J. Exp. Biomed. Sci. 12 (2006) 161-170

A Novel Anti-cancer Agent, SJ-8029, Inhibits Angiogenesis and Induces Apoptosis

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A new piperazine derivative, SJ-8029, is a synthetic anti-cancer agent which exhibits both microtubule and topoisomerase II inhibiting activities. In this study, we investigated the ability of SJ-8029 for anti-angiogenesis and apoptosis. SJ-8029 decreased the bFGF-induced angiogenesis in the CAM and the mouse Matrigel implants, *in vivo*. SJ-8029 inhibited the proliferation, migration, invasion, tube formation, and expression of MMP-2 in BAECs. In addition, SJ-8029 reduced the cell viability in HepG2 cells, caused the production of fragmented DNA and the morphological changes corresponding to apoptosis. SJ-8029 also elicited the release of cytochrome c and the activation of caspase-3. Taken together, these results suggest SJ-8029 may be a candidate for anti-cancer agent with the ability to inhibit the angiogenesis of endothelial cells and to induce the apoptosis of tumor cells.

Key Words: Angiogenesis, Apoptosis, Microtubule and topoisomerase II inhibitor

INTRODUCTION

Angiogenesis is the formation of new capillaries from preexisting vasculature by migration and proliferation of endothelial cells (Risau., 1997), and a fundamental process required for many physiologic and pathologic processes (Auerbach et al., 1994). Under physiologic conditions, angiogenesis is tightly regulated by the control of balance between angiogenic stimulators and inhibitors. Unregulated angiogenesis is observed under pathologic conditions such as solid tumor growth, diabetic retinopathy, psoriasis and rheumatoid arthritis (Ferrara et al., 1999; Carmeliet et al., 2000). As close to the central regions of tumor larger than a few cubic millimeters in size, pH, oxygen tension, concentration of glucose and growth factors are lower than boundary (Crowther et al., 2001). Under these conditions, normal cell should die by apoptosis. However, in tumor cells, this condition triggers the release of angiogenic activators and survival factors, which interact with endothelial cells and then angiogenesis is induced (Folkman, 1992). As new blood vessels are recruited, tumor cells can escape from apoptosis and rapidly grows in sizes (Folkman, 1990).

Apoptosis, or programmed cell death, is high conserved and regulated program by which cells commit suicide under a variety of internal and external conditions (Isaacs, 1993; Kerr, 1994; Thompson, 1995). Apoptosis has been reported to be involved in the carcinogenic process and in tumor therapy and prevention (Bursch et al., 1992; Miyashita et al., 1993).

Generally, there is higher level of topoisomerase II in tumor cells than that in normal cell. Topoisomerase II has therefore become the main target of several potent anticancer agents, even though the exact mechanism of cell killing remains elusive. DNA topoisomerase II inhibitors either stabilize DNA-topoisomerase II complexes by blocking DNA religation or the enzyme's catalytic activity, and then induce apoptosis (Beck et al., 2001).

Many crucial endothelial cell activities relevant to angiogenesis, including migration, proliferation, secretion, alignment, and formation of capillary-like structure, require a

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Accepted after revision: September 7, 2006
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functional cytoskeleton. It has been known that agents affecting the cytoskeleton are inhibitors of angiogenesis (Chaplin et al., 1999). Many of them initially isolated from natural sources promote either microtubule polymerization (e.g., taxanes and epothilones) or depolymerization (e.g., colchicines and Vinca alkaloids). Despite their opposite final effects, the main activity of these compounds is likely to be kinetic stabilization of the microtubule dynamics (Jordan et al., 1998; Wood et al., 2001). Additionally, many of these microtubule-affecting agents induce tumor cell apoptosis by disrupting the dynamics of cytoskeleton.

SJ-8029, 4-(3, 5-dimethylphenyl) piperazine-1-carboxylicacid (5-{1-[3-(acridine-9-yl-amino)-5-hydroxymethylphenylcarbamoyl]-ethylcarbamoyl}-6-ethyl-2-ethoxypyridine -3-yl), is a complex of SJ-8026 (topoisomerase II inhibitor) and SJ-8031 (microtubule inhibitor). So SJ-8029 exhibits both microtubule and topoisomerase II inhibiting activities.

We therefore investigated whether this compound inhibits endothelial cell angiogenesis and induces tumor-cell apoptosis using bovine aortic endothelial cells (BAECs) and human hepatocellular carcinoma cells (HepG2). Here, we demonstrate that SJ-8029 inhibits the angiogenesis of endothelial cells and induces the apoptosis of tumor cells.

MATERIALS AND METHODS

1. Materials

SJ-8029 was obtained from Samjin Pharm (Hwasung, Korea). FBS, penicillin and streptomycin was purchased from Gibco/BRL Life Technologies. Thermanox coverslips were purchased from Nunc. Intralipose was purchased from Green Cross (Seoul, Korea). Seven-week-old, specific pathogen-free (SPF) male C57BL/6 mice were supplied from Hyochang Science (Taegu, Korea). Drabkin reagent kit 525 and DAPI purchased from Sigma. [^{3}H]-methylthymidine and ECL kit were purchased from Amersham (Aylesbury, UK). 8 μm pored transwell filter chambers was purchased from Corning-Costar (Cambridge, UK). Anti-cytochrome c monoclonal antibody and anti-caspase-3 polyclonal antibody were purchased from PharMingen (San Diego, USA).

