survival for non small lung cancer (NSCLC) was extremely low. This poor prognosis suggests the occurrence of micrometastasis, therefore, it may be important to detect and treat occult micrometastasis (van Rens *et al.*, 2000).

Metastasis comprises of a series of sequential steps that begins with a tumor cell leaving a primary site and concludes with the formation of a metastatic tumor in a remote site (Townson *et al.*, 2003). Proteolysis, migration, adhesion and proliferation are critical steps controlling malignant invasion and metastatic processes (Mignatti and Rifkin, 1993; Woodhouse *et al.*, 1997). The principal barriers to tumor growth and spread are the extracellular matrix (ECM) compartments. During the metastatic process, tumor cells need to attach to other cells and ECM proteins. Translocation of neoplastic cells across ECM barriers is also a part of the metastatic processes and lysis of matrix proteins by specific proteinases is required for invasion (Woodhouse *et*

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al., 1997; Fiore *et al.*, 2002; Lee *et al.*, 2003).

During the process of metastasis, tumor cells are inevitably encountered with various apoptotic stimuli, such as low nutrients, hypoxia, loss of cell-matrix interactions, cytokines, interactions with natural killer cells, nitric oxide anions and mechanical stresses (Weiss, 1992; Takasu *et al.*, 1999). Therefore, it is thought that successful metastatic cells must acquire a decreased sensitivity to apoptotic stimuli in addition to genetic changes that promote unregulated proliferation (Townson *et al.*, 2003).

Shikonin, a major component of *Lithospermum erythrorhizon*, was reported to exert antitumor activity with poor solubility and strong deleterious effect. Thus, 6-(1-hydroxyimino-4-methylpentyl)5,8-dimethyoxy 1,4-naphthoquinone S-52 (DMNQ S-52) was synthesized to obtain the increased efficacy and low side effect (Song *et al.*, 2001). Our previous study indicated that DMNQ S-52 was very effective antitumor agent and had apoptosis-inducing activities against LLC cells.

Based on these findings, in the present study, we investigated the anti-tumor and anti-metastatic activities of DMNQ S-52 on tumor metastasis models *in vitro* and *in vivo* using LLC cells.

MATERIALS AND METHODS

Chemicals and reagents

Shikonin was isolated from *Lithospermum erythrorhizon* SIEB. et ZUCC. and DMNQ S-52 was synthesized at College of Pharmacy, Chungnam University, Taejeon, Korea (Fig. 1) (Song *et al.*, 2001). DMNQ S-52 and shikonin were dissolved in dimethylsulfoxide (DMSO) for the *in vitro* study and dissolved in saline and adjusted to pH 7.0 for the *in vivo* study. Eagle's Minimal Essential Medium (EMEM) was purchased from Gibco BRL (NY, USA). Fetal bovine serum (FBS) was purchased from INC Biomedicals (CA, USA). 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) Cell Counting Kit was purchased from Dojindo

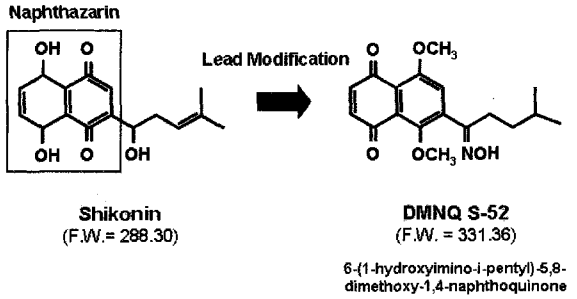

Fig. 1. Chemical structures and molecular weights of DMNQ S-52 and shikonin.

Laboratories (Kumamoto, Japan). Transwell cell culture chambers were purchased from Corning Costar (No. 3422, MA, USA). Polyvinylpyrrolidone-free polycarbonate filters (8.0 μm pore size) was purchased from Nuclepore (CA, USA). Matrigel was purchased from BD Biosciences (MA, USA). Fibronectin was purchased from Asahi Technoglass (Tokyo, Japan). RNeasy Mini kit was purchased from QIAGEN (CA, USA). Superscript™ II RNase reverse transcriptase and oligo(dT) primer were purchased from Invitrogen Life Technologies (CA, USA). Ex Taq™ HS PCR kit was purchased from Takara (Shiga, Japan). The anti-matrix metalloproteinase (MMP)-9 antibodies were purchased from Cell Signaling Technology (MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from DAKO (Glostrup, Denmark). Enhanced chemiluminescence (ECL) Western Blotting Detection Reagents was purchased from Amersham Biosciences (Buckinghamshire, UK). An Immobilon-P membrane was purchased from Millipore (MA, USA). Block Ace was purchased from Dainipponseiyaku (Osaka, Japan). Bovine serum albumin (BSA), ethidium bromide and other reagents were purchased from Sigma (MO, USA)

