Xylitol inhibits *in vitro* and *in vivo* angiogenesis by suppressing the NF-κB and Akt signaling pathways

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Abstract. Angiogenesis is an important process involved in tumor growth and metastasis. Many studies have investigated the use of natural compounds such as angiogenic inhibitors. Xylitol is a 5-carbon sugar alcohol and is an artificial sweetener that has been used in chewing gums to prevent tooth decay. Xylitol has been also known to inhibit inflammatory cytokine expression induced by lipopolysaccharide (LPS). Since angiogenesis and inflammation share a common signaling pathway, we investigated the role of xylitol in angiogenesis. Xylitol inhibited the migration, invasion and tube formation of human umbilical vein endothelial cells (HUVECs). Xylitol also inhibited *in vivo* angiogenesis in a mouse Matrigel plug assay. Furthermore, mRNA expression of vascular endothelial growth factor (VEGF), VEGFR-II (KDR), basic fibroblast growth factor (bFGF), bFGFR-II, matrix metalloproteinase-2 (MMP-2) and MMP-9 of HUVECs decreased following treatment with xylitol. These anti-angiogenic effects of xylitol are exerted through inhibition of NF-κB and Akt activation. Taken together, these results suggest that xylitol acts as a beneficial angiogenesis inhibitor.

Introduction

Angiogenesis is the development of new capillaries from pre-existing vasculature during tumor growth and metastasis (1,2) and is regulated by the balanced action of angiogenic activators and inhibitors (3,4). This process is complex and involves diverse cellular actions, such as degradation of the extracellular matrix (ECM), proliferation and migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes (5).

Angiogenesis and inflammation are important processes involved in tumor growth and expansion (6-8). Natural compounds with anti-inflammatory properties can also have anti-angiogenic activities since these processes share a common signaling pathway such as NF-κB and Akt (9,10). One of these natural compounds is xylitol. Xylitol, first derived from Birch trees in Finland in the 20th century (11), is a 5-carbon sugar alcohol (12) (Fig. 1). It has been widely used as a sugar substitute and is found in the fibers of many fruits and vegetables (13). Xylitol can also be used as an artificial sweetener in chewing gums to prevent tooth decay (14,15).

In a recent study, xylitol was shown to inhibit lipopolysaccharide (LPS)-induced inflammatory cytokine expression (16). Because inflammation and angiogenesis share a common signaling pathway (17,18), we tested whether xylitol has anti-angiogenic effects by using *in vitro* and *in vivo* angiogenesis assays. We found that xylitol suppressed tube formation, migration and invasion of human umbilical vein endothelial cells (HUVECs). Xylitol also inhibited *in vivo* angiogenesis in a Matrigel plug assay. Additionally, xylitol downregulated the mRNA expression of vascular endothelial growth factor (VEGF), VEGFR-II (KDR), basic fibroblast growth factor (bFGF), bFGFR-II, matrix metalloproteinase-2 (MMP-2) and MMP-9 of HUVECs. We also found that xylitol suppressed NF-κB and Akt activation in HUVECs.

Materials and methods

Materials and reagents. HUVECs were purchased from InnoPharmaScreen (Chungnam, Korea). Matrigel was obtained from Collaborative Biomedical Products (Bedford, MA, USA) for the mouse Matrigel plug assay. Basic fibroblast growth factor (bFGF) and heparin were obtained from PeproTech (Gaithersburg, MD, USA). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from JBI (Daegu, Korea). Drabkin reagent kit 525 was purchased from Sigma (St. Louis, MO, USA). The 8-μm pore Transwell filter chambers were purchased from Corning-Costar (Corning, NY, USA). Antibodies for NF-κB, phosphor-NF-κB, Akt, and phosphor-Akt were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Xylitol and gelatin were purchased from Sigma.

HUVECs. HUVECs were grown in M199 supplemented with heat-inactivated 20% fetal bovine serum (JBI), 20 ng/ml of bFGF, 10 U/ml of heparin, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a 37˚C incubator with a humidified atmosphere containing 5% CO_2.

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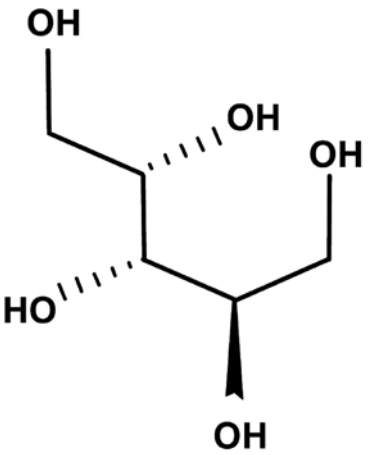

Figure 1. Structure of xylitol.