2. Cell culture

BAECs and HepG2 cells were grown in DMEM and MEM, respectively. Each medium was supplemented with heat-inactivated 10% FBS, 100 units/ml of penicillin and 100 g/ml of streptomycin in a 37℃ incubator with a humidified atmosphere containing 5% CO_2.

3. Chorioallantoic membrane (CAM) assay

Fertilized chick eggs were incubated under conditions of constant humidified egg breeder at 37℃ On the 3rd day of incubation, about 2 ml of egg albumin were aspirated by an 18-gauge hypodermic needle to detach the developing CAM from the shell. After incubation for 2 more days, sample-loaded thermanox coverslips were air-dried and applied to the CAM surface for testing of anti-angiogenic activity of SJ-8029. Two days later, 1 to 2 ml of 10% intralipose was injected into the chorioallantois and the newly formed blood vessels were observed under a microscope.

4. Animals

Seven-week-old, specific pathogen-free (SPF) male C57BL/6 mice were provided with auto-claved tap water and lab chow *ad libitum* and were housed at 23±0.5℃ 10% humidity in a 12 h light-dark cycle.

5. *In vivo* Matrigel plug assay

C57BL/6 mice (7 weeks of age) were injected subcutaneously 500 μl Matrigel (Collaborative Biomedical Products, Bedford, MA) at 4℃ containing bFGF (100 ng/ml) and heparin (50 units/ml) without or with SJ-8029 (100 μg/ml). After injection, the Matrigel rapidly formed a plug. After seven days, the skin of the mouse was pulled back to expose the Matrigel plug, which remained intact. After quantitative differences were noted and photographed, hemoglobin was measured using the Drabkin method and Drabkin reagent kit 525 for the quantitation of blood vessel formation (Drabkin et al., 2001). The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel.

6. [^{3}H]-Thymidine incorporation assay

BAECs grown to near confluence in 24-well culture plates were made quiescent and treated with SJ-8029 for 24 h. Cells were labeled with 1 μl of [^{3}H]-methylthymidine (25 mCi/mM Amersham, Aylesbury, United Kingdom) for 4 h before the assay. After labeling, unincorporated [^{3}H]-methylthymidine was removed by washing with 10% trichloroacetic acid and then incorporated [^{3}H]-methylthymidine was extracted in 0.2 M NaOH and 0.1% SDS at 37℃ or

1 h. The cpm values from cultures were counted with a liquid scintillation counter (Beckman Instruments, Fullerton, CA).

7. Wounding migration assay

BAECs, plated on 60 mm culture dishes at 90% confluence, were wounded with a razor blade 2 mm in width and marked at the injury line. After wounding, the cultures were washed with serum-free medium and further incubated in DMEM with 1 mM thymidine and/or SJ-8029. BAECs were allowed to migrate for 18 h and rinsed with serum-free medium, followed by fixing with absolute methanol and staining with Giemsa. Migration was quantitated by counting the number of cells that moved beyond the reference line.

8. Cell invasion assay

The 8 μm pored Transwell filter inserts were coated with a solution of Matrigel (0.5 mg/ml), and type IV collagen (0.5 mg/ml) at room temperature for 1 h. SJ-8029 dissolved in DMEM containing 0.1% bovine serum albumin (BSA) was added in the bottom chamber. BAECs (3×10^4 cells/well) suspended in DMEM containing 0.1% BSA were placed in upper chamber. After 4 h incubation at 37℃ cells on both sides of the membrane were fixed with methanol and stained with Hematoxylin and Eosin. Cells on the upper surface of the membrane were removed by wiping with cotton swab, and the average number of cells on the lower side of the membrane was counted with optical microscopy at × 40 magnification.

9. Tube formation assay

BAECs (5×10^5 cells) were seeded on a layer of previously polymerized Matrigel with or without SJ-8029. Madrigal cultures were incubated at 37℃ After 6 h, changes of cell morphology were observed under a phase contrast microscope and photographed at × 40 magnification.

10. Gelatin-based zymography

Sub-confluent BAECs cultures were incubated for 4 h in the absence or presence of indicated amount of SJ-8029 in serum-free DMEM, and aliquots of the resultant conditioned medium were analyzed for gelatinolytic activity. Gelatinolytic activity was determined on gelatin impregnated sodium dodecyl sulfate (SDS) 10% polyacrylamide gels with gelatin (0.33 mg/ml) as described previously. After electrophoresis, the gel was rinsed twice with 2.5% triton X-100 for 15 min and incubated at 37℃ for 24 h in incubation buffer (0.05 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.01 M $CaCl_2$, 1 M $ZnCl_2$, 0.02% NaN_3). Gelatinase was identified following staining of the gel with Coomassie brilliant blue R250 and destaining with 7% acetic acid.

11. Cell viability

Cell viability of HepG2 was assessed by the MTT staining as described (Mosmann, 1986). Briefly, cells were incubated with or without SJ-8029 for indicated period in 1 ml of medium in 24-well plates at an initial density of 2×10^4 cells/well. After each period of incubation, 100 μl of MTT solution (5 mg/ml) were added and cells were incubated for another 4 h. Cell viability was determined by scanning with an ELISA reader (Molecular Devices, Sunnyvale, CA) with a 540 nm filter.

