Betaine inhibits *in vitro* and *in vivo* angiogenesis through suppression of the NF-κB and Akt signaling pathways

EUI-YEUN YI[1,2] and YUNG-JIN KIM[1]

[1]Department of Molecular Biology, [2]Research Institute for Genetic Engineering, Pusan National University, Busan 609-735, Republic of Korea

Received May 29, 2012; Accepted July 23, 2012

DOI: 10.3892/ijo.2012.1616

Abstract. Angiogenesis is defined as the formation of new blood vessels form existing vessels surrounding a tumor. The process of angiogenesis is an important step for tumor growth and metastasis, as is inflammation. Thus, angiogenesis inhibitors that suppress inflammation have been studied as an anticancer treatment. Recently, many research groups have investigated the anti-angiogenic activity of natural compounds since some have been demonstrated to have anticancer properties. Among many natural compounds, we focused on betaine, which is known to suppress inflammation. Betaine, trimethylglycine (TMG), was first discovered in the juice of sugar beets and was later shown to be present in wheat, shellfish and spinach. In Southeast Asia, betaine is used in traditional oriental medicine for the treatment of hepatic disorders. Here, we report the anti-angiogenic action of betaine. Betaine inhibited *in vitro* angiogenic cascade, tube formation, migration and invasion of human umbilical vein endothelial cells (HUVECs). Betaine also inhibited *in vivo* angiogenesis in the mouse Matrigel plug assay. The mRNA expression levels of basic fibroblast growth factor (bFGF), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in HUVECs were decreased by betaine treatment. In addition, betaine suppressed NF-κB and Akt activation.

Introduction

Angiogenesis is an essential step in new blood vessel formation for tumor growth and metastasis (1,2), and it is regulated by the balance between angiogenic activators and inhibitors (3,4). In addition, complex and diverse cellular actions are implicated in angiogenesis, such as degradation of the extracellular matrix (ECM), proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells into tubes (5,6).

Correspondence to: Professor Yung-Jin Kim, Department of Molecular Biology, College of Natural Science, Pusan National University, Busan 609-735, Republic of Korea
E-mail: yjinkim@pusan.ac.kr

Key words: betaine, angiogenesis, Akt, NF-κB

A critical role for inflammation in tumorigenesis was postulated by Rudolf Virchow in 1863; generally, chronic inflammation is linked to various steps involved in tumorigenesis, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis (7-10). Various inflammatory mediators, chemokines and growth factors have been shown to enhance the proliferation and spread of tumor cells with necessary nutrients and oxygen (11-13).

Both angiogenesis and inflammation are important processes in tumor growth and expansion (7,14), and these processes share common signal pathways in NF-κB and Akt (15,16). Thus, we speculate that some compounds with anti-inflammatory properties could also have anti-angiogenic activities.

Recently, scientists have become interested in the potential antitumor effects of nutrients due to their safety and general acceptance. Many publications have demonstrated that a large class of natural compounds, including pharmaconutrients, exhibits antitumoral activities against selected cancer types (17,18). We focused on the natural compound betaine (Fig. 1). Betaine is an essential biochemical molecule of the methionine/homeocystein cycle and is synthesized by conversion of choline. It was first discovered in the juice of sugar beets (*Beta vulgaris*) in the 19th century (19), and since then has been found in various microorganisms, plants and animals (20,21). In addition to being used as a food source, water extracts of betaine from *Lyciumchinensis* have been used in traditional oriental medicine for the treatment of hepatic disorders in Southeast Asia (22). The biological function of betaine was proposed to act as an osmolyte to protect cells under stress as a catabolic source of methyl through trans-methylation in many biochemical pathways (23-25).

Recent studies have shown that betaine suppresses inflammation through NF-κB activation during aging (26). Since NF-κB signalling is common to inflammation and angiogenesis, we investigated the anti-angiogenic activities of betaine using *in vitro* and *in vivo* angiogenesis assays. As a result, we found that betaine suppressed tube formation, migration, and invasion of human umbilical vein endothelial cells (HUVECs). Betaine also inhibited *in vivo* angiogenesis in the mouse Matrigel plug assay. In addition, betaine downregulated the mRNA expression levels of basic fibroblast growth factor (bFGF), matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) in HUVECs. We also found that betaine inhibited NF-κB and Akt activation in HUVECs.