Cell culture

Lewis lung carcimoma (LLC) cell line was maintained as monolayer cultures in EMEM supplemented with 10% FBS. LLC cells were collected by brief treatment with EDTA, and then used for the

experiments. All cultures were maintained at 37°C in a humidified atmosphere of a 5% CO_2/95% O_2 air.

Animals

Six-week old, specific-pathogen-free female C57BL/6 mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). The mice were maintained under specific pathogen-free conditions and used according to institutional guidelines.

Cytotoxicity assay

Log-phase cell cultures of LLC (1×10^4 cells/well) were seeded in 100 ml of complete medium on 96-well culture plates. After preincubation for 24 h, the medium was replaced with new medium supplemented with 0.1% BSA containing with various concentration of DMNQ S-52 or shikonin. The cultures were incubated for various time periods and then cytotoxicic activity was determined by WST-1 colorimetric method (Ishiyama *et al.*, 1996). Briefly, before 2 h of the termination of incubation, 10 μl of the working solution containing WST-1 reagent was added to each well. After the termination of the culture, the absorbance was measured at 450 nm in an immuno-reader (Immuno Mini NJ-2300, Nippon InterMed K.K., Tokyo, Japan).

Invasion assay

The invasion assay was carried out using Transwell cell culture chambers as described previously with some modifications (McCarthy *et al.*, 1983; Saito *et al.*, 1997). Briefly, polyvinylpyrrolidone-free polycarbonate filters were precoated with 1 μg of fibronectin on the lower surface, and 5 μg of Matrigel on the upper surface. Cell suspensions (1×10^5 cells/100 μl) were added to the upper compartment of the chamber with various concentrations of DMNQ S-52. After 12 h incubation, the filters were fixed with 30% methanol and cells that had invaded to the lower surface were stained with crystal violet and colorimetrically assessed by measuring its absorbance at 595 nm in an immuno-reader (Immuno Mini NJ-2300).

Haptotactic migration assay

Haptotatic cell migration assay was carried out using Transwell cell culture chambers according to the methods previously reported with some modifications (McCarthy *et al.*, 1983; Saito *et al.*, 1997). Briefly, the lower surface of the filters was precoated with 1 mg of fibronectin. Cell suspensions (1×10^5 cells/100 μl) were added to the upper compartment of the chamber with various concentrations of DMNQ S-52. After 4 h incubation, the filters were fixed with 30% methanol and cells that had migrated to the lower surface were stained with crystal violet and colorimetrically assessed by measuring its absorbance at 595 nm in an immuno-reader (Immuno Mini NJ-2300).

Gelatin zymography

To prepare conditioned medium, LLC cells were grown to subconfluence in complete medium. After several washes with PBS, LLC cells were recultured for 12 h in EMEM containing 0.1% BSA and various concentrations of DMNQ S-52. The culture media were then collected and centrifuged to remove debris and the supernatants were subjected to gelatin zymography as reported previously (Heussen and Dowdle, 1980) with some modifications. Briefly, aliquots of the samples were applied directly to SDS-polyacrylamide gels (7.5% w/v) copolymerized with 0.1% (w/v) gelatin. After electrophoresis, the gels were rinsed twice (30 min each) in 50 mM Tirs-HCl containing 2.5% Triton X-100, 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.05% NaN_3 at room temperature to remove SDS. The gels were incubated in reaction buffer (50 mM Tris-HCl containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.05% NaN_3) for 24 h at 37°C. The gelatin gels were stained with 0.1% Commassie Brilliant Blue containing 10% acetic acid and 10% isopropanol, destained in the same solution in the absence of dye, and dried. Enzyme-digested regions were identified as a clear band in the background of uniform staining.

