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Betulinic Acid Inhibits Growth Factor-induced *in vitro* Angiogenesis via the Modulation of Mitochondrial Function in Endothelial Cells

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Betulinic acid (BetA), a pentacyclic triterpene, is a selective apoptosis-inducing agent that works directly in mitochondria. Recent study has revealed that BetA inhibits *in vitro* enzymatic activity of aminopeptidase N (APN, EC 3.4.11.2), which is known to play an important role in angiogenesis, but the anti-angiogenic activity of BetA has not been reported yet. Data presented here show that BetA potently inhibited basic fibroblast growth factor (bFGF)-induced invasion and tube formation of bovine aortic endothelial cells (BAECs) at a concentration which had no effect on the cell viability. To access whether the anti-angiogenic nature of BetA originates from its inhibitory action against aminopeptidase N (APN) activity, the effect of BetA on APN was investigated. Surprisingly, BetA did not inhibit *in vivo* APN activity in endothelial cells or APN-positive tumor cells. On the other hand, BetA significantly decreased the mitochondrial reducing potential, and treatment with mitochondrial permeability transition (MPT) inhibitors attenuated BetA-induced inhibition of endothelial cell invasion. These results imply that anti-angiogenic activity of BetA occurs through a modulation of mitochondrial function rather than APN activity in endothelial cells.

Key words: Betulinic acid — Angiogenesis — Aminopeptidase N — Mitochondrial permeability transition

Betulinic aicd (BetA), a pentacyclic triterpene, has been isolated from the stembark of *Betula* ssp. and from many other plants.[1, 2)] It was first identified as a selective apoptosis-inducing agent in human melanoma cells.[3)] In an *in vivo* animal model system, BetA showed highly effective tumor growth inhibition in mice injected subcutaneously with human melanoma MEL-2 cells. Furthermore, it also induces apoptosis in neuroectodermal tumor and malignant glioma cells.[4, 5)] Extensive studies have shown that BetA-induced apoptosis occurs through the perturbation of mitochondrial function, such as loss of membrane potential ($\Delta\psi_m$), reactive oxygen species (ROS) production, and permeability transition (PT) pore opening.[5)] These mitochondrial events trigger a sequential apoptotic cascade including the release of mitochondrial apoptogenic factors, activation of caspases, and DNA fragmentation.[6)] All of these effects are CD95- and p53-independent, suggesting that BetA may induce apoptosis via a direct effect on mitochondria.[7, 8)]

Melzig and Bormann reported that BetA inhibited *in vitro* enzymatic activity of aminopeptidase N (APN, EC 3.4.11.2).[9)] APN is identical to CD13 (gp150), a myeloid cell surface glycoprotein, and is a widely distributed membrane-bound, zinc-dependent metalloproteinase.[10, 11)] Previous studies have shown that APN, as a zinc-dependent metalloproteinase, plays an important role in metastatic tumor invasion.[12, 13)] Recently, Bhagwat *et al.* reported that APN was activated by angiogenic stimuli and was essential for the endothelial cell tube formation.[14)] Several inhibitors of APN significantly inhibited retinal neovascularization, *in vivo* angiogenesis of chorioallantoic membrane, and xenograft tumor growth, suggesting that APN plays an important role in the progression of tumor vasculogenesis and may be involved in a crucial step of the angiogenic process.[15)]

These interesting results led us to investigate whether BetA, a mitochondrial function disruptor as well as an inhibitor of APN, could inhibit growth factor-induced angiogenesis. We performed *in vitro* angiogenesis assays including cell proliferation, chemoinvasion and tube formation of endothelial cells. Data presented here show that BetA potently inhibits growth factor-induced angiogenesis *in vitro* without showing any cytotoxic effect on endothelial cells. Surprisingly, *in vivo* enzymatic analysis of APN revealed that BetA does not inhibit the enzyme activity in endothelial cells or APN-positive tumor cells. These results indicate that anti-angiogenic activity of the compound is not derived from the inhibition of APN. Instead, we found that mitochondrial function in endothelial cells is major target of BetA.

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MATERIALS AND METHODS

Materials BetA was purchased from Aldrich Chem. Co. (Milwaukee, WI). The basic fibroblast growth factor (bFGF) was obtained from Upstate Biotechnology (Lake Placid, NY), bestatin, aminopeptidase N, ala-7-amido-4-methylcoumarin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma (St. Louis, MO), cell culture media from Life Technology (Grand Island, NY), the Matrigel from Collaborative Biomedical Products (Bedford, MA), and the Transwell plate from Corning Costar (Cambridge, MA). All chemicals used in this study were of the highest grade commercially available.