12. DNA isolation and electrophoresis

HepG2 cells were treated with or without SJ-8029 for 72 h and collected by centrifugation, washed twice in PBS at pH 7.4, and resuspended at a density of 1×10^7 cells/ml of lysis buffer containing 50 mM NaCl, 5 mM EDTA, and 150 mM Tris-HCl at pH 8.0. To each 200 μl of cell suspension, 20 μl of proteinase K (25 μg/μl) and 20 μl of 10% SDS were added. Samples were incubated at 50℃ or 24 h. RNase A (10 μl, 100 units/200 μl) was added, and the incubation continued for 30 min. DNA was then extracted with phenol: chloroform, 24:1, precipitated by addition of 0.1 volume of 5 M NaCl and 1 volume of isopropyl alcohol, and separated by electrophoresis in 1.5% agarose. The pattern of DNA fragmentation was photographed under UV light after staining the gel with ethidium bromide (0.5 μg/ml in TAE buffer).

13. Morphologic assessment of apoptosis

Nuclear morphology was observed using 4, 6-diamidino-2-phenylindole (DAPI). Briefly, HepG2 cells were seeded on cover glass and incubated with or without SJ-8029 for 72 h. Cells were fixed in 3.7% paraformaldehyde, and then stained with DAPI (5 μM) for 5 min at room temperature in the dark. Cells were mounted in crystal mount and viewed by fluorescence microscopy.

14. Flow cytometry analysis of apoptosis

HepG2 cells were seeded on cover glass and incubated with or without SJ-8029 for 72 h. Cells were harvested by centrifugation (1,000 rpm, 5 min, 4℃, and fixed in 70% ethanol at 4℃ for 16 h. Fixed cells were centrifuged as above, and resuspended in 1 ml of PBS. 100 μl of DNase-free RNase A (200 μg/ml) and 100 μl of propidium iodide (1 mg/ml) were added and incubated for 30 min. The number of apoptotic sub-diploid cells was determined using FACScan flow cytometry with EPICS-XL. The numbers of apoptotic cells were expressed as percent of sub-diploid cells.

15. Preparation of cytosolic extracts and immunoblotting

HepG2 cells were incubated with or without SJ-8029 for 72 h. The cells were collected and resuspended in 500 μl of extraction buffer (50 mM Pipes-KOH, 220 mM mannitol, 68 mM sucrose, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitors). After 30 min incubation on ice, cells were homogenized using a glass dounce and a B pestle (50 strokes). Cell homogenates were centrifuged, and 10 μg of protein was loaded on 15% SDS-polyacrylamide gels. Mitochondrial cytochrome c was detected with anti-cytochrome c monoclonal antibody and visualized using an ECL kit according to the manufacturer's instructions.

16. Western analysis

HepG2 cells were treated with or without SJ-8029 for 72 h and lysed with lysis buffer containing 40 mM Tris-Cl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitors. Total proteins (50 μg) were used for western blot analysis. Western blot analysis was performed using 15% reducing polyacrylamide gels, and the protein was transferred to nitrocellulose by electroblotting. The membrane was probed with anti-caspase-3 polyclonal antibody and visualized using an ECL kit according to the manufacturer's instructions.

17. Data analysis and statistics

Data are presented as means ± SD or as percentage of control. Statistical comparisons between groups were performed using the Student's *t* test. $*P<0.05$ was considered statistically significant.

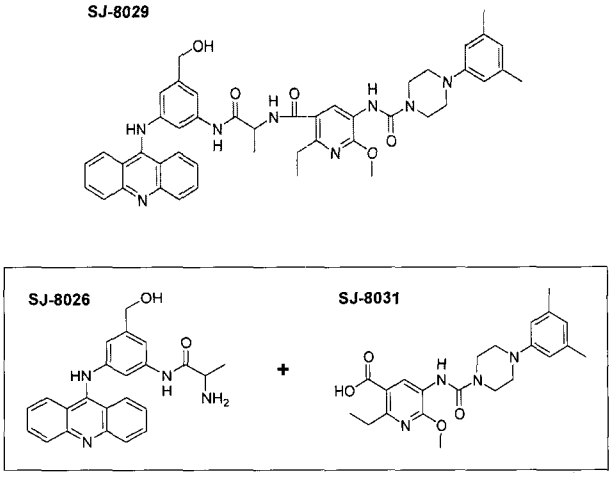
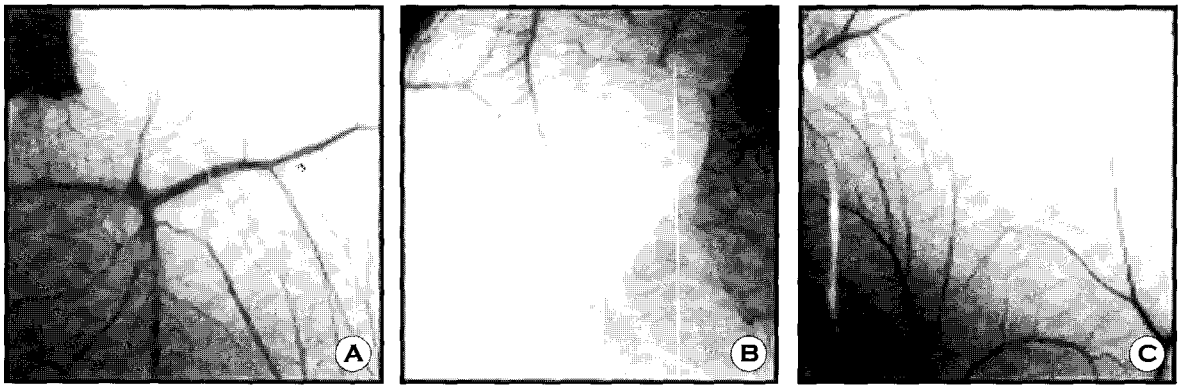


Fig. 1. Chemical structure of SJ-8029.