RT-PCR

LLC cells were cultured in complete medium for 24 h. The medium was replaced with fresh medium containing 0.1% BSA and DMNQ S-52 and then incubation was continued for a further 24 h. Total RNA was isolated using RNeasy Mini kit according to manufacturer's instructions. Two micrograms of isolated RNA was reverse-transcribed using a Superscript™ II RNase reverse transcriptase with oligo (dT) primer at 42°C for 50 min to maximize the first strand cDNA synthesis, and terminated by heating at 70°C for 15 min. PCR amplification of the cDNAs was performed using specific oligonucleotide primers and Ex Taq™ HS PCR kit. The sequence of primers was as follows: MMP-2, 5′-CCTGATGTCCAGCAAGTAGATGC-3′ and 5′-TTAAGGTGGTGCAGGTATCTGG-3′; MMP-9, 5′-TTCTCTGGACGTCAAATGTGG-3′ and 5′-CAAAGAAGGAGCCCTAGTTCAAGG-3′; MT1-MMP, 5′-CCTGCATCCATCAATACTACTGC-3′ and 5′-GCGTCTGAAGAAGAAGACAGC-3′; urokinase-type plasminogen activator (uPA), 5′-CGAATACTAC-AGGGAAGAC-3′ and 5′-GACATTTTCAGGTT-CTTTGG-3′; uPA receptor (uPAR), 5′-ATTGCCTCT-CTGCTCCTGAC-3′ and 5′-GAGACCCAACCTT-ATTCACTGCACTG-3′ and glyceraldehydes 3-phosphate dehydrogenase (GAPDH), 5′-GGTGAAGGT-CGGTGTCAACGGATTT-3′ and 5′-GATGCCAAAG-TTGTCATGGATGACC-3′. PCR was performed in a thermocycler (Hybaid Ltd., Middx, UK) for 30 cycles of denaturation (94°C, 30 sec), annealing (60°C, 1 min), and extension (72°C, 90 sec). The PCR products were electrophoresed on 1.5% agarose gels and detected by ethidium bromide staining.

Western blot analysis

LLC cells were cultured in complete medium for 24 hr. The cells were then incubated with DMNQ S-52. After indicated treatment, the cells were then rinsed with ice-cold PBS and then whole cell lysates were prepared with lysis buffer (25 mM HEPES (pH 7.7), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 μM β-glycerophosphate, 0.1 μM sodium orthovanadate, 0.5 μM phenylemethylsulfonyl fluoride (PMSF), 1 μM dithiothreitol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were subjected to SDS-polyacrylamide gel and transferred to Immobilon-P membranes. The membrane was treated with Block Ace overnight at 4°C and probed with various antibodies for 2 h. The primary antibodies were detected using HRP-conjugated goat anti-rabbit IgG and visualized by ECL Western Blotting Detection Reagents.

Intrapulmonary implantation of LLC cells and evaluation of anti-metastatic activity

Orthotopic implantation of LLC cells into the lung was performed as described previously (Doki *et al.*, 1999) with some modifications. Briefly, the left chest of anesthetized mice was incised (approximately 5 mm incision), and 20 μl aliquots of LLC cell suspension (3×10^3 cells) admixed with 20 μg of Matrigel were injected into the left lung parenchyma through the intercostal space. The skin incision was closed with a surgical skin clip. Mice were sacrificed on day 18 after tumor implantation, and the weights of the tumor at the implantation site in the lung and the metastasized tumor in the mediastinal lymph node were measured. DMNQ S-52 and shikonin were administered by intraperitoneal (i.p.) injections of 5 mg/kg for 7 days starting on 1 day after the intrapulmonary implantation of LLC cells. Doxorubicin was administered by intravenous (i.v.) injection of 7 mg/kg on 7 days after tumor cell inoculation.

Statistical analysis

Representative data from each experiment are presented as mean values ± SD, as described in the figure legends. The statistical differences between the groups were determined by applying the Student's two-tailed *t*-test. The Dunnett's test was performed to decrease the multiplicity in comparisons of drug-treated groups with control group. Statistical significance was defined as a P value < 0.05.

RESULTS

Inhibitory effects of DMNQ S-52 on the growth of various cell lines

Cell growth was monitored in terms of reduction of WST-1. LLC cells were incubated with various concentrations of DMNQ S-52 or shikonin for 6, 12, 24, and 48 h, respectively. As shown in Fig. 2, shikonin, at concentrations of more than 1 μM, showed direct cytotoxicity against LLC cells for 24, 48 hr incubation, whereas the cytotoxicity of DMNQ S-52 was much less than that of shikonin. For example, IC_{50} of 24 h incubation was 12.3 ± 1.6 μM on DMNQ S-52 and 6.5 ± 8.5 μM on shikonin (Fig. 2A and B).

