Midkine, a heparin-binding growth factor, promotes growth and glycosaminoglycan synthesis of endothelial cells through its action on smooth muscle cells in an artificial blood vessel model

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Accepted 26 April 2002
Journal of Cell Science 115, 2659-2667 (2002) © The Company of Biologists Ltd

Summary

To study the interactions between smooth muscle cells and endothelial cells in vitro, we developed an artificial blood vessel model, which consisted of collagen gel containing human aortic smooth muscle cells and human umbilical vein endothelial cells grown on the gel. The blood vessel model was utilized to investigate the role of midkine, a heparin-binding growth factor, in the intercellular interactions that are important in angiogenesis. In the blood vessel model, midkine induced stratification of the endothelial cells and increased their proliferation and glycosaminoglycan synthesis. However, midkine had no effect on the smooth muscle cells or endothelial cells when they were cultured separately. Increased proliferation of the endothelial cells was also attained by coculturing them with smooth muscle cells in the presence of midkine or culturing endothelial cells with the conditioned medium of the smooth muscle cells, which had been treated with midkine. These experiments indicate that the target of midkine was smooth muscle cells, which secreted factor(s) acting on the endothelial cells. We identified interleukin-8 as one such factor; the synthesis of interleukin-8 by the smooth muscle cells was increased by exposure to midkine, and anti-interleukin-8 inhibited the midkine action. Furthermore, interleukin-8 caused stratification of the endothelial cells in the blood vessel model. These results provided evidence that midkine is one of the factors involved in epithelial-mesenchymal interactions.

Key words: Midkine, Collagen gel, Endothelial cell, Interleukin-8, smooth muscle cell

Introduction

Epithelial-mesenchymal interactions play fundamental roles in organogenesis and oncogenesis (Arias, 2001). Although several growth factors, such as fibroblast growth factors and bone morphogenic proteins, have been revealed as key regulators of epithelial-mesenchymal interactions, the factors so far identified are limited in number (Arias, 2001).

Angiogenesis is one example of epithelial-mesencymal interactions, in which endothelial cells and smooth muscle cells interact with each other. L'Heureux et al. reported that they made an artificial blood vessel equivalent constructed with human vascular smooth muscle cells, endothelial cells and fibroblasts (L'Heureux et al., 1993). Their tissue-like structure was obtained by the contraction of a tubular collagen gel by vascular smooth muscle cells, generating a media-like structure, and endothelium was established within the tubular structure after intraluminal cell seeding. We modified their artificial blood vessel equivalent, and developed an artificial blood vessel model (BVM), which is described in this paper. Our BVM was made up of collagen gel populated with human aortic smooth muscle cells (HASMC) and human umbilical vein endothelial cells (HUVEC), seeded on the gel. This model enables simple construction of a blood vessel-like structure, and may be used to examine the activities of exogenously added factors in tissue interactions.

Here, we utilized the BVM to analyze the role of midkine (MK), a heparin-binding growth factor (Kadomatsu et al., 1988; Tomomura et al., 1990), in interactions between HASMC and HUVEC. MK promotes angiogenesis (Choudhuri et al., 1997), neurite outgrowth (Muramatsu et al., 1993), survival of neurons (Owada et al., 1999), cell growth (Muramatsu and Muramatsu, 1991), fibrinolysis (Kojima et al., 1995) and cell migration (Takada et al., 1997; Maeda et al., 1999; Horiba et al., 2000). MK has been suggested to have roles in epithelial-mesenchymal interactions in two systems. First, anti-MK antibody inhibited development of tooth germ in vitro (Mitsiadis et al., 1995a). Second, during branching morphogenesis of embryonic lung in vitro, MK added to the medium enhanced mesenchymal development (Toriyama et al., 1996). However, whether MK is involved in inter-tissue interactions remains to be clarified.

Materials and Methods

Materials

Human MK produced by yeast *Pichia pasrotis* (Ikematsu et al., 2000)

was a gift from S. Sakuma, Meiji Milk Co. (Kanagawa, Japan). Human interleukin-8 (IL-8), anti-human IL-8 antibody, ELISA kits for human hepatocyte growth factor (HGF), human basic fibroblast growth factor (bFGF) and human vascular endothelial growth factor (VEGF) were obtained from R&D systems Inc (Minneapolis, MN). Anti-human protein tyrosine phosphatase ζ (PTPζ) antibody was obtained from BD Transduction Laboratories (Lexington, KY). Anti-mouse LDL-receptor-related protein antibody was prepared as follows. cDNA encoding the N-terminal portion of mouse LRP (879 bp, nucleotide number 505-1383, GenBank accession number X67469) was ligated into the expression vector pGEX-5x-1(Pharmacia Biotech AB). LRP-GST (glutathione-S-transferase) fusion protein was purified by glutathione-Sepharose column chromatography and SDS-PAGE followed by electroelution as described previously (Salama et al., 2001). The LRP-GST fusion protein was produced by immunizing a New Zealand white rabbit, and the antibody was affinity purified on a 2 ml Sepharose 4B column coupled with 10 mg of the fusion protein. Anti-human MK antibody was prepared as described previously (Muramatsu et al., 1996). Chondroitin 4-sulfate, chondroitin sulfate E, dermatan sulfate, heparin, chondroitinase ABC and heparitinase I and II were purchased from Seikagaku Kogyo Co. (Tokyo, Japan).

Culture of HASMC and HUVEC

HASMC and HUVEC were purchased from Kurabo (Tokyo, Japan). HASMC were maintained in HuMedia-SG (Kurabo) supplemented with 5% fetal calf serum (FCS; Hyclone, Logan, UT), 1 ng/ml amphotericin B, 1 ng/ml gentamycin, 1 ng/ml human recombinant epidermal growth factor (EGF), 2 ng/ml bFGF and 5 μg insulin. HUVEC were maintained in HuMedia-KG (Kurabo), supplemented with 2.5% FCS, 1 ng/ml amphotericin B, 1 ng/ml gentamycin, 10 ng/ml EGF, 5 μg/ml bFGF, 1 ng/ml hydrocortisone and 10 μg/ml heparin. They were grown at 37°C in an atmosphere of 5% CO_2/95% air. Cells were used for experiments between the third and fourth passage.

Preparation of BVM

Collagen gels containing HASMC were prepared according to the procedure described previously (Sumi et al., 2000). Briefly, medium with 0.2% collagen was prepared by mixing 0.3% pepsin-processed type I atelocollagen solution (Koken Co. Ltd., Tokyo, Japan), six-fold concentrated minimal essential medium (MEM) (Gibco BRL Life Technologies Inc., Rockville, MD) and FCS at a ratio of 4:1:1 (V/V/V). HASMC were dispersed with 0.05% trypsin and 0.02% EDTA (Gibco) in Dulbecco's phosphate buffered saline (PBS). Cells were suspended at a density of 1.0×10^5 cells/ml in the medium with 0.2% collagen solution and dispensed at 3.0 ml/dish into six-well tissue culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). After incubation at 37°C in 5% CO_2/95% air for 2 hours, cultures were carefully washed twice with Dulbecco's modified Eagle's medium (DMEM) (Gibco) and then with 1 ml of DMEM supplemented with 10% FCS.