Cell culture The early passages (5–7 passage) of bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Jo at the NIH of Korea and by Dr. Kwon at Kangwon Natl. Univ., respectively. BAECs were grown in MEM supplemented with 10% fetal bovine serum (FBS). HUVECs were maintained in M199 supplemented with 20% FBS, heparin, and 1.5 ng/ml bFGF. HT1080 and MDA-MB-231 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), and B16/BL6 and HCT116 cells in RPMI1640 containing 10% FBS. All cells were grown at 37°C in a humidified atmosphere of 5% CO_2.

Cell growth assay BAECs were seeded at a density of 2×10^4 cells/well in a 48-well plate. The cells were incubated in growth media for 24 h. Various concentrations of BetA were added to the wells and incubation was continued for 24 or 48 h. The cells in each well were trypsinized and pelleted by centrifugation. Each pellet was resuspended in 10 μl of phosphate-buffered saline (PBS) and trypan blue dye was added. Cells were observed under a microscope and counted with a hemocytometer. Cell viability was accessed as unstained cells/total cells×100.

Chemoinvasion assay The invasiveness of the endothelial cells was examined *in vitro* using a Transwell chamber system with 8.0-μm-pore polycarbonate filter inserts. The lower side of the filter was coated with 10 μl of gelatin (1 mg/ml), and the upper side was coated with 10 μl of Matrigel. Cells (1×10^5 cells) were placed in the upper part of the filter and BetA was applied to the lower part for 30 min at room temperature before seeding. The chamber was then incubated at 37°C for 18 h. The cells were fixed with methanol and stained with hematoxylin/eosin. The cell invasion was determined by counting whole cells on the lower side of the filter using an optical microscope at ×100 magnification.

Capillary tube formation assay Matrigel (250 μl, 10 mg/ml) was placed in a 24-well culture plate and polymerized for 30 min at 37°C. The BAECs (1×10^5 cells) were seeded on the surface of the Matrigel and treated with bFGF (30 ng/ml). Then, BetA was added and incubation was continued for 6–18 h. The morphological changes in the cells and tubes formed were observed under a microscope and photographed at ×100 magnification using JVC digital camera (Victor, Yokohama).

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR) Total cellular RNA was isolated from cultured cells with an RNeasy mini kit (Qiagen, Inc., Valencia, CA) and reverse-transcribed by Molony murine leukemia virus reverse transcriptase (Life Technologies, Rockville, MD) using Oligo-d$(T)_{15}$ primer (Life Technologies). For the determination of APN mRNA content in each cell line, a standard PCR was performed using 5′-CCTTCAACCTGGCCAGTGC-3′ and 5′-CGTCTTCTCCAGGGCTTGCTCC-3′ (sense and antisense primers common to murine and human aminopeptidase N (APN)) as primers. GAPDH mRNA was quantified using the RT primer pair commercially available from Stratagene (Heidelberg, Germany) and used to normalize the cDNA content. The PCR products were resolved by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

Assay of APN enzymatic activity The activity of APN was determined according to Saiki *et al.*[12] Briefly, the enzyme substrate, ala-7-amido-4-methylcoumarin was dissolved (2 m*M*) in PBS as a stock solution. The substrate (0.1 m*M*) was added to PBS with or without inhibitors. The reaction was started by adding an enzyme solution (final conc., 0.2 mU) and continued in dark room at 37°C. After 1 h, the reaction mixture was centrifuged and the supernatant was collected for measurement of fluorescence using an FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, Vermont) at the excitation wavelength of 360 nm, and the emission wavelength of 460 nm. For *in vivo* enzyme assay, cells (2×10^5) were seeded in a 24-well culture plate and after incubation for 24 h, culture media were replaced with 500 μl of PBS in each well. Aliquots of substrate (2 m*M*) were added directly to each well with or without inhibitors. The plate was incubated in dark room at 37°C for 1 h. The supernatant from each well was collected and enzyme activity was determined as described above.

MTT reduction assay BAECs were seeded at a density of 5×10^3 cells/well in a 96-well plate and incubated for 24 h. Various concentrations of drugs were added to the wells and incubation was continued for 12 h. After 12 h, 50 μl of MTT (0.4 mg/ml, final conc.) was added and the plate was incubated for an additional 4 h. After removal of the culture supernatant, 150 μl of dimethylsulfoxide (DMSO) was added to dissolve MTT-formazan. The plate was read at 540 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT).