Effect of DMNQ S-52 on invasion and migration of LLC cells

To examine the anti-metastatic properties of DMNQ S-52, invasion and migration assays were performed using LLC cells. Treatment with DMNQ S-52 for less than 12 h, at the concentrations ranging from 1 to 10 μM, did not significantly inhibit the growth of LLC cells (Fig. 2A). Therefore, we used DMNQ S-52 at concentrations of less than 10 μM in the following experiments for evaluating anti-metastatic properties of DMNQ S-52. DMNQ S-52 caused a concentration-dependent inhibition of the invasion of LLC through Matrigel/fibronectin-coated filters and migration through fibronectin-coated filters (Fig. 3A and B).

Effect of DMNQ S-52 on the expression of mRNAs for the metastasis-related molecules

Next, we examined the effect of DMNQ S-52 on the expression of the mRNAs for the metastasis-related molecules, such as MMPs and uPA/plasminogen system, in LLC cells. RT-PCR showed that LLC cells expressed mRNAs for MMP-2, MMP-9, membrane type (MT)1-MMP, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-2, uPA, uPAR and inhibitor of plasminogen activator (PAI)-1. Treatment with DMNQ S-52 for 12 hr resulted in a decrease of the expression of mRNAs for MMP-2, MMP-9, MT1-MMP, uPA and uPAR. Specially, DMNQ S-52 strongly decreased the mRNA expression of MMP-9 and MT1-MMP (Fig. 4). However, the treatment with DMNQ S-52 did hot show significant decrease of the expression of mRNAs for TIMP-1, TIMP-2 and PAI-1 (data not shown). Therefore, the reduced

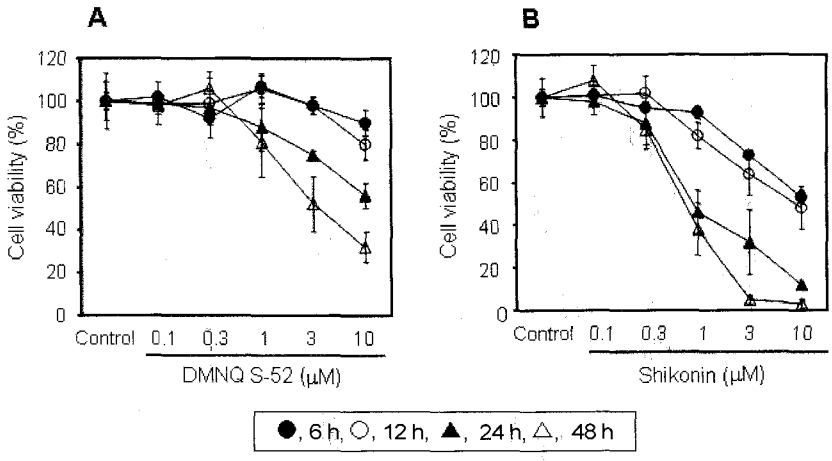

Fig. 2. Cytotoxic effect of DMNQ S-52 and shikonin against LLC cells. LLC cells were seeded in 96-well culture plates. After a 24 h preincubation, various concentrations of DMNQ S-52 and shikonin were added to the cultures and the cultures were employed a further incubation. Tow hr before the end of incubation, WST solution was added into each well and after the end of incubation, the absorbance was measured at 450 nm. The data were expressed as the mean ± SD of triplicate cultures. A, the cytotoxicity of DMNQ S-52; B, the cytotoxicity of shikonin.