Animals. Seven-week-old, specific pathogen-free (SPF) male C57BL/6 mice were supplied by Hyochang Science and Samtako (Daegu and Kyung-gi, 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 xylitol. Matrigel culture was incubated at 37˚C. After 6 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 was 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 xylitol. 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 Transwell 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 xylitol in serum-free medium was placed in the upper part of the filters, and full medium was treated in the lower parts. Cells were incubated at 37˚C for 24 h, fixed with methanol, and then stained with hematoxylin and eosin. 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, Bedford, MA, USA) containing bFGF (100 ng/ml) and heparin (50 U/ml) without or with xylitol. 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, Sungnam, Korea). Amplification products were electrophoresed on 1% 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 xylitol 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.

Results

Xylitol inhibits vascular network formation in HUVECs. Because differentiation of endothelial cells into a capillary-like network is important for the process of angiogenesis, we tested the effect of xylitol on the morphological differentiation of endothelial cells *in vitro*. HUVECs were placed on a Matrigel-coated plate and incubated. Endothelial cells formed weak capillaries on Matrigel beds, and these tubes became stronger and more robust with elongated networks over 6-24 h. HUVECs on Matrigel formed a blood vessel-like network in the absence of xylitol (Fig. 2), whereas treatment with xylitol for 18 h resulted in broken, shortened and narrow tube

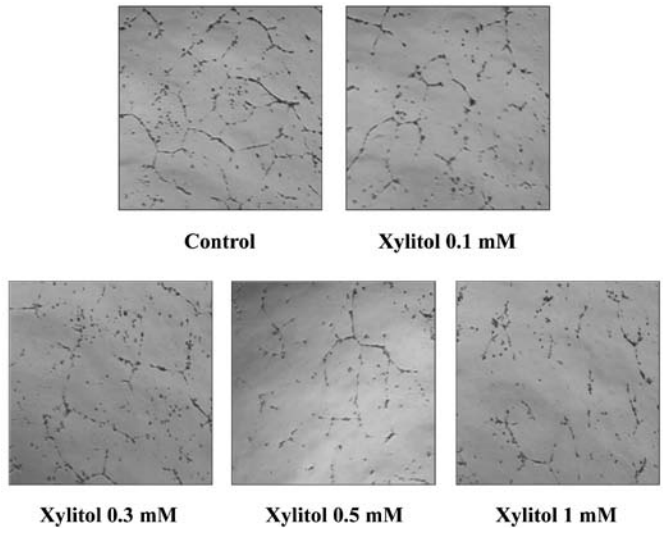

Figure 2. Xylitol inhibits tube formation of HUVECs. The effect of xylitol on tube formation of HUVECs was examined using an *in vitro* tube formation assay. Indicated amounts (0.1, 0.3, 0.5 and 1 mM) of xylitol were added and the cells were incubated for 18 h. Changes in cell morphology were observed using a phase contrast microscope (x40) and photographed. Representative photographs of tube formation of endothelial cells cultured on polymerized Matrigel layers revealed the inhibitory effect of xylitol on the formation of capillary-like structures. This experiment was repeated three times.

networks. This result shows that xylitol inhibits tube formation of HUVECs.

Xylitol inhibits migration and invasion activity in HUVECs. Migration and invasion in endothelial cells is a critical feature in forming new blood vessels and repairing injured vessels (19,20). Thus, we examined the effect of xylitol on the movement of HUVECs from a wounded edge to the open area by using a wound migration assay. Exposure to xylitol for 20 h significantly decreased HUVEC migration compared with that of control cells in a dose-dependent manner (Fig. 3A). To examine the effect of xylitol on HUVEC invasiveness, we performed an invasion assay by using a Transwell system. As shown in Fig. 3B, xylitol suppressed HUVEC invasiveness compared with that of control cells in a dose-dependent manner after 24 h of incubation. These inhibitory activities of xylitol on the migration and invasion of HUVECs indicate that xylitol suppresses *in vitro* angiogenesis.