Materials and methods

Materials and reagents. HUVECs were purchased from Innopharma Screen (Chungnam, Korea). Matrigel was from Collaborative Biomedical Products (Bedford, MA, USA) for the mouse Matrigel plug assay. Basic fibroblast growth factor (bFGF) and heparin were from Peprotech (Gaithersburg, MD, USA). FBS, penicillin and streptomycin were purchased from JBI (Daegu, Korea). Drabkin reagent kit 525 was purchased from Sigma (St. Louis, MO, USA). Trans-well filter chambers (8-μm pores) were purchased from Corning-Costar (Cambridge, MA, USA). Antibodies for NF-κB, phospho-NF-κB, Akt and phospho-Akt were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Betaine and gelatin were purchased from Sigma.

Cell culture. HUVECs were grown in M199, supplemented with heat-inactivated 20% fetal bovine serum (JBI), 20 ng/Ml of bFGF, 100 U/Ml of penicillin and 100 μg/Ml of streptomycin in a 37˚C incubator with a humidified atmosphere containing 5% CO_2.

Animals. Seven-week-old, specific pathogen-free (SPF) male C57BL/6 mice were supplied from Hyochang Science and Samtako (Daegu, Korea). They were provided with autoclaved tap water and lab chow *ad libitum* and were housed at 23±0.5˚C, 10% humidity under a 12 h light-dark cycle. The animal protocol used in this study was reviewed on the ethical procedures and scientific care, and approved by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC).

In vitro tube formation assay. HUVECs ($2x10^4$ cells) were seeded on a layer of previously polymerized matrigel and treated with or without betaine. Matrigel culture was incubated at 37˚C. After 18 h, changes in cell morphology were captured through a phase contrast microscope and photographed at x40 magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

In vitro wounding migration assay. HUVECs were seeded onto 24-well culture plates until confluence and left overnight. Media were aspirated the next day, and cells were scratched with a 200 μl pipette tip along the diameter of the well. Cells were washed twice with PBS and incubated at 37˚C and 5% CO_2. After wounding, the cultures were washed with serum-free medium and further incubated in M199 with 1% serum, 1 mM thymidine, and/or betiane. These culture conditions minimized proliferation of HUVECs. Wound diameters were photographed at 18 to 24 h. Wound closure was determined by measurement with optical microscopy at x40 magnification. Migration was quantitated by counting the number of cells that moved beyond the reference line. Each sample was assayed in duplicate, and independent experiments were repeated three times.

In vitro invasion assay. Invasiveness of HUVECs was performed *in vitro* using a trans-well chambers system (Corning-Costar) with 8.0-μm pore polycarbonate filter inserts. The upper side was coated with 10 μl of matrigel (0.5 mg/Ml) at room temperature for 1 h. Cells ($2x10^4$ cells) and betaine in serum-free media were placed in the upper part of the filters, and full media was treated in the lower parts. Cells were incubated at 37˚C for 24 h, fixed with methanol, and then stained with H&E. Cells on the upper surface of the membrane were removed by wiping with a cotton swab. Cell invasion was determined by counting whole cell numbers in a single filter by optical microscopy at x40 magnification. Each sample was assayed in duplicate, and independent experiments were repeated three times.

In vivo mouse Matrigel plug assay. C57BL/6 mice (7 weeks of age) were injected subcutaneously into 500 μl of matrigel (Collaborative Biomedical Products) containing bFGF (100 ng/Ml) and heparin (50 U/Ml) without or with betaine. After injection, the matrigel rapidly formed a plug. After 7 days, skin of the mouse was pulled back to expose the matrigel plug, which remained intact. After quantitative differences were noted and photographed, hemoglobin content was measured using the Drabkin reagent kit 525 (Sigma) for quantification of blood vessel formation. The amount of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel. Independent experiments were repeated twice and at least five mice in each experiment were used.

Reverse transcription-polymerase chain reaction (RT-PCR). Total-RNA from HUVECs was isolated using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. First-stranded cDNA was synthesized by M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) with 2 μg each of DNA-free total-RNA sample and oligo (dT)15 (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. Equal amounts of cDNA were subsequently amplified by PCR in a 20 μl reaction volume containing 1X PCR buffer, dNTP mixture, 10 μM of each specific primer and i-Taq™ DNA polymerase (iNtRON Biotechnology, Korea). Amplification products were electrophoresed on 2% agarose gels and visualized by GelRed (Biotium Inc, Hayward, CA, USA) staining under ultraviolet trans-illumination.