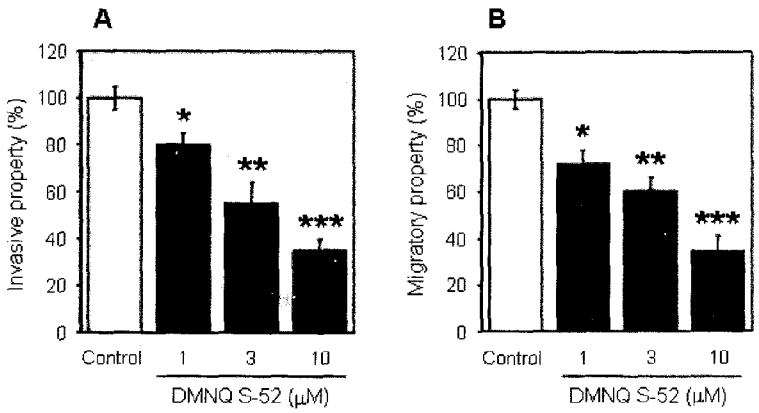

Fig. 3. Effect of DMNQ S-52 on the tumor cell invasion and migration through Matrigel/fibronectin-or fibronectin-coated filters. LLC cells were seeded into Transwell chambers in quadruplicate and incubated with or without DMNQ S-52. After 12 h, invaded and migrated cells were detected by the crystal violet staining method. A, invasive property of LLC cells with or without DMNQ S-52. B, migratory property of LLC cells with or without DMNQ S-52. All data are represented as the mean ± SD. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

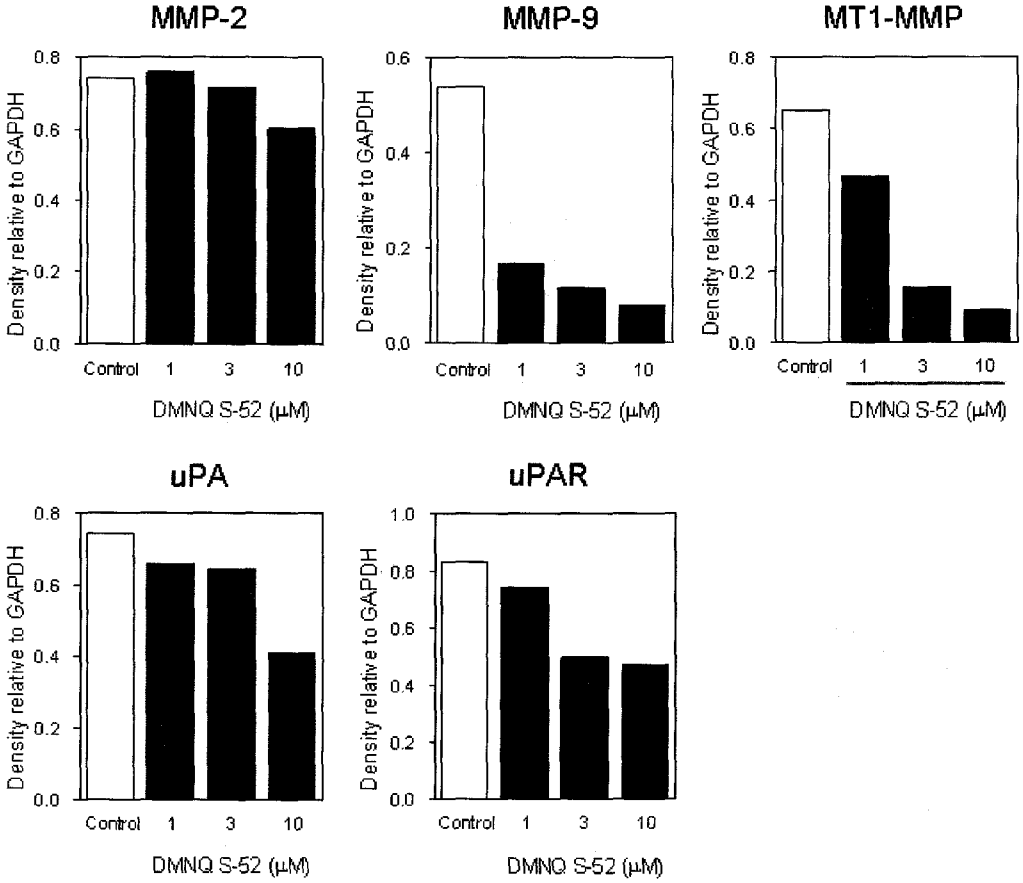

Fig. 4. Effect of DMNQ S-52 on the expression of mRNAs for the metastasis-related molecules. LLC cells were incubated with DMNQ S-52 for 12 h. RT-PCR was performed using primers and conditions described in "Materials and Methods" and PCR products were electrophoresed on 1.5% agarose gels.

expression by DMNQ S-52 may be involved in the inhibition of invasive and migratory abilities of LLC cells.