Xylitol inhibits in vivo angiogenesis. To examine the effect of xylitol on *in vivo* angiogenesis, we performed a mouse Matrigel plug assay, an established *in vivo* angiogenesis model. As shown in Fig. 4A, Matrigel plugs containing bFGF were abundantly filled with intact red blood cells, indicating formation of a functional vasculature inside the Matrigel, whereas vessels were not

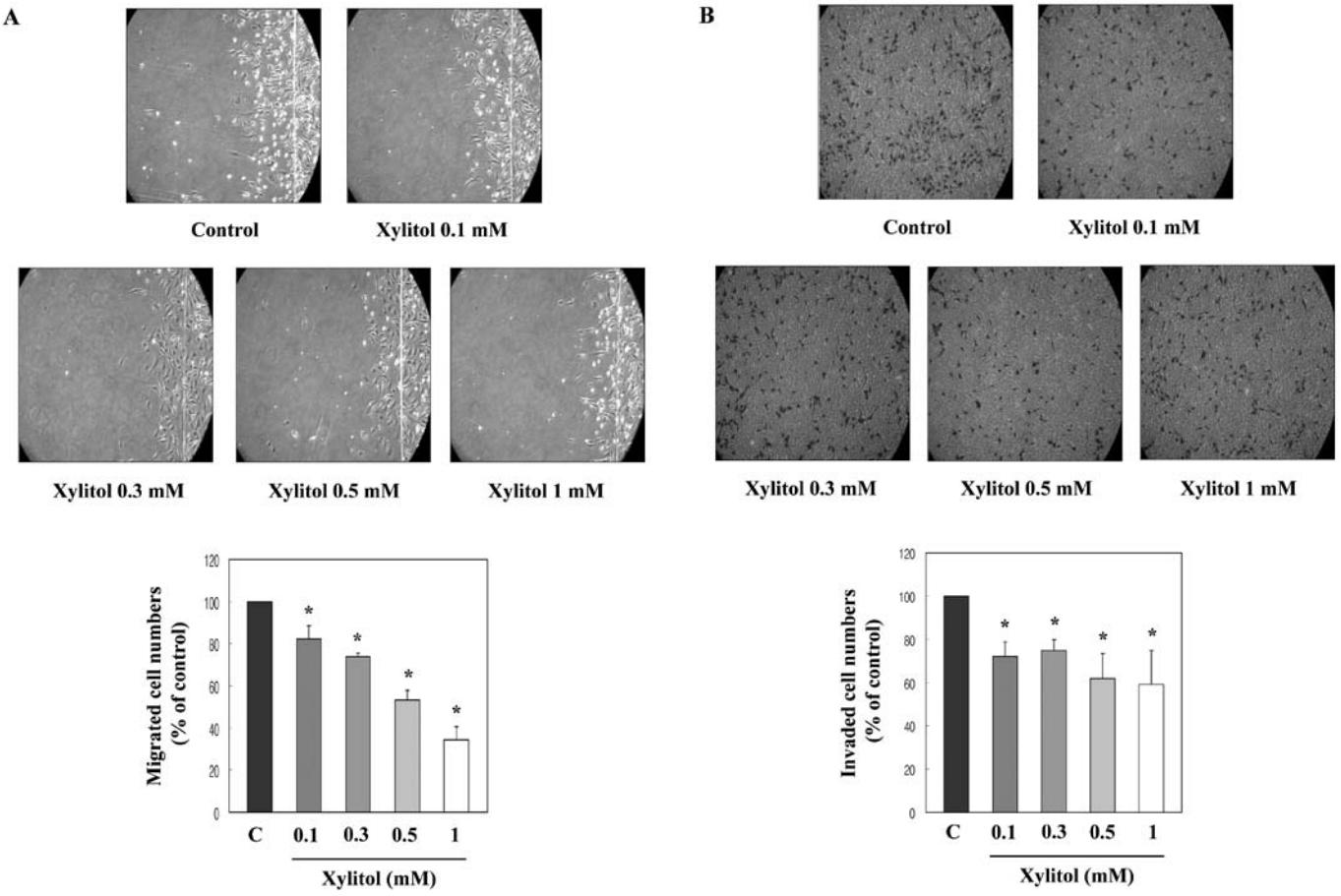

Figure 3. Xylitol inhibits HUVEC migration and invasion. (A) Migration ability of HUVECs was measured using a wound migration assay. (B) Invasion capacity was examined using the Transwell system in which the wells were coated with Matrigel. Data are mean ± SD of three independent experiments performed in triplicate. $^{*}p<0.05$ vs. control.