Western blot analysis. HUVECs were treated with or without betaine for 24 h in medium. Total cell lysates were prepared by addition of PRO-PREP Protein Extraction Solution (iNtRON Biotechnology), including 1 mM sodium orthovanadate. Equal amounts (30 μg) of samples were resolved by electrophoresis on a 10% SDS-polyacrylamide gel, transferred to a membrane, and sequentially probed with antibody. The following primary antibodies were used at the indicated dilutions: total NF-κB and anti-phospho-NF-κB, total Akt and anti-phospho-Akt, 1:1,000 in 5% BSA in TBS-T.

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

Results

Betaine inhibits vascular network formation of HUVECs. We carried out tube formation assay to examine the effects of betaine on the morphological differentiation of endothelial

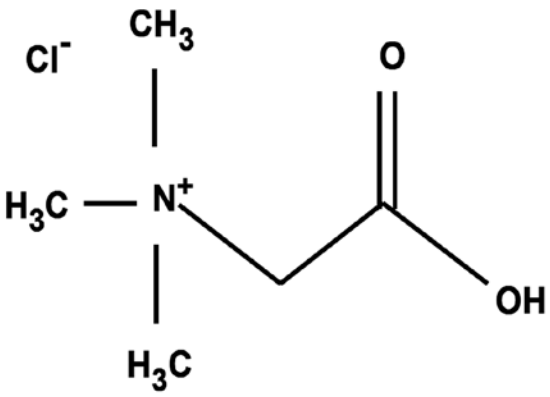

Figure 1. Structure of betaine.

cells into a capillary-like structure, an important step in the process of angiogenesis. HUVECs were placed on a matrigel-coated plate and incubated. Endothelial cells formed hollow capillaries on matrigel beds, and these tubes became stronger and more robust with elongated networks between 6-24 h. As shown in Fig. 2, HUVECs on matrigel formed a typical blood vessel network in the absence of betaine. However, treatment with betaine for 18 h resulted in broken, shortened and much narrower tube networks. This finding demonstrates that betaine inhibits tube formation of HUVECs.

Betaine inhibits migration and invasion of HUVECs. The migration and invasion of endothelial cells are critical features in the formation of new blood vessels and in the repair of injured vessels. Therefore, we investigated the effects of betaine on the movement of HUVECs from a wounded edge to an open area using wounding migration assay (Fig. 3). Treatment with betaine for 20 h profoundly decreased the migration of HUVECs compared with that of control in a dose-dependent manner (Fig. 3A). Migration of endothelial cells was decreased by about 70% by betaine treatment compared with that of control.

To examine the effects of betaine on the invasiveness of HUVECs, we performed invasion assay with a trans-well system. Trans-wells were prepared such that the upper sides of the filter were coated with matrigel and used for invasion assay. Betaine suppressed the invasiveness of HUVECs compared with that of control in a dose-dependent manner after 24 h of incubation (Fig. 3B). The invasiveness of endothelial cells was inhibited by about 60% by betaine treatment compared with that of control. Taken together, betaine strongly suppressed migration and invasion of HUVECs, which are crucial steps of angiogenesis.

Betaine inhibits angiogenesis in vivo. We demonstrated the *in vitro* anti-angiogenic activities of betaine as above. We then performed the mouse Matrigel plug assay to examine the effects of betaine on angiogenesis *in vivo*. As shown in Fig. 4A, matrigel plugs containing bFGF were abundantly filled with intact red blood cells, which indicates the formation of a functional vasculature inside the matrigel, whereas vessels were not observed in matrigel alone (blank). Matrigel plug containing betaine produced fewer vessels compared with plug containing bFGF, indicating that betiane inhibited the formation of bFGF-induced neomicrovessels. In addition, we measured the hemoglobin contents inside the matrigel plugs in order to quantify the anti-angiogenic effects of betaine. The hemoglobin content of bFGF-treated plug was 32 g/dl, whereas that of 1 mM betaine-treated plugs was profoundly diminished to about 2 g/dl (Fig. 4B). These results indicate that betaine has strong anti-angiogenic activity *in vivo.*