Inhibitory effects of DMNQ S-52 on the production of MMPs

Changes in the MMP levels can markedly affect the invasive behavior of tumor cells and their ability to metastasize in experimental animal models (Coussens *et al.*, 2002). As shown in Fig. 4, DMNQ S-52 strongly inhibited the expression of mRNA of MMP-9. Therefore, we next investigated the inhibitory effect of DMNQ S-52 on the production of MMP-9 in the protein levels using the gelatin zymography and Western blot analysis. Conditioned serum-free media of LLC cells were used in gelatin zymography. The degradation of gelatin substrate by MMP-9 was inhibited by decrease of synthesis and secretion of MMP-9 by DMNQ S-52 in a concentration-dependent manner (Fig. 5). In contrast, the synthesis and secretion of MMP-2 were not affected in DMNQ S-52-treated LLC cells. Also, gelatinolytic activities of MMP-9 and MMP-2, analyzed by overnight gel incubation in buffer containing DMNQ S-52, were not affected by DMNQ S-52-treatment (data not shown). Western blotting analysis showed the similar result supporting the zymography results (Fig. 5).

Effects of DMNQ S-52 on the tumor growth and mediastinal lymph node metastasis of LLC

Finally, the *in vivo* efficacy of DMNQ S-52 was examined in a lymph node metastasis model by orthotopic implantation of LLC cells. Mice were treated with DMNQ S-52 or shikonin (5 mg/kg/day, i.p.) for 7 days from 1 day after tumor inoculation. The tumor growth and mediastinal lymph node metastasis were assessed 18 days after the inoculation (Fig. 6). Administration of DMNQ S-52 and shikonin significantly inhibited the growth of LLC tumors at the implantation site (Fig. 6A and B). Moreover, metastasis of LLC cells to the mediastinal lymph nodes was significantly inhibited by DMNQ S-52 and shikonin in a dose-dependent manner (Fig. 6C). DMNQ S-52 did not cause any adverse effects in the mice such as decrease of body weight during the *in vivo* experiments (Fig. 6D).

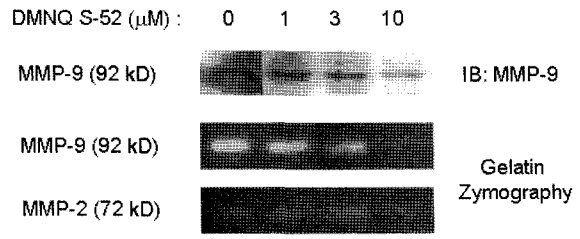

Fig. 5. Effect of DMNQ S-52 on the production of MMPs. Gelatin zymography and western blotting were performed as described in "Materials and Methods."

DISCUSSION

Several shikonin derivatives, 2- or 6-substituted naphthazarins were synthesized for both enhancing the antitumor activity of shikonin and decreasing the deleterious effects. DMNQ S-52 is one of these systhetic shikonin derivatives and it showed anti-tumor efficacy such as inhibition of topoisomerase I activity and prolongation of the life span in sarcoma 180 tumor bearing mice (T/C 241%) (Song *et al.*, 2001). Furthermore, our previously study have exhibited that DMNQ S-52 as well as shikonin was potent apoptosis-inducing molecule (submitted data). Notwithstanding apoptosis-inducing potential of DMNQ S-52, it showed lower cytotoxicity than that of shikonin (Fig. 2) in parallel with the purpose of DMNQ S-52 synthesis.

It is well known that apoptosis plays a crucial role in oncogenesis and tumor progression (Kaufmann and Earnshaw, 2000). Apoptosis has been reported to be the cause of tumor cell death during chemotherapy, radiation therapy and even immunotherapy (Tsuchida *et al.*, 1998; Hersey, 1999). In the metastatic process, tumor cells are subjected to a number of apoptotic stimuli (Takasu *et al.*, 1999; Townson *et al.*, 2003) and several metastasis suppressor genes can induce apoptosis