RESULTS

Effect of BetA on the cell viability and proliferation of BAECs To determine the optimum dose of BetA in angiogenesis assays, endothelial cell viability assay was performed using the trypan blue exclusion method. BAECs were treated with various doses of BetA for 24 or 48 h and stained with trypan blue. BetA (1–20 μM) for 24 h did not significantly affect the viability of BAECs (Fig. 1A). However, the viability of BAECs was slightly decreased at high concentrations of BetA (15–20 μM) for 48 h. We next examined the effect of BetA on the proliferation of BAECs. BAECs were treated with various doses of BetA and stained with trypan blue at each time point. BetA showed a weak inhibition of BAECs proliferation at a concentration of 20 μM without showing any cytotoxicity, as in the cell viability assay (Fig. 1B). However, no inhibition of proliferation was observed at 10 μM BetA up to 72 h. Therefore, angiogenesis assays were performed in a concentration range of 1 to 10 μM of BetA within 24 h.

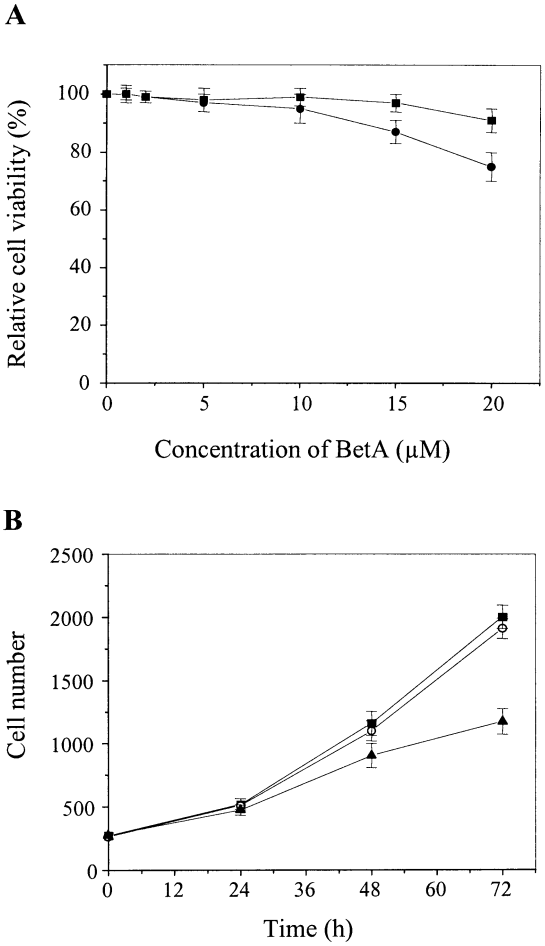

Fig. 1. Dose response of betulinic acid (BetA) on bovine aortic endothelial cells (BAECs). (A) Effect of BetA on the viability of BAECs. Cell viability was determined by trypan blue exclusion assay as described in "Materials and Methods." ■ 24 h, ● 48 h. (B) Effect of BetA on the proliferation of BAECs. Cells were treated with BetA (0–20 μM) and cell growth was measured at various time points. ■ Control, ○ BetA (10 μM), ▲ BetA (20 μM).

BetA potently inhibits growth factor-induced angiogenesis *in vitro* Angiogenic endothelial cells secrete several protein-degrading enzymes, including matrix metalloproteinases that degrade the basement membrane, favoring the formation of new blood vessels.[16, 17] Thus, endothelial cell invasion is a crucial step for angiogenesis.[18] For *in vitro* invasion assay with endothelial cells, BAECs were starved for 24 h and stimulated by bFGF in the presence or absence of BetA. The assay was performed using polycarbonate-filter Transwells coated with the Matrigel to prevent the migration of non-invasive cells. BetA potently inhibited bFGF-induced invasion of BAECs in a dose-dependent manner (Fig. 2A). An inhibitory effect on BAECs invasion was seen at a concentration as low as 2 μM BetA (data not shown). We next examined the effect of BetA on capillary tube formation. In the presence of bFGF, cultured BAECs on the Matrigel formed an extensive network of thick tubes (Fig. 2C). Treatment of BAECs with BetA resulted in dose-dependent inhibition of tube formation induced by bFGF. To rule out the possibility that the inhibition of tube formation is merely due to a cytotoxic effect of BetA on BAECs, trypan blue staining was performed after the complete formation of tubes. The highest concentration (10 μM) of BetA did not affect the endothelial cell viability in the formed tubes (Fig. 2C). In addition, 10 μM BetA did not significantly inhibit the proliferation of BAECs, as shown in Fig. 1B. These results suggest that BetA selectively and potently inhibits angiogenesis *in vitro*, and the anti-angiogenic activity of the compound may mostly originate from a specific effect on the angiogenic differentiation of endothelial cells, rather than anti-proliferative activity.