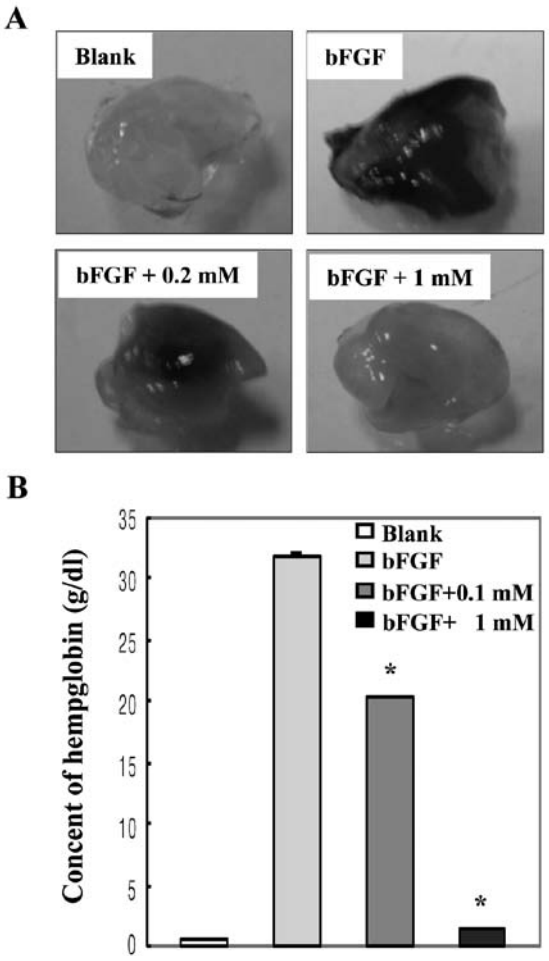

Figure 4. Xylitol suppressed *in vivo* angiogenesis in mouse Matrigel plug assay. (A) Matrigel plugs were photographed (x40). (B) Quantification of hemoglobin content. Each value represents the mean ± SD of at least five animals, and the experiment was performed twice. Blank, Matrigel alone. $^{*}p<0.05$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

observed in the Matrigel alone (blank). Matrigel plugs containing xylitol produced fewer vessels compared with plugs containing bFGF, indicating that xylitol inhibits formation of bFGF-induced neo-microvessels. We then measured the hemoglobin content inside the Matrigel plugs to quantify the anti-angiogenic effect of xylitol. The amount of hemoglobin indicates the degree of formation of a functional vasculature inside the Matrigel. The hemoglobin content of bFGF-treated plugs was 31.8 g/dl, whereas that of 1 mM xylitol-treated plugs was profoundly lowered to approximately 1.4 g/dl (Fig. 4B). These results indicate that xylitol has strong anti-angiogenic activity *in vivo*.

Xylitol downregulates mRNA expression of angiogenesis-related genes. To determine which molecules are involved in the anti-angiogenic activity of xylitol, we examined mRNA expression of angiogenic factors and their receptors in HUVECs following xylitol treatment by using RT-PCR. As shown in Fig. 5A, VEGF mRNA expression was significantly reduced in the presence of xylitol. VEGFR-II (KDR) mRNA expression was also downregulated following treatment with xylitol in a dose-dependent manner. mRNA expression of other angiogenic molecules, including bFGF and bFGR-II, was remarkably reduced by xylitol (Fig. 5B).