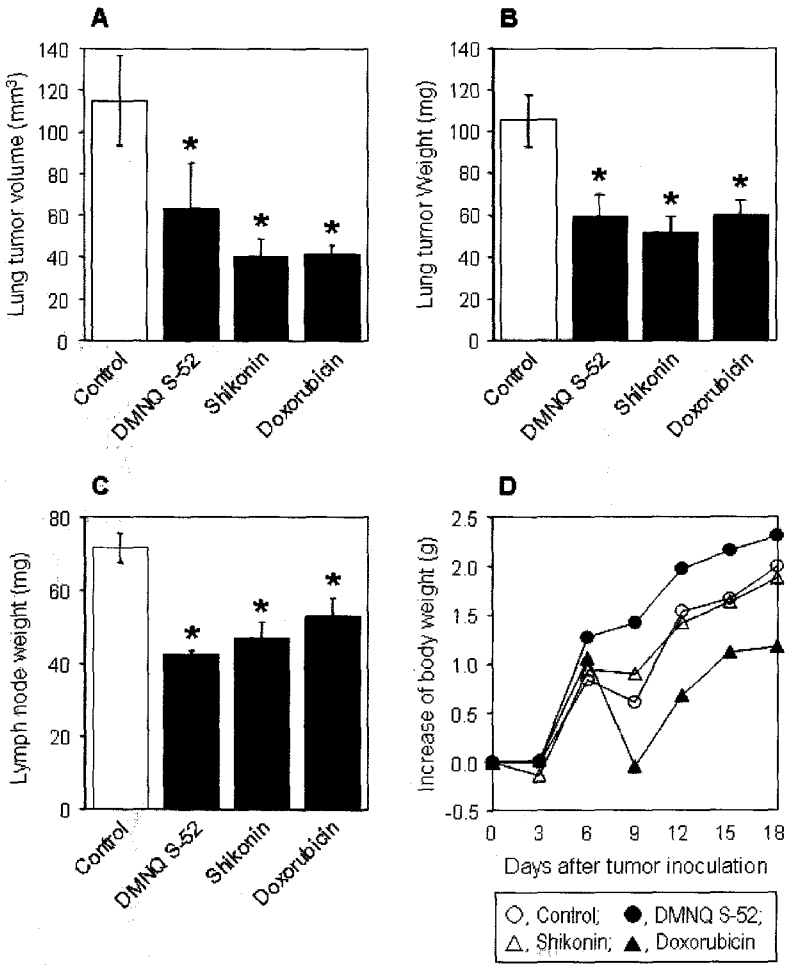

Fig. 6. Tumor growth and mediastinal lymph node metastasis after orthotopic implantation of LLC cells. LLC cell suspension was orthotopically implanted into the left lungs of mice followed by subcutaneous administration of DMNQ S-52, shikonin or doxorubicin over 7 days from 1 day after tumor inoculation. The volume and weight of the tumor at the implanted site (A and B) and the metastasized tumor at the mediastinal lymph node (C) were measured on 18 days after implantation. The body weight was measured every 3 days (D). Data are represented as the mean ± SD of 6 mice in each group. $^*P < 0.05$.

(Bhoumik *et al.*, 2002; Schoenfeld *et al.*, 2004). Actually, the occurrence of metastatic spread depends on many factors both the condition of the patient and the properties of the tumor. In this investigation, the association between proliferation and apoptosis and the incidence of lymph node involvement of patients with non-small cell lung carcinomas was analyzed.

The high mortality rates associated with cancer can be attributed to the metastatic spread of tumor cells from the site of their origin. Cells from malignant primary tumors spread from their sites of origin to invade local tissue and enter the systemic circulation (Fidler, 2001). During the metastatic formation of tumors, a complex series of events occur and the metastasizing tumor cells interact with various host cells and ECM components. Such interactions may positively or negatively influence the invasion, adhesion, migration and proliferation of tumor cells. Therefore, several

attempts have been made to control cell functions such as adhesion, migration and invasion of tumor cells during the metastatic process (McCarthy and Furcht, 1984; Terranova *et al.*, 1986; Silvestri *et al.*, 2002).

Invasion and metastasis require the disruption of several collagen-endowed tissue barriers, and therefore most of the initial interest focused on proteinases capable of degrading collagen IV, the major collagen constituent of basement membrane (BM), which are gelatinases A (MMP-2) and B (MMP-9) (Stamenkovic, 2000; Coussens *et al.*, 2002). DMNQ S-52, at non-cytotoxic concentrations, inhibited a number of *in vitro* metastatic functions of tumor cells, including invasion and migration (Fig. 3). Also, DMNQ S-52 could strongly inhibit the production of MMP-9 and MT1-MMP in LLC cells (Fig. 4 and 5). Moreover, DMNQ S-52 inhibited the mRNA expression of uPA and uPAR (Fig. 4). Many reports have shown that the degradation of extracellular matrix by uPA correlates well with the tumorigenicity and metastatic ability of various types of tumor cells (Silvestri *et al.*, 2002; Lee *et al.*, 2003). The uPA/plasminogen system is thought to be involved in the development of metastatic disease through fibrinolytic degradation of ECM, and the activation of growth factors and mitogen for tumor cells and vascular endothelial cells (Andreasen *et al.*, 1997).