BetA inhibits APN enzymatic activity *in vitro* but not *in vivo* As mentioned above, BetA is an inhibitor of APN, a potent angiogenesis inducer.[14, 15] We examined whether the anti-angiogenic activity of BetA originates from the inhibition of enzymatic activity of APN. *In vitro* APN activity assay was carried out as previously described by Saiki *et al.*[12] Partially purified APN (Sigma) was used as an enzyme source for *in vitro* analysis. As shown in Fig. 3A, BetA as well as bestatin, a known APN competitive inhibitor, potently inhibited APN activity with an IC_{50} of 7 μM. The *in vitro* inhibition of APN by BetA is consistent with the report by Melzig and Bormann.[9] Next, we investigated the effect of BetA on the *in vivo* enzymatic activity of APN. Endothelial cells were seeded in a 24-well culture plate and a fluorogenic substrate was added directly to

each well with or without inhibitors. After reaction, the supernatant from each well was collected to determine *in vivo* APN activity. Surprisingly, BetA-treated cells still showed strong APN activity, at the same level as the drug-untreated control cells (Fig. 3B), whereas the enzyme activity from bestatin-treated cells was inhibited. These results indicate that BetA does not inhibit *in vivo* APN activity in endothelial cells.

BetA does not inhibit *in vivo* APN activity in APN-positive tumor cells We observed that BetA does not inhibit *in vivo* APN activity in endothelial cells. To investigate the possibility that this lack of effect of BetA is limited to endothelial cells, we next examined the effect of BetA on the APN activity in APN-positive tumor cells. RT-PCR analysis showed that APN is up-regulated in HUVECs in the presence of bFGF (Fig. 4A). In addition, B16/BL6, murine melanoma, and HT1080, human fibrosarcoma cells, constitutively express high levels of APN, whereas HCT116, colon cancer, and MDA-MB-231, human breast cancer cells do not express APN (Fig. 4B). So, we examined the *in vivo* APN activity of HT1080 cells treated with various concentrations of BetA. BetA did not inhibit *in vivo* APN activity in HT1080 cells, while bestatin potently inhibited the enzyme activity (Fig. 4C). These data demonstrate that BetA is not an *in vivo* inhibitor of APN in tumor or endothelial cells.

BetA strongly decreases mitochondrial reducing potential in endothelial cells Several investigations have