RESULTS

1. SJ-8029 inhibits angiogenesis *in vivo*

SJ-8029 is a piperazine derivative showing microtubule and topoisomerase II inhibiting activity (Cho et al., 2002). The chemical structure of this compound is shown in Fig. 1. To determine whether SJ-8029 has anti-angiogenic activity, CAM assay was first carried out. Upon the dissection of the CAM of 7-day-old chick embryo, the spontaneous angiogenesis in CAM was clearly observed (Fig. 2A). The treatment of retinoic acid (RA) blocked completely the neovascularization of chick embryo (Fig. 2B). After topical application of SJ-8029 for 48 h, SJ-8029 inhibited the spontaneous angiogenesis in CAM (Fig. 2C). As shown in Fig. 2D, the treatment of 20 and 30 μg of SJ-8029 showed the inhibitory effect of 36% and 67%. In addition, the effect of SJ-8029 on the ongoing angiogenesis *in vivo* was evaluated by using *in vivo* mouse Matrigel plug assay (Fig. 3). Plugs with Matrigel alone were pale in their color indicating no or less blood vessel formation (Fig. 3A). In contrast, bFGF addition significantly increased capillary density as compared with that of the blank (Fig. 3B). However, the capillary area in the Matrigel implant containing both bFGF and SJ-8029 was lower than that in the bFGF alone (Fig. 3C). We also measured the hemoglobin content inside the Matrigel plugs to quantify the angiogenesis inhibited by SJ-8029. Fig. 3D shows SJ-8029 strongly inhibited that bFGF-induced angiogenesis. Quantitative results of hemoglobin assay were



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Compound	Dose (μg/egg)	Egg showing anti-angiogenesis	Total eggs tested	% of inhibition
Blank	-	0	11	0
RA	1	10	11	91*
SJ-8029	20	4	11	36*
	30	6	9	64*

Fig. 2. Anti-angiogenic effect of SJ-8029 on the chick CAM. Coverslip alone and retinoic acid (RA, 1 μg/egg) were used as a negative and positive control, respectively. **(A)** Normal neovascularization in the absence of SJ-8029. **(B)** Angiogenesis was almost blocked by RA. **(C)** SJ-8029 (30 μg/egg) showed the anti-angiogenic effect similar to that observed in RA-treated CAM. **(D)** Angiogenic responses were scored as positive when the SJ-8029-treated CAM showed a vascular zone similar to RA-treated CAM, which had very small vessels compared with blank, and calculated by the percentage of positive eggs. *, $P<0.05$ compared to blank.

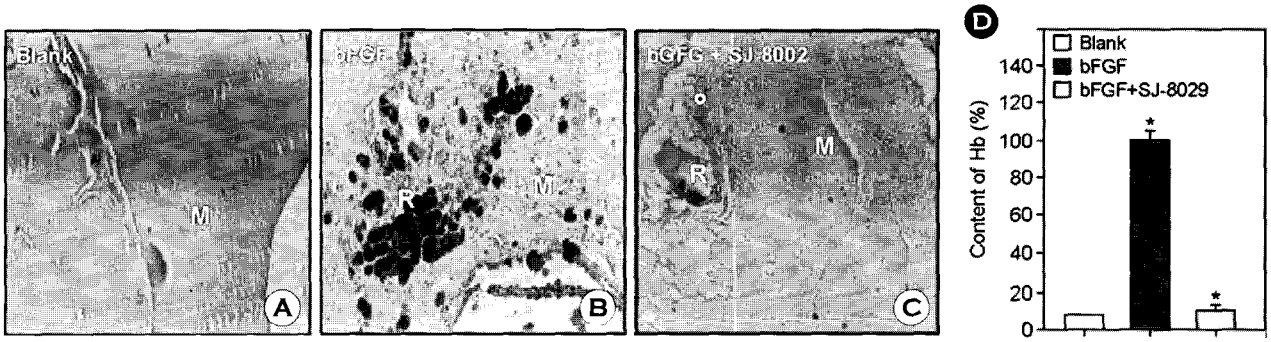

Fig. 3. Anti-angiogenic effect of SJ-8029 on the mouse Matrigel plug. Matrigel alone and bFGF were used as a negative and positive control, respectively. **(A)** The Matrigel without SJ-8029 did not show any migration and invasion of endothelial cells. **(B)** With Matrigel containing bFGF, many blood vessels appeared in the gel. **(C)** SJ-8029 strongly inhibited bFGF-induced angiogenesis. **(D)** Quantitation of active vasculature inside the Matrigel by measurement of hemoglobin content. Each value represents the mean ± SD of at least five animals, and similar results were obtained in three different experiments. *M*, Matrigel *R*, red blood cells. *, $P<0.05$ compared to blank.

consistent with those of morphologic analysis in the Matrigel implants. These data indicate that SJ-8029 suppresses angiogenesis *in vivo*.