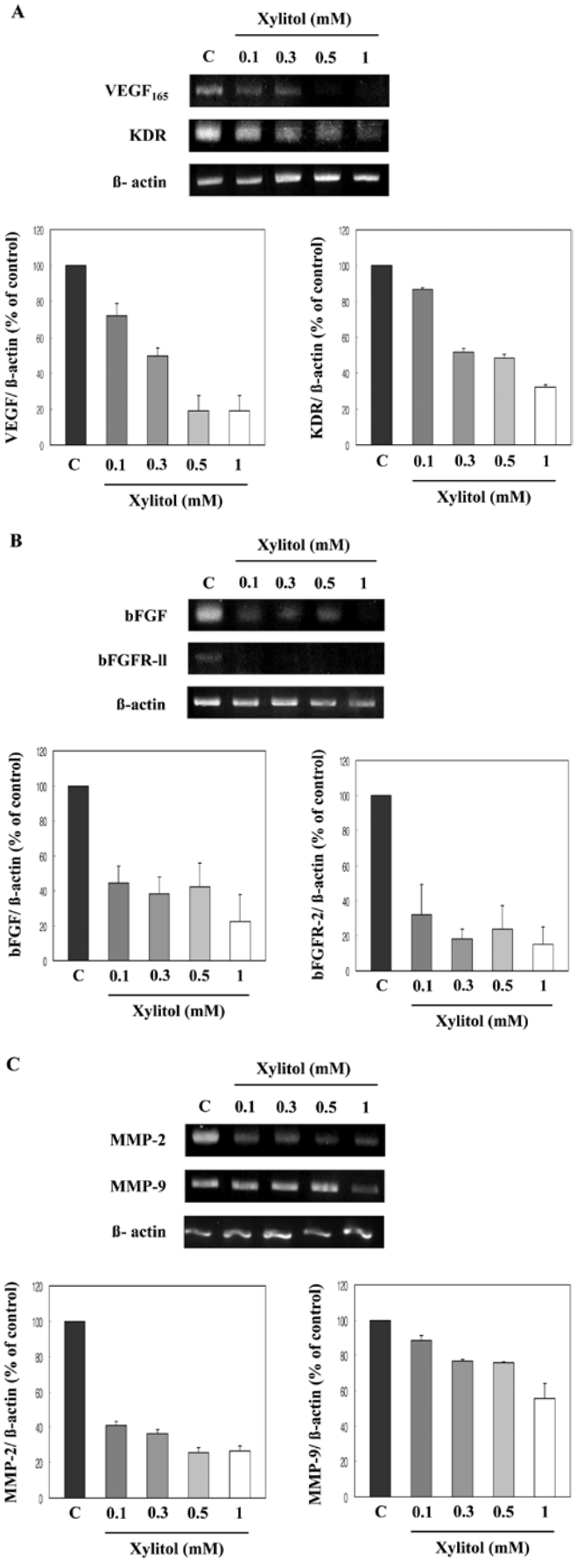

Figure 5. Xylitol downregulated angiogenesis-related gene expression. mRNA expression in xylitol-treated HUVECs was analyzed using RT-PCR. (A) VEGF and KDR, (B) bFGF and bFGFR-II, (C) MMP-2 and MMP-9. Amplification of β-actin demonstrates comparable RNA amount and quantity among samples.

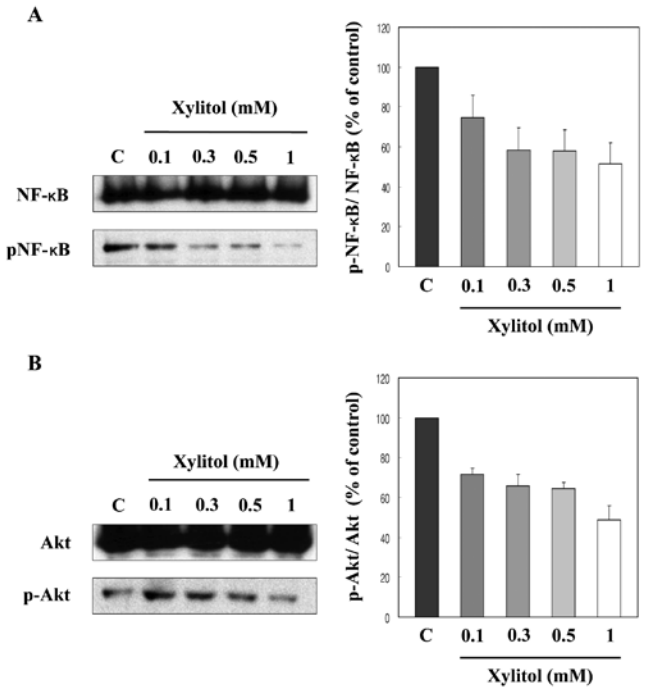

Figure 6. Downregulation of NF-κB and Akt activation by xylitol in HUVECs. HUVECs were treated with the indicated amounts of xylitol for 24 h. Total proteins were prepared and analyzed by western blot analysis for each primary antibody. (A) Phosphor-Akt and total Akt. (B) Phospho-NF-κB and total NF-κB. Each value was normalized to the total form and α-tubulin.

Regulation of extracellular proteolytic activity is important for endothelial cell migration and invasion, and MMP-2 and MMP-9 are major functional molecules involved in the degradation of the extracellular membrane (21). Thus, we examined the effect of xylitol on the mRNA expression of MMP-2 and MMP-9. Expression of both molecules was markedly downregulated following treatment with xylitol in a dose-dependent manner (Fig. 5C). These data suggest that downregulation of VEGF, KDR, bFGF, bFGFR-II, MMP-2 and MMP-9 mRNA expression may be responsible for the anti-angiogenic action observed in HUVECs treated with xylitol.