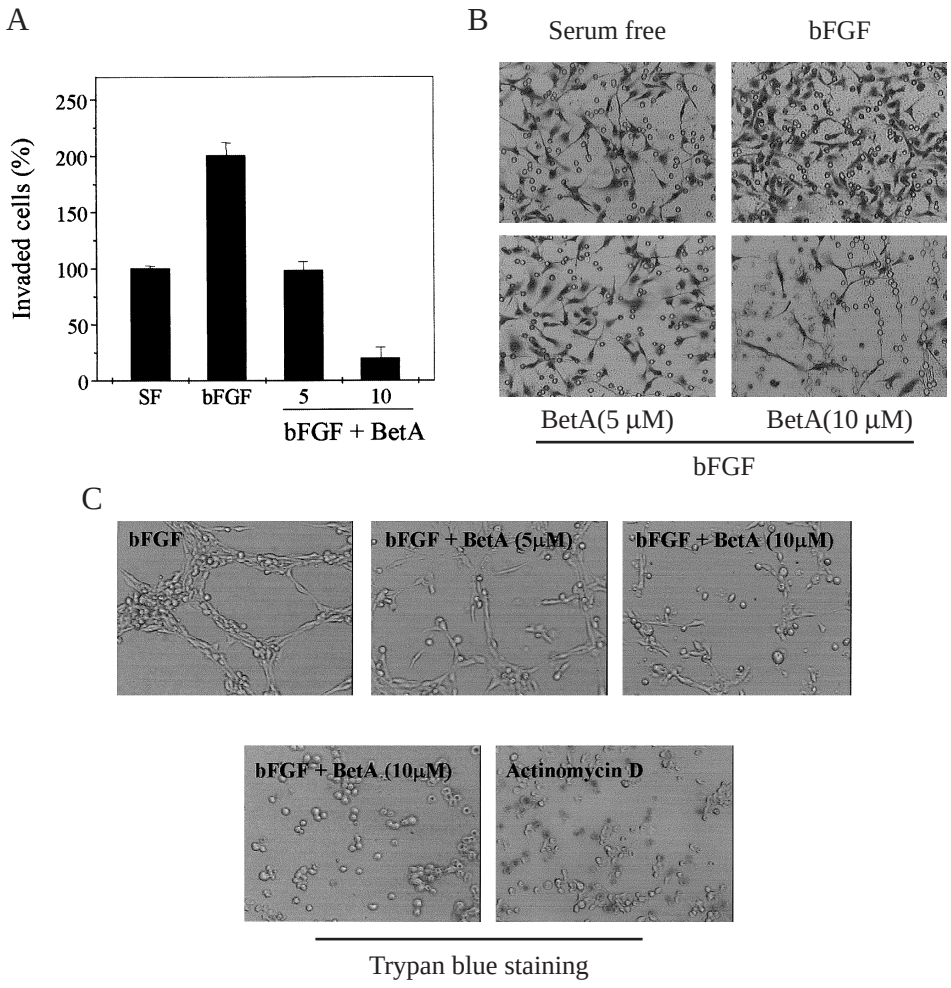

Fig. 2. *In vitro* anti-angiogenic activity of BetA. (A) Effect of BetA on the basic fibroblast growth factor (bFGF)-induced invasion of BAECs. Serum-starved cells left untreated in serum-free medium (SF) or treated with BetA were treated with bFGF. (B) Microscopic observation of invaded BAECs. (C) Effect of BetA on the capillary tube formation of BAECs. Cytotoxic effect of BetA on tube-formed BAECs was evaluated by trypan blue staining. Actinomycin D, a cytotoxic drug, was used as a control. Figures were selected as representative scenes from two independent experiments.

shown that mitochondria are likely to be a direct target for BetA in tumor cells.[6–8] This effect of BetA on the mitochondrial function may contribute to certain changes in endothelial cell metabolism. Therefore, we next investigated the effect of BetA on the mitochondrial reducing potential in BAECs. The assay was performed with MTT, which is reduced by mitochondrial dehydrogenases, especially by succinate dehydrogenase.[19] BAECs treated with or without BetA and structurally related compounds including ursolic acid (UA) and dexamethasone (Dex), were incubated for 12 h and MTT was added to the cells. After 4 h, cells were observed under a microscope and MTT reduction was measured at 540 nm using a microplate reader. Cell viability assay using trypan blue staining was performed in parallel. BetA potently decreased MTT reduction in endothelial cells, while UA and Dex did not (Fig. 5, A and C). Microscopic observation of cells after MTT administration showed the mitochondrial localization in cells as black dots (Fig. 5B). Interestingly, cell proliferation was not affected at the same concentration of BetA

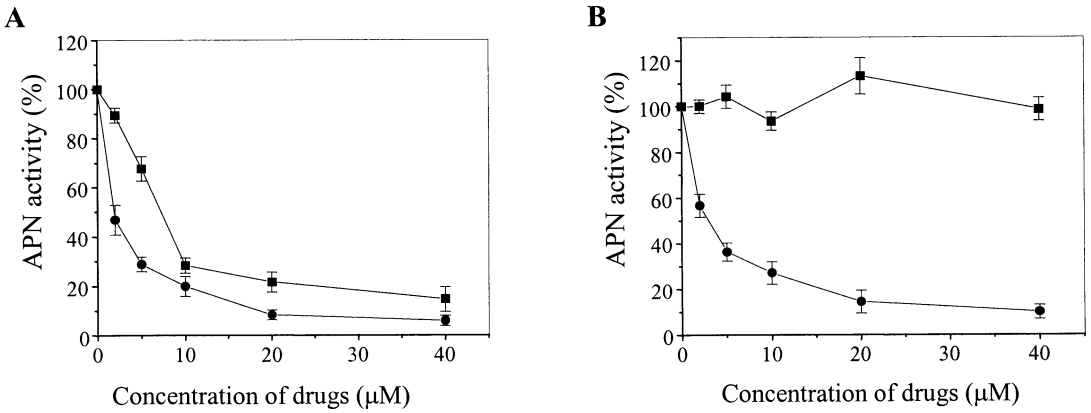

Fig. 3. Effect of BetA on the enzymatic activity of aminopeptidase N (APN). (A) *In vitro* analysis of APN activity. Commercially available leucine aminopeptidase was used as an enzyme source. Bestatin, a competitive inhibitor of APN, was used as a positive control. (B) *In vivo* analysis of APN activity. BAECs grown in culture plates were used as an enzyme source and APN activity was determined as described in "Materials and Methods." ■ BetA, ● Bestatin.