2. SJ-8029 inhibits *in vitro* angiogenesis of BAECs

Based on the *in vivo* studies, we tested the effect of SJ-8029 on each step of angiogenesis by using *in vitro* angiogenesis assays. At first, we tested the effect of SJ-8029 on the viability of BAECs by performing MTT assay. SJ-8029 did not show any cytotoxicity on the BAECs at this range (from 0.01 to 0.1 μM, data not shown). To determine the effect of SJ-8029 on the proliferation of BAECs, [^{3}H]-thymidine incorporation assay was performed. As shown in Fig. 4A, SJ-8029 significantly inhibited the proliferation of BAECs in a dose of 0.1 μM. The migration of endothelial cells is one of the critical features in angiogenesis. Therefore,

we examined the effect of SJ-8029 on the migration ability of endothelial cells. SJ-8029 decreased the migration of BAECs from the edge of the wound into the open area (Fig. 4B). Through *in vitro* invasion assay, we also studied the effect of SJ-8029 on the invasive capacity of endothelial cells. The treatments of SJ-8029 (0.01 and 0.1 μM) decreased the invasion of BAECs into Matrigel by 20% and 55% (Fig. 4C). MMP-2 is important in the development of the angiogenic phenotype (Herron et al., 1986). Therefore, we conducted a gelatin based zymography to determine the effect of SJ-8029 on the production of MMP-2 in BAECs. The cells with SJ-8029 (0.1 μM) produced lower level of pro-MMP-2 as compared with that of the blank. (Fig. 4D). In addition, we tested whether the treatment of SJ-8029