Xylitol suppresses NF-κB and Akt activation of HUVECs. NF-κB and Akt are the main signaling pathways involved in tumor progression and angiogenesis (22,23). Therefore, we examined the effect of xylitol on NF-κB and Akt activation in HUVECs. In this experiment, total proteins were prepared to detect NF-κB and Akt phosphorylation and then analyzed by western blot analysis. As shown in Fig. 6A, NF-κB phosphorylation was suppressed by xylitol in a dose-dependent manner. Because NF-κB activation is known to be mediated by the Akt signal pathway, we then examined Akt activation (17,24). Akt phosphorylation was inhibited in the presence of xylitol in a dose-dependent manner (Fig. 6B). These results indicate that xylitol interferes with NF-κB and Akt phosphorylation in HUVECs.

Discussion

Angiogenesis is required for tumor growth and expansion. Angiogenesis is the formation of new blood vessels from existing vessels; these processes involve several cascades (19,20,25). Inflammation is also known to be a central process in many cancers during tumorigenesis (26). Therefore, signal transduction of angiogenesis and inflammation can occur simultaneously (27).

Xylitol was first derived from Birch trees in Finland, and it is used as a safe sweetener for diabetic patients (11). Xylitol is obtained from natural sources, including plums, strawberries, raspberries, and rowanberries (12,28,29). It is used in various food products such as chewing gum, candy and soft drinks (30). Xylitol has also been used to prevent tooth decay since it inhibits the growth of *Streptococcus mutans* (31,32).

Recently, xylitol was reported to have an inhibitory effect on LPS-induced inflammatory cytokine expression (16). Since inflammation and angiogenesis share a common signaling pathway (17,18), molecules that show anti-inflammatory activity also show anti-angiogenic activity (8,27). Therefore, we examined whether xylitol has anti-angiogenic effects by using *in vitro* and *in vivo* angiogenesis assays. In this study, we found that xylitol inhibited *in vitro* and *in vivo* angiogenesis. Xylitol strongly inhibited tube formation (Fig. 2), migration (Fig. 3A), and invasion (Fig. 3B) of HUVECs. Xylitol prominently inhibited the formation of neo-microvessels in the Matrigel assay (Fig. 4A), and reduced the hemoglobin content in the Matrigel plug (Fig. 4B). These results suggest that xylitol suppresses angiogenesis both *in vitro* and *in vivo.*

Tumors produce various angiogenic molecules during the angiogenesis process. VEGF, bFGF and their receptors are well known as the main stimuli for angiogenesis (33-35). Therefore, we examined the involvement of xylitol in the expression of major angiogenic factors and their receptors. Xylitol decreased the mRNA expression of key angiogenic molecules and their receptors (VEGF, KDR, bFGF and bFGFR-II) in a dose-dependent manner (Fig. 5).

MMPs are secreted as proenzymes and they regulate extracellular matrix degradation. Regulation of extracellular proteolytic activity is important for cell migration and invasion (21). Thus, we tested the effect of xylitol on mRNA expression of MMP-2 and MMP-9 by using RT-PCR. As shown in Fig. 5C, xylitol decreased the mRNA expression of both MMP-2 and MMP-9.

NF-κB and Akt comprise a multiunit transcription factor that plays a central role in tumorigenesis and is involved in tumor cell invasion and metastasis (24,36). We examined the effect of xylitol on NF-κB and Akt activation by western blot analysis. Protein expression of NF-κB and Akt did not change following treatment with xylitol; however, NF-κB and Akt phosphorylation was suppressed in a dose-dependent manner (Fig. 6).

In summary, we found that xylitol inhibited *in vitro* and *in vivo* angiogenesis. These effects of xylitol are linked with the mRNA expression of VEGF, KDR, bFGF, bFGFR-II, MMP-2 and MMP-9. Xylitol inhibited angiogenesis by inhibiting the NF-κB and Akt signal pathway. Furthermore, xylitol is widely used as a sugar substitute as well as an artificial sweetener in chewing gums to prevent tooth decay. Therefore, xylitol may be a promising candidate as an inhibitor of angiogenesis-related diseases.

Acknowledgements

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