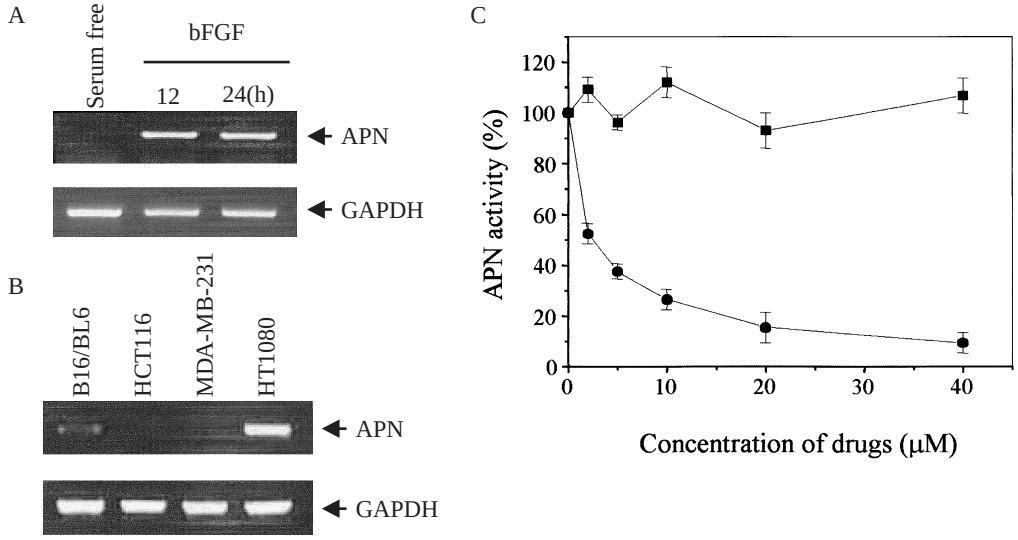

Fig. 4. Effect of BetA on *in vivo* APN activity in APN-positive tumor cells. (A) RT-PCR analysis of the expression of APN in endothelial cells with or without bFGF stimulation. (B) Analysis of APN expression in various tumor cells using RT-PCR. (C) Effect of BetA on *in vivo* enzymatic activity of APN in HT1080 cells. ■ BetA, ● Bestatin.

that showed a potent decrease in MTT reduction in endothelial cells. In addition, BetA-induced inhibition of MTT reducibility by endothelial cells was fully reversible (data not shown). These results suggest that BetA may affect the mitochondrial redox potential of endothelial cells in reversible manner without inhibiting cell proliferation.

Mitochondrial permeability transition (MPT) inhibitors attenuate BetA-induced inhibition of endothelial cell invasion It was previously observed that isolated mitochondria treated with BetA could induce an apoptotic cascade, including caspase activation and nuclear fragmentation.[6] These apoptotic events were preceded by the induction of mitochondrial permeability transition (MPT) and sequential loss of mitochondrial membrane potential ($\Delta\psi_m$). Several MPT inhibitors significantly inhibited BetA-induced mitochondria-derived apoptosis, suggesting that the compound is a potent inducer of MPT.[20] These effects of BetA on mitochondria may not be limited in tumor cells. Thus, we next examined the effect of MPT inhibitors including bongkrekic acid (BA), an inhibitor of adenosine nucleotide translocater,[21] cyclosporine A (CsA), a transient inhibitor of MPT,[22] and z-VAD-fmk, a broad range caspase inhibitor, on the BetA-induced inhibition of endothelial cell invasion. BAECs pretreated with MPT inhibitors were seeded on the upper chamber of Transwells in the presence or absence of BetA, and invasion assay was performed as described in "Materials and Methods." bFGF stimulated the invasion of BAECs and this invasiveness was potently inhibited by BetA (Fig. 6). Surprisingly, BA as well as CsA attenuated BetA-induced