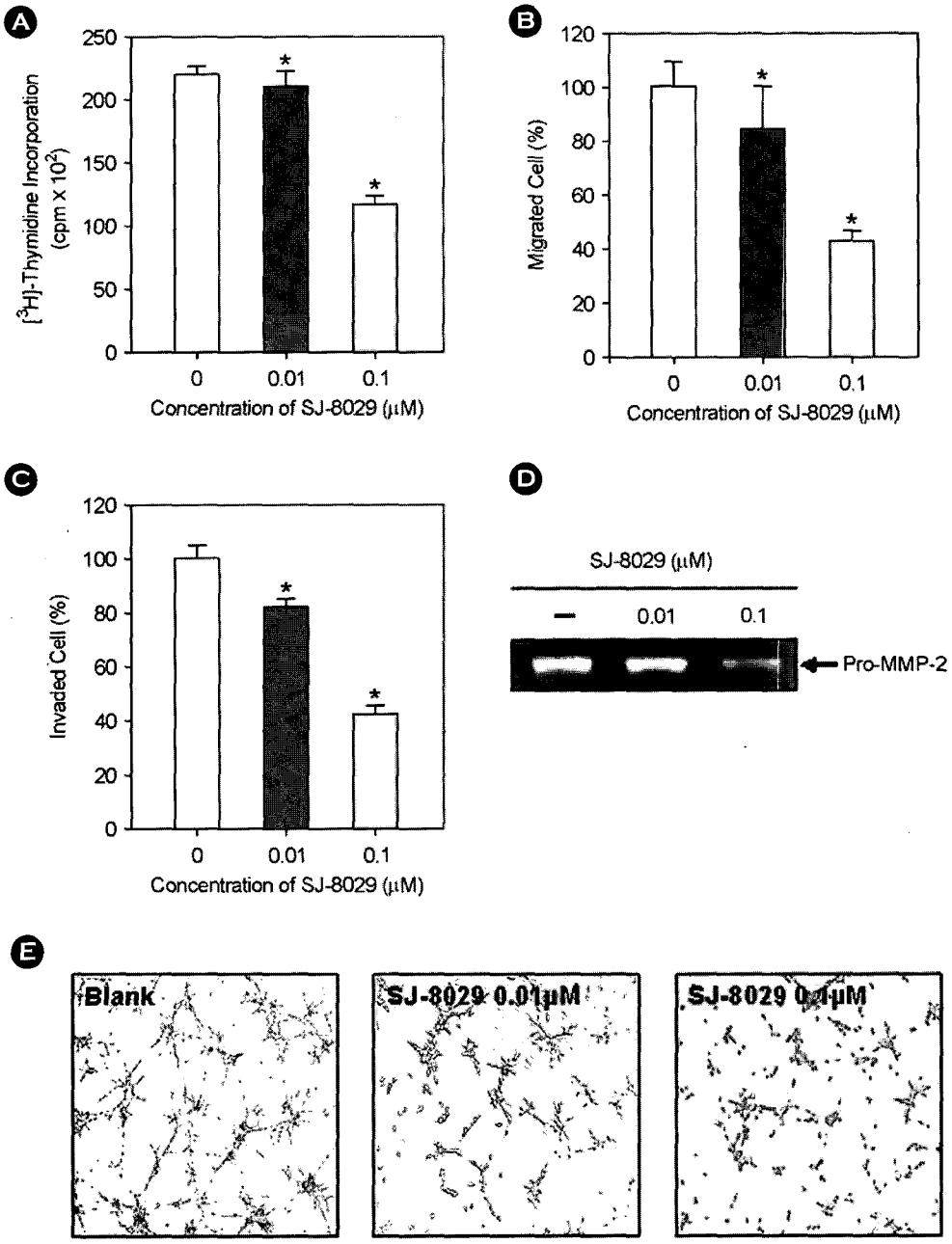

Fig. 4. Effect of SJ-8029 on *in vitro* angiogenesis of BAECs. BAECs were incubated with the indicated concentrations of SJ-8029 (A~E). Effect of SJ-8029 on the **(A)** proliferation. **(B)** migratory ability. **(C)** invasive capacity. **(D)** activation of pro-MMP-2 using gelatin-based zymography. **(E)** development of capillary-like structures (×40). Data represent the mean ± SD of triplicate, and similar results were obtained in three different experiments. C, untreated blank cells. *, $P<0.05$ compared to blank.

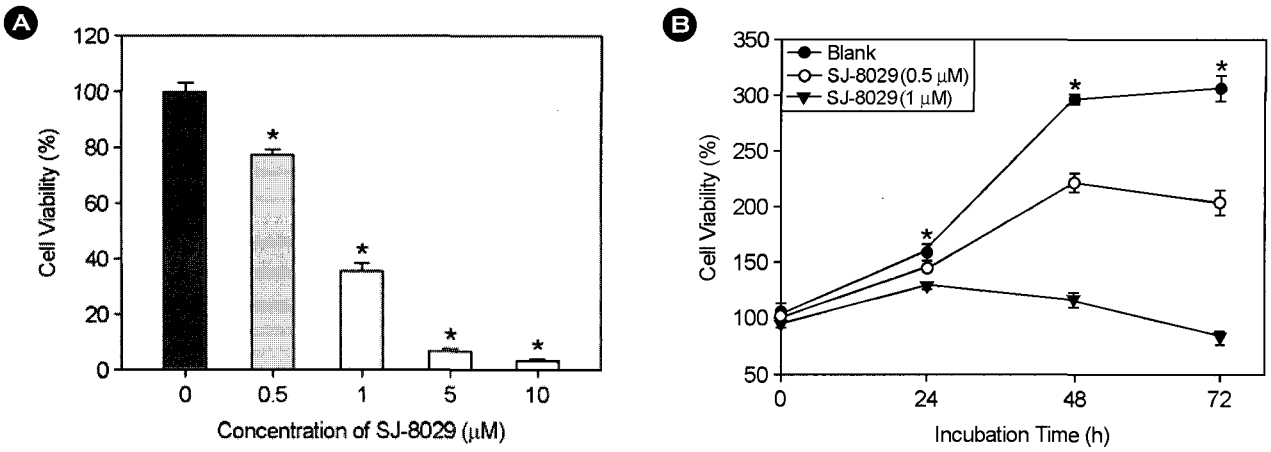

Fig. 5. Decreased viability of HepG2 cells by SJ-8029. The cell viability was assessed by MTT assay. **(A)** HepG2 cells were treated with 0.5 to 10 μM of SJ-8029 for 48 h. **(B)** HepG2 cells were treated with 0.5 and 1 μM SJ-8029 for 0, 24, 48, and 72 h. Results are expressed as the percent change of the controlled condition. Data represent the mean ± SD of triplicate. *, $P<0.05$ compared to blank.