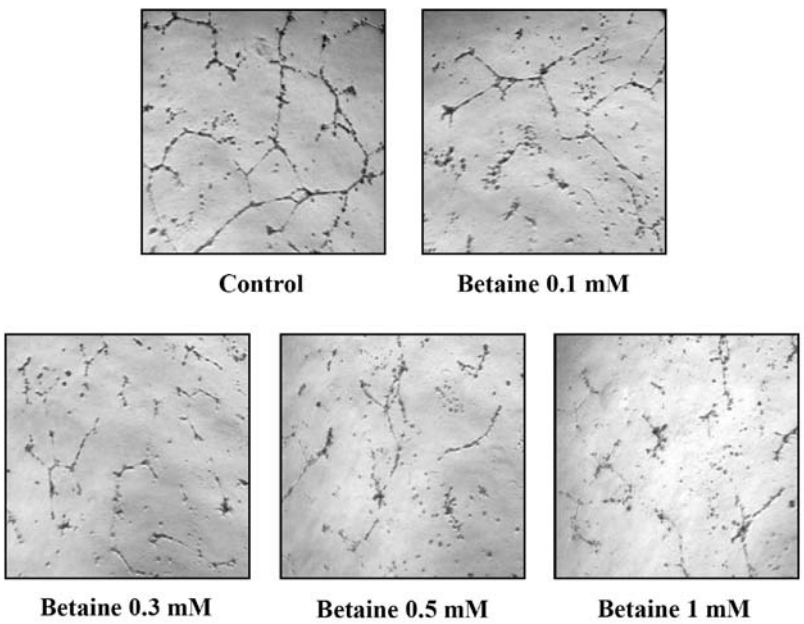

Figure 2. Betaine inhibits tube formation of HUVECs. HUVECs were grown on matrigel in the absence or presence of the indicated amount of betaine for a period of 18 h. Changes in cell morphology were captured through a phase contrast microscope (x40) and photographed. Representative photographs of tube formation of endothelial cells cultured on polymerized matrigel layers reveal an inhibitory effect of betaine on formation of a capillary-like structure. This experiment was repeated independently three times.