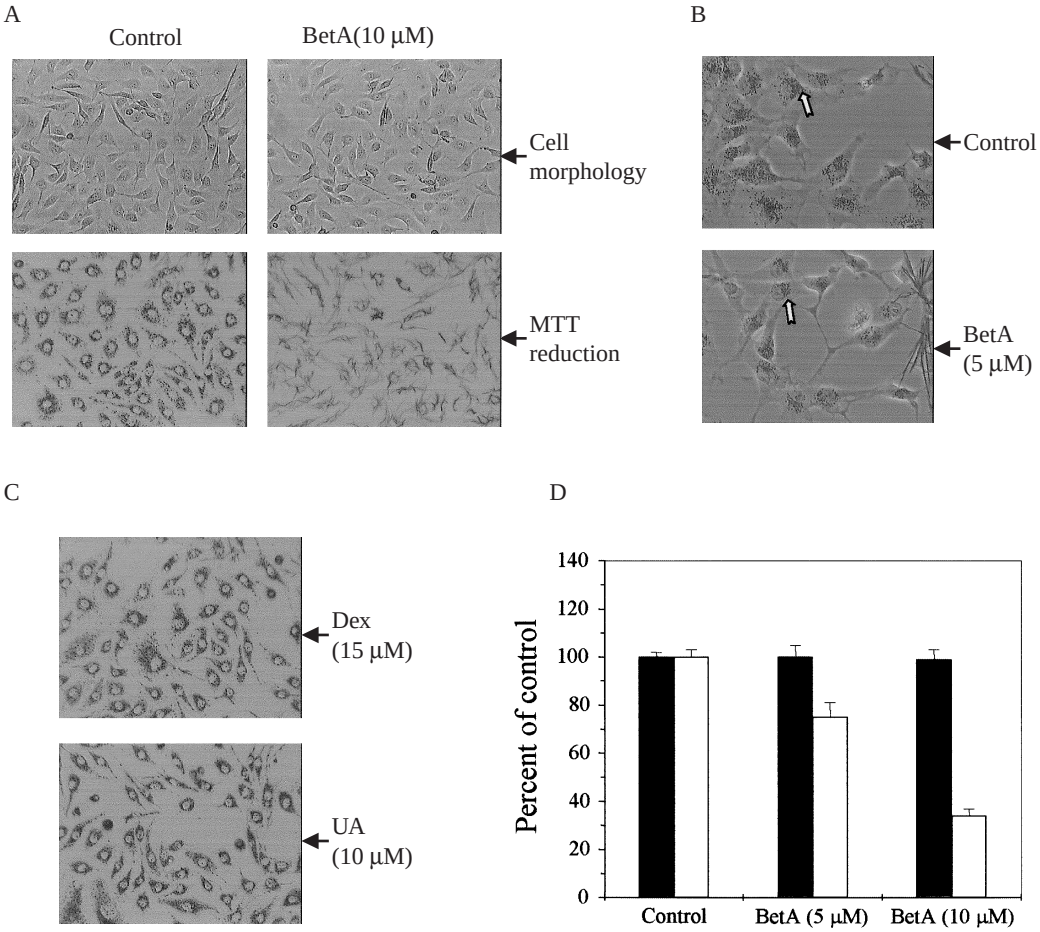

Fig. 5. Effect of BetA on the mitochondrial reducing potential in endothelial cells. (A) Effect of BetA on the morphology of BAECs and their capacity for MTT reduction. Cells treated with BetA for 12 h were exposed to MTT solution and observed under a microscope at ×100 magnification. In parallel, a group of cells was treated with BetA for 12 h and observed without addition of MTT solution. (B) Microscopic observation of MTT reduction in BAECs (×400 magnification). Black dots indicated by white arrows represent mitochondria localization in BAECs. BetA (5 μM) significantly reduced MTT reduction. (C) Effect of dexamethasone (Dex) or ursolic acid (UA) on the MTT reduction in BAECs. (D) Quantitative analysis of MTT reduction in BetA-treated BAECs using a 540 nm filter-equipped microplate reader. Cell viability was determined in parallel by trypan blue exclusion assay. ■ Cell viability, □ MTT reducibility.