Activation of the mitogen-activated protein kinase (MAPK) pathway is a frequent event in tumorigenesis. MAPKs have been implicated in cell migration, proteinase-induction, regulation of apoptosis and angiogenesis which are essential for metastatic spread (Reddy *et al.*, 2003). MMP-9 gene expressions was induced by the activation of ERKs and PI3K/AKT (Reddy *et al.*, 2003) and the up-regulation of uPA/uPAR was through phosphorylation of MEK/ERK and p38 kinase (Ahmed *et al.*, 2003; Lee *et al.*, 2004). Our previous report showed that DMNQ S-52 regulated mitogen-activated protein (MAP) kinases such as ERK, JNK and p38 kinase and induced apoptosis against tumor cells (submitted data). Therefore, the inhibition of tumor cell invasion and migration as well as the decrease of mRNA expressions in MMP-9, MT1-MMP, uPA and uPAR may be related the MAPK alteration and balance regulated by DMNQ S-52.

Since the metastatic spread of many tumors such as breast (Weidner *et al.*, 1991), nasopharyngeal (Wakisaka *et al.*, 1999) and lung cancers (Wakisaka *et al.*, 1999) relies on lymphangiogenesis to some extent, their regulation is one of the important features in understanding cancer metastasis. The extent of lymphatic metastasis is the most important influencing factor in the prognosis of non-small cell lung cancer (NSCLC) (Macchiarini *et al.*, 1992). Therefore, suppression of lymphatic metastasis provides an improvement in the survival of lung cancer patients (Ishikura *et al.*, 2001). The distribution of basement membrane collagen has been shown to correlate significantly with the presence of lymph node metastasis (Hirota *et al.*, 1990). Therefore, we employed the *in vivo* mediastinal lymph node metastasis model using LLC cells expressing collagenases (MMP-1), gelatinases (MMP-2 and 9) and tissue inhibitor of metalloproteinase (TIMP)-1 and -2 (Pitzel *et al.*, 2000). The present lymph node metastasis model showed that DMNQ S-52 reduced *in vivo* mediastinal lymph node metastasis as well as primary tumor growth. Therefore, the possibility cannot rule out that the inhibition of lymph node metastasis by DMNQ S-52 might be because of inhibition of tumor growth, which is induction of apoptosis. Shikonin and doxorubicin, which are well known apoptosis-inducing molecules, also showed strong reduction of the primary tumor volume and weight (Fig. 6A and B). It is very interesting that DMNQ S-52 exhibited the strongest inhibition of mediastinal lymph node metastasis among DMNQ S-52, shikonin and doxorubicin (Fig. 6C). There is possible explanation that DMNQ S-52 inhibited MMP-9, MT1-MMP, uPA, and uPAR production at non toxic concentration and the doses of shikonin and doxorubicin were harmful to mice. The body weight of doxorubicin-

treated group showed doxorubicin had toxic effect to living things because the body weight was extremely decreased right after doxorubicin administration. However, the body weight of shkonin-treated group was not much decreased in comparison with DMNQ S-52-treated group. Therefore, these results proposed that DMNQ S-52 might have anti-metastatic potential as well as apoptosis-inducing activity.

In conclusion, this study demonstrated that DMNQ S-52, a new synthetic shikonin derivative, not only induced apoptosis but also inhibited metastatic properties of tumor *cells in vitro* and *in vivo*. DMNQ S-52 carries safety in comparison with the shikonin and the biological activities of DMNQ S-52 are more plentiful. These findings suggest that DMNQ S-52 may have therapeutic potential for controlling tumor metastasis based on its inhibitory effects on invasion and migration *via* suppression of production of MMPs and uPAR. Therefore, DMNQ S-52 can be a useful antitumor candidate suppressing primary tumor growth and metastasis.

ACKNOWLEDGEMENTS

This study was supported by the grants from Korean Ministry of Health and Welfare, Korean Biogreen 21 project, and Korea Foundation of Science and Engineering.

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