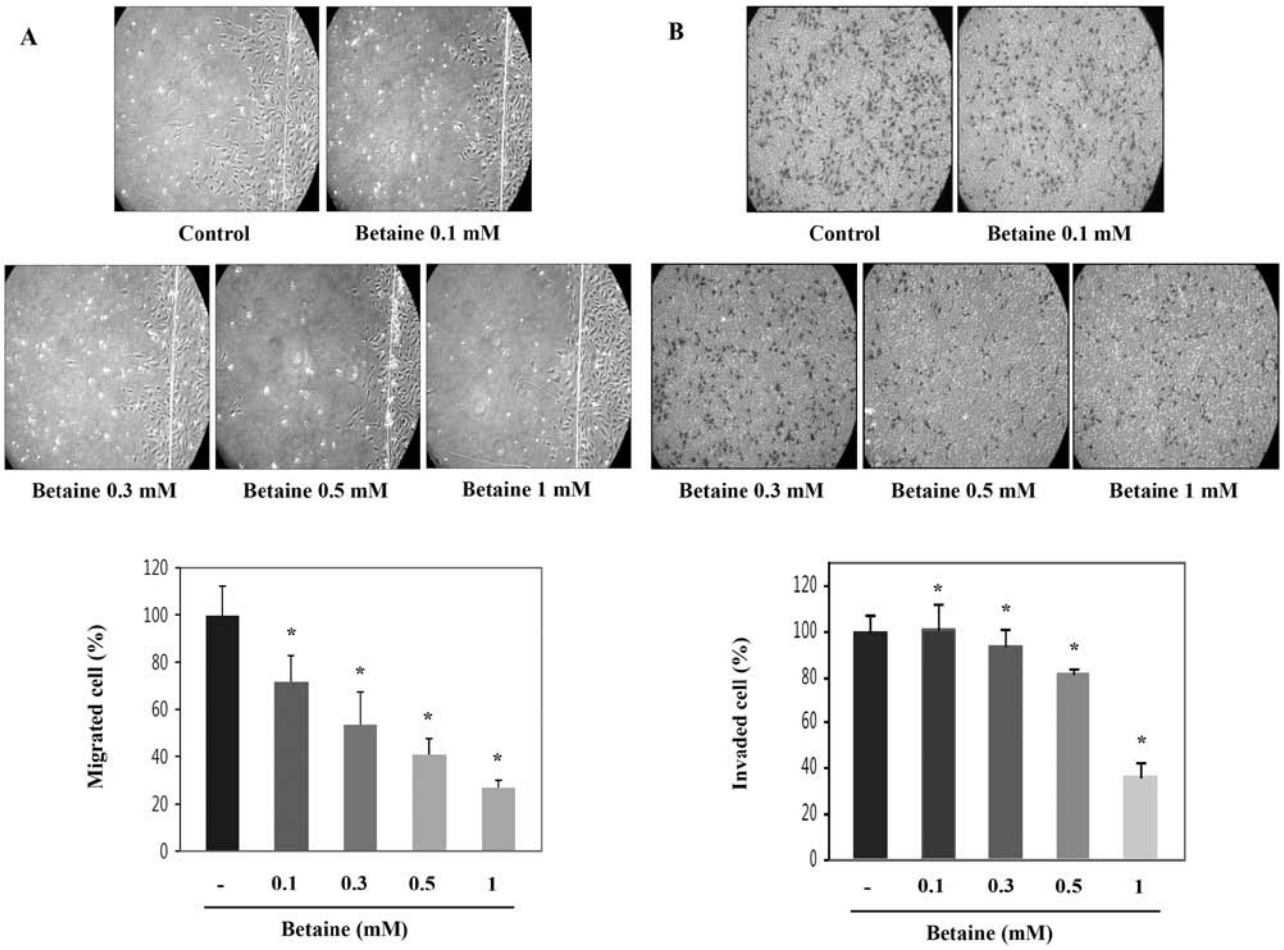

Figure 3. Betaine inhibits migration and invasion of HUVECs. (A) Migration ability of HUVECs was measured by wounding migration assay. (B) Invasion capacity was examined using a trans-well system coated with matrigel. Data are mean ± SD from three independent experiments performed in triplicate. *p<0.05 vs control.

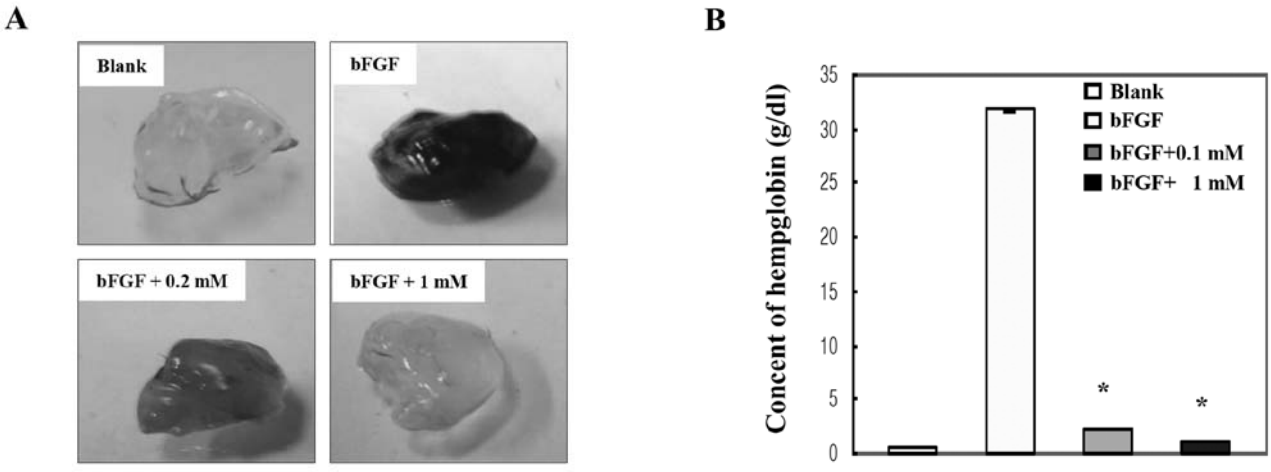

Figure 4. Betaine suppresses *in vivo* angiogenesis in the mouse Matrigel plug assay. (A) Matrigel plugs were photographed (x40). (B) Quantification of hemoglobin content. Each value represents the mean of ± SD of at least five animals, and the experiment was performed twice. Blank, matrigel alone. *p<0.05 vs hemoglobin content of bFGF-induced implants.