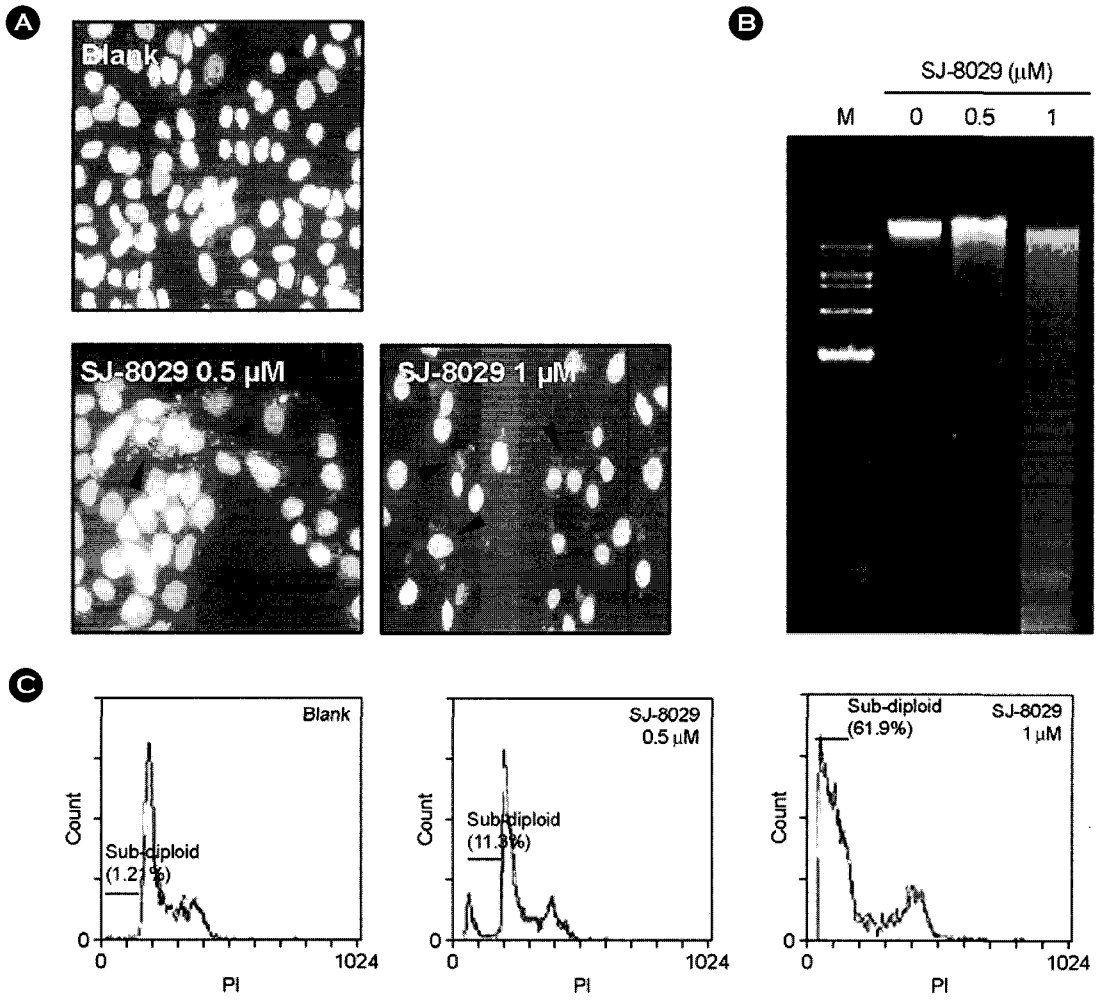

Fig. 6. SJ-8029 induces apoptotic cell death in HepG2 cells. HepG2 cells were treated for 48 h without or with SJ-8029. **(A)** Cells were stained with DAPI. Untreated cells (blank) showed normal distribution of chromatin. However, SJ-8029-treated cells showed local distribution of condensed chromatin. Arrowheads indicate spots of condensed chromatin in nuclei. **(B)** DNA was isolated from designated cells and analyzed by 1.5% agarose gel electrophoresis. Lane M presents DNA marker. **(C)** Flow-cytometric analysis was done to quantify the percentage of cells undergoing apoptosis. After SJ-8029 treatment, the percentage of hypodiploid cells was determined.

(0.01 and 0.1 μM) also prevented endothelial tube formation. The cells formed blood vessel on Matrigel in the absence of SJ-8029, whereas the addition of SJ-8029 resulted in the inhibition of tube formation (Fig. 4E). These findings further confirmed that SJ-8029 is able to modulate endothelial cell functions at concentrations and exposure times at which cell viability is not affected and suggest that it indeed inhibits the processes of angiogenesis.

3. SJ-8029 inhibits proliferation of HepG2 cells

To investigate the effect of SJ-8029 on the viability of HepG2 cells, we performed MTT assay. As shown in Fig. 5A, the treatment of SJ-8029 (1 to 10 μM) for 48 h significantly decreased their viability. In addition, we performed the proliferation assay to examine the anti-proliferative effect of SJ-8029 on the proliferation of HepG2 cells. As shown in Fig. 5B, the proliferation of SJ-8029-treated cells was inhibited at 48 and 72 h, whereas untreated cells maintained in an exponential proliferation state.

4. SJ-8002 causes apoptotic features in HepG2 cells

To determine whether SJ-8029-induced cell death occur through an apoptotic pathway, we performed several assays. The morphological changes with DAPI staining were observed. Morphological changes, corresponding to apoptosis, including nuclear condensation and chromatin fragmentation, were elicited by the treatment of SJ-8029 (1 μM) (Fig. 6A). At this concentration, cell viability was extensively decreased. As shown in Fig. 6B, SJ-8029 produced DNA fragmentation, indicating that SJ-8029 induced the apoptosis of HepG2 cells. In addition, the degree of DNA fragmentation was also measured by flow cytometric measurement of the percentage of nuclei with a hypodiploid DNA content. In Fig. 6C, untreated HepG2 cells (blank) showed a normal cell cycle, consisting of a major diploid peak (G_0/G_1), a small hyperdiploid region (S), and a minor tetraploid peak (G_2/M). However, SJ-8029 pretreatment (0.5 and 1 μM) led to the increase of the percentage of hypodiploid cells, reflecting that cells had undergone apoptosis-associated DNA degradation.

5. SJ-8029 enhances release of cytochrome c and induces activation of caspase-3 in HepG2 cells

During apoptosis, cytochrome c is released from mitochondria to cytosol, and then initiates caspase activation. To examine whether SJ-8029 induce the release of cytochrome c, we performed Western blot analysis. SJ-8029 increased the release of mitochondrial cytochrome c from mitochondria to cytosol (Fig. 7). The expression of caspase-3 was also determined by Western blot analysis. The pro-caspase-3 (32 kDa) exists in the cytosol, which is activated by proteolytic cleavage. Also, we detected active-caspase-3 (17 kDa) after SJ-8029 treatment for 48 h. These results indicate that SJ-8029-induced apoptosis is mediated by the release of cytochrome c and the activation of caspase-3.