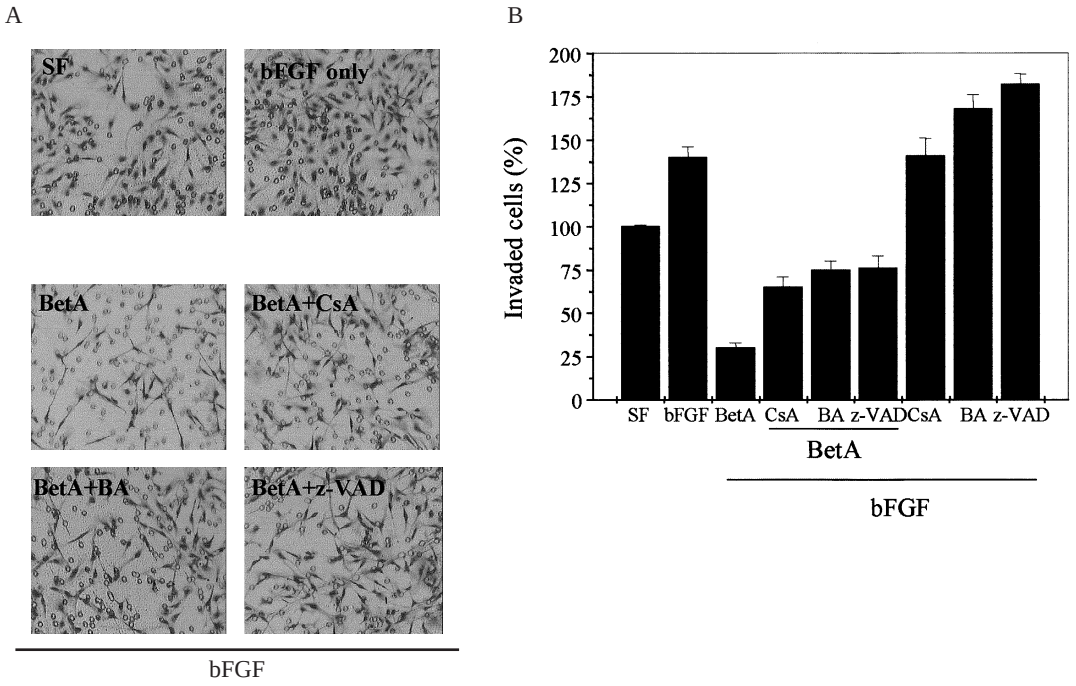

Fig. 6. Effect of mitochondrial permeability transition (MPT) inhibitors on BetA-induced inhibition of endothelial cell invasion. (A) BAECs pretreated with various MPT inhibitors including cyclosporine A (CsA) and bongkrekic acid (BA), and caspase inhibitor, z-VAD-fmk for 2 h were exposed to BetA and bFGF, and invasion assay was carried out. Invaded cells were observed under a microscope at ×100 magnification. (B) Invaded cells were counted under a microscope and analyzed quantitatively.

inhibition of BAECs invasion. z-VAD-fmk also partially prevented the effect of BetA. These results suggest that the anti-angiogenic activity of BetA arises, at least in part, through modulation of the mitochondrial function in endothelial cells.

DISCUSSION

Angiogenesis is a key process for the outgrowth of cancer cells and their spread into other tissues. Therefore, the specific inhibition of angiogenesis may be a powerful means to suppress angiogenesis-related diseases including cancer. Extensive studies have been carried out to identify the cellular target proteins for angiogenesis, and several of these target proteins have been identified, i.e., matrix metalloproteinases,[23] vascular endothelial growth factor receptors,[24, 25] methionine aminopeptidase-2,[26, 27] and histone deacetylases.[28, 29] APN has been highlighted recently by many investigators as having possible involvement in angiogenesis.[14, 15] In this respect, the specific inhibition of APN activity may be a novel approach to angiogenesis therapy.

The present data show that BetA selectively and potently inhibits growth factor-induced angiogenesis *in vitro*. Furthermore, BetA strongly inhibits *in vitro* enzymatic activity of APN. This result is consistent with the previous observation by Melzig and Bormann.[9] However, BetA does not inhibit *in vivo* enzymatic activity of APN in endothelial cells or APN-positive tumor cells. These results demonstrate that BetA is not an *in vivo* inhibitor of APN. Although, the reason for the different activity spectrum of BetA *in vivo* and *in vitro* is not clear, APN may not be related to the anti-angiogenic activity of the compound.

Several investigations have demonstrated that BetA might directly target mitochondrial function in various tumor cells.[6–8] Thus, it is probable that BetA directly affects the mitochondrial function in endothelial cells. The present data show that BetA strongly decreases the mitochondrial reducing potential in BAECs. In addition, several MPT inhibitors can attenuate the inhibition of angiogenesis induced by the compound. These data strongly support the idea that BetA may inhibit angiogenesis via the modulation of mitochondrial function in endothelial cells.

The MPT pore is known to possess several redox-sensitive sites.[30] An enhanced generation of reactive oxygen species could induce changes in cellular redox potential.[31] These changes, including depletion of nonoxidized glutathione or of NADPH, facilitate MPT. In this respect, the

BetA-induced decrease in mitochondrial reducing potential may facilitate MPT in endothelial cells. The induction of MPT in cells may cause the release of apoptogenic factors that can directly trigger cell death.[32] However, these apoptogenic factors may not contribute the anti-angiogenic activity of the compound, since the BetA-induced decrease in the mitochondrial reducing potential is fully reversible and results in no significant inhibition of the cell viability.

On the other hand, Lee *et al.* reported very recently that pyruvate, the end metabolite of glycolysis could induce angiogenesis both *in vivo* and *in vitro*.[33] This study shows that an increase in mitochondrial oxidative phosphorylation can enhance angiogenic differentiation of endothelial cells. Thus, it is possible that the effect of BetA on MPT may cause the reversible inhibition of the mitochondrial respiration. To investigate this possibility, we examined the effect of BetA on the enzymatic activity of succinate dehydrogenase (SDH), a component of mitochondrial respiratory chain complex II. BetA did not significantly inhibit SDH activity *in vivo* or *in vitro* (data not shown), suggesting that SDH is not associated with the inhibition of mitochondrial reducing potential and the inhibition of angiogenesis by BetA. Further investigations of the effect of BetA on other mitochondrial respiratory complexes and dehydrogenases are needed to account for the anti-angiogenic activity of the compound.

In conclusion, betulinic acid potently inhibits growth factor-induced angiogenesis, at least in part through the modulation of mitochondrial function in endothelial cells.

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