Betaine downregulates mRNA expression levels of bFGF, MMP-2 and MMP-9. To determine which factors are involved in the anti-angiogenic activity of betaine, we examined the mRNA expression levels of angiogenic factors (VEGF and bFGF) in HUVECs treated with betaine using RT-PCR. As shown in Fig. 5, treatment with betaine decreased the mRNA expression

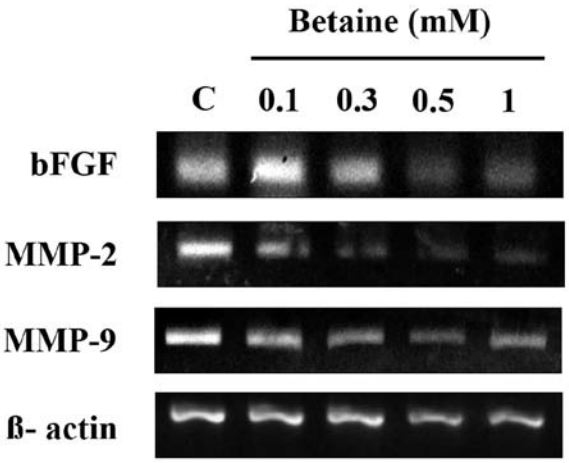

Figure 5. Betaine downregulates angiogenesis-related gene expression. The mRNA expression levels of bFGF, MMP-2 and MMP-9 in betaine-treated HUVECs were analyzed by RT-PCR analysis. Amplification of β-actin demonstrates comparable RNA amount and quantity among the samples.

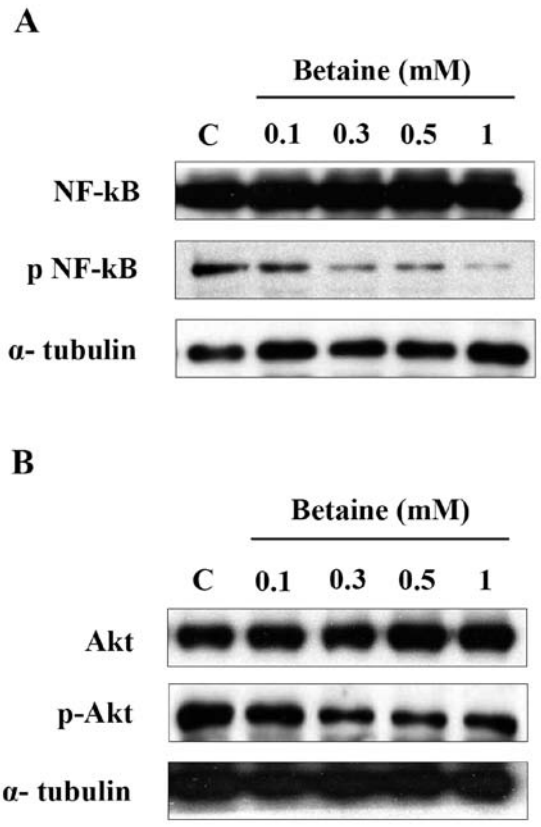

Figure 6. Betaine suppresses NF-κB and Akt activation of HUVECs. HUVECs were treated with the indicated concentrations of betaine for 24 h. Total proteins were prepared and analyzed by western blotting using each primary antibody. (A) Phospho-NF-κB and total NF-κB. (B) Phospho-Akt and total Akt. Each value was normalized by total form and α-tubulin based on total protein concentration.

of bFGF in a dose-dependent manner. However, treatment with betaine did not produce any significant change in mRNA levels of VEGF (date not shown).

Regulation of extracellular proteolytic activity is important in the process of endothelial cell migration and invasion through the basement membrane and capillary morphogenesis. Thus, the mRNA expression levels of both MMP-2 and MMP-9, which are major functional molecules in the degradation of the extracellular membrane, were examined. The mRNA expression levels of both MMP-2 and MMP-9 were markedly downregulated by treatment with betaine in a dose-dependent manner (Fig. 5). These data suggest that the downregulated expression of bFGF, MMP-2 and MMP-9 mRNA may be responsible for the anti-angiogenic activity observed in HUVECs treated with betaine.

Betaine suppresses NF-κB and Akt activation in HUVECs. Since NF-κB and Akt activation are two of the main signaling pathways for tumor progression and angiogenesis, we examined the effects of betaine on NF-κB and Akt activation in HUVECs. In this experiment, total proteins were prepared and analyzed using western blotting for detection of NF-κB and Akt phosphorylation. As shown in Fig. 6A, NF-κB phosphorylation was suppressed by betaine in a dose-dependent manner. As NF-κB activation is mediated by the Akt signaling pathway, we next examined Akt activation. Phosphorylation of Akt was inhibited by the presence of betaine in a dose-dependent manner (Fig. 6B). These results indicate that betaine interferes with NF-κB phosphorylation and Akt phosphorylation in HUVECs.