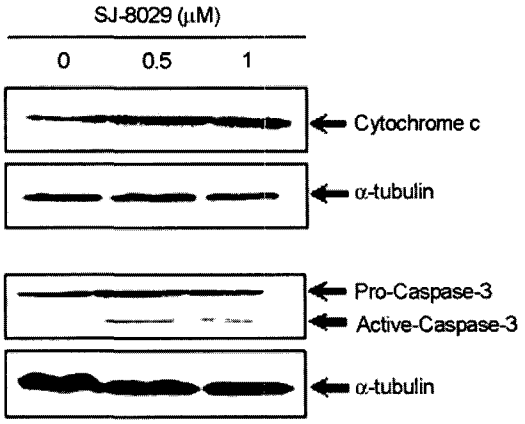

Fig. 7. SJ-8029 enhances cytochrome c release and induces caspase-3 activation in HepG2 cells. HepG2 cells were treated with the indicated concentrations of SJ-8029 for 48 h. The expressions of released cytochrome c and activated caspase-3 were detected by Western blot analysis. α-tubulin was used as an internal control.

DISCUSSION

In the present study, we demonstrated that SJ-8029 restrained the angiogenesis of BAECs and caused the apoptosis of HepG2 cells. We clearly elucidated the anti-angiogenic activity of SJ-8029 by performing both *in vivo* and *in vitro* angiogenesis assays. We observed that the treatment of SJ-8029 significantly inhibits the development of capillary networks in CAM (Fig. 2). We also confirmed the anti-angiogenic activity of SJ-8029 by performing *in vivo* mouse Matrigel plug assay (Fig. 3). The angiogenic process is a strongly regulated phenomenon that includes at least four sequential stages: (a) proliferation of endothelial cells; (b) enzymatic degradation of basement membrane and interstitial matrices by endothelial cells: (c) migration of endothelial cells; and (d) formation of capillary loops by endothelial cells (Ausprunk et al., 1975; Folkman, 1986). The treatment of SJ-8029 did not show any cytotoxicity at lower concen-

trations than 0.1 μM. At this range of concentration, we observed that SJ-8029 inhibited the proliferation of BAECs (Fig. 4A). Our results showed that the treatment of SJ-8029 significantly inhibited the migration (Fig. 4B) and invasion (Fig. 4C) of BAECs, and the expression of MMP-2 (Fig. 4D) in BAECs. In the presence of SJ-8029, BAECs hardly formed capillary-like networks, whereas BAECs established tube-like structure when in incubated in the absence of SJ-8029 (Fig. 4E). According to the data obtained from both *in vivo* and *in vitro* angiogenesis assays, we concluded that SJ-8029 has strong anti-angiogenic effect on these sequential angiogenic cascades.

VEGF and bFGF are well known as key stimulus for angiogenesis (Pepper et al., 1996; Lamoreaux et al., 1998). To test whether expression of these factors were altered in the anti-angiogenic activity of SJ-8029, the expression of both factors in BAECs was tested using RT-PCR analysis. However, SJ-8029 did not significantly down-regulate the expressions of VEGF and bFGF mRNA (data not shown). These data suggest a possibility that SJ-8029 might modulate the expressions of other angiogenesis-associated genes rather than them.

The data obtained from *in vivo* and *in vitro* angiogenesis assays clearly suggest that SJ-8029 has a strong anti-angiogenic activity. This anti-angiogenic activity of SJ-8029 *in vivo* and *in vitro* urged us to examine the apoptosis-inducing activity of SJ-8029 in HepG2 cells, since many of microtubule and topoisomerase inhibitor affecting agents with anti-angiogenic activity induces tumor cell apoptosis. Consistent with the idea that SJ-8029 with strong anti-angiogenic activity could induce apoptosis of tumor cells, SJ-8029 causes apoptosis of HepG2 cells by decreasing cell viability, and increasing chromatin condensation, DNA fragmentation and the number of sub-diploid cells (Fig. 5 and 6). These data support that SJ-8029 induced cell death through apoptotic process in HepG2 cells. Cytochrome c released from mitochondria forms complexes with several caspases in the presence of dATP or ATP and causes the activation of caspase cascade (Kiechle et al., 1998). The release of cytochrome c is the pivotal event in the initiation of the apoptotic pathway (Mullauer et al., 2001). Caspases can be thought of as the central executioners of the apoptotic pathway, since they induce most of the visible changes that characterize apoptotic cell death. Among caspase family, caspase-3 is a distal executioner, which is transform pro-enzyme to active-form enzyme by apoptotic triggers-induced caspase-cascade activation, and then mediates the apoptotic cascade. We tried to detect the release of cytochrome c and the activation of caspase-3 by Western-blot analysis. As expected, SJ-8029 enhanced the release of cytochrome c and induced the activation of caspase-3 in HepG2 cells (Fig. 7). Therefore, we conclude that SJ-8029 could induce tumor cell death via apoptotic pathway through the release of cytochrome c and activation of caspase-3.

In conclusion, our findings strongly suggest that SJ-8029 has not only anti-angiogenic effect on endothelial cells, but also apoptosis-inducing activity on tumor cells. Therefore, SJ-8029 might be a multi-potent anti-cancer drug that can act at multiple levels of cancer development.

Acknowledgements

This work was supported for two years by Pusan National University Research Grant.

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