Discussion

Angiogenesis, the formation of new blood vessels from previously existing vessels, is required for tumor growth and metastasis. Angiogenic processes involve several steps: i) enzymatic degradation of vascular basement membranes by endothelial cells, ii) directed migration and proliferation of endothelial cells to provide cells for new vessels, iii) invasion of endothelial cells and iv) formation of capillary-like networks by endothelial cells (27).

Inflammation is also known to be a key process in many cancers during tumorigenesis (28). Both angiogenesis and inflammation are initiated by secreted chemokines/cytokines, growth factors and other mediators (13). These processes often share a common signal pathway. Thus, anti-inflammation in the form of angiogenesis inhibitors has been investigated as an anticancer therapy (29,30). For example, anti-inflammatory drugs such as nonsteroidal anti-inflammatory drugs (NAIDs) lead to the inhibition of angiogenesis (31).

One of the approaches to anti-angiogenesis therapy is using natural compounds from various organisms. Paclitaxel (Taxol), doxorubicine and citarabine are examples of anticancer agents originating from plants, microbes and a marine source, respectively (17,32). Natural compounds from medicinal plants display diverse pharmacological activities and have advantages over synthetic drugs, such as smoother action and better tolerance (33,34). Thus, the search for novel anticancer drugs from natural sources has continued through the collaboration of scientists looking for new bio-active compounds (17,35).

Recently, betaine was demonstrated as having anti-inflammatory effects by modulating NF-κB activation in the kidney tissue of aged rats (26). Since the NF-κB signal pathway participates both in inflammation and angiogenesis, we exam-

ined the anti-angiogenic activity of betaine by performing *in vitro* and *in vivo* angiogenesis assays. Firstly, we observed the anti-angiogenic effects of betaine in each step of angiogenesis *in vitro*. Treatment with betaine clearly inhibited tube formation (Fig. 2), migration (Fig. 3A) and invasion (Fig. 3B) of HUVECs. To demonstrate the *in vivo* anti-angiogenic effects of betaine, *in vivo* mouse Matrigel plug assay was performed. Betaine excellently inhibited the formation of neomicrovessels in matrigel (Fig. 4A), and we noted that these newly synthesized vessels in matrigel plug containing betaine were not abundantly filled with intact red blood cells (Fig. 4B). Taken together, these results show that betaine suppressed angiogenesis *in vitro* and *in vivo*.

In addition, tumors produce a wide array of angiogenic molecules during angiogenesis (4). VEGF and bFGF are well known as key stimuli for angiogenesis (36,37). Therefore, we determined the involvement of betaine in the expression of major angiogenic factors. As shown in Fig. 5, mRNA expression of bFGF was remarkably reduced by treatment with betaine. However, we could not observe any change in VEGF mRNA expression (date not shown).

Matrix metalloproteinases (MMPs) are secreted as proenzymes and act as key regulators of extracellular matrix turnover through degradation of a wide variety of extracellular matrix proteins (38). The regulation of extracellular proteolytic activity is important in the process of endothelial cell migration and invasion, both of which occur during angiogenesis, and is an essential step in tumor invasion and metastasis. Based on our finding that motility of HUVECs was profoundly suppressed by betaine, we examined the effects of betaine on the expression of MMP-2 and MMP-9 using RT-PCR. As shown in Fig. 5, betaine downregulated the expression of both MMP-2 and MMP-9.

NF-κB and Akt are important mediators in tumor cell invasion and metastasis (15,39). Since betaine was recently reported to modulate the NF-κB signaling pathway during aging (26), we examined the activation of NF-κB and Akt by treatment with betaine using western blot analysis. In the presence of betaine, activation of NF-κB and Akt was suppressed in a dose-dependent manner (Fig. 6).

In summary, betaine inhibited angiogenesis *in vitro* and *in vivo*. These effects of betaine are linked with decreased mRNA expression of bFGF, MMP-2 and MMP-9. Furthermore, betaine suppressed angiogenesis through the NF-κB and Akt signal pathways. Therefore, we conclude that betaine possesses strong anti-angiogenic activity and could be developed as a useful inhibitor of angiogenesis.

Acknowledgements

This study was supported in part by National Research Foundation of Korea Grant funded by the Korean Government (Ministry of Education, Science and Technology. NRF-2010-355-C00044), and by a 2-Year Research Grant of Pusan National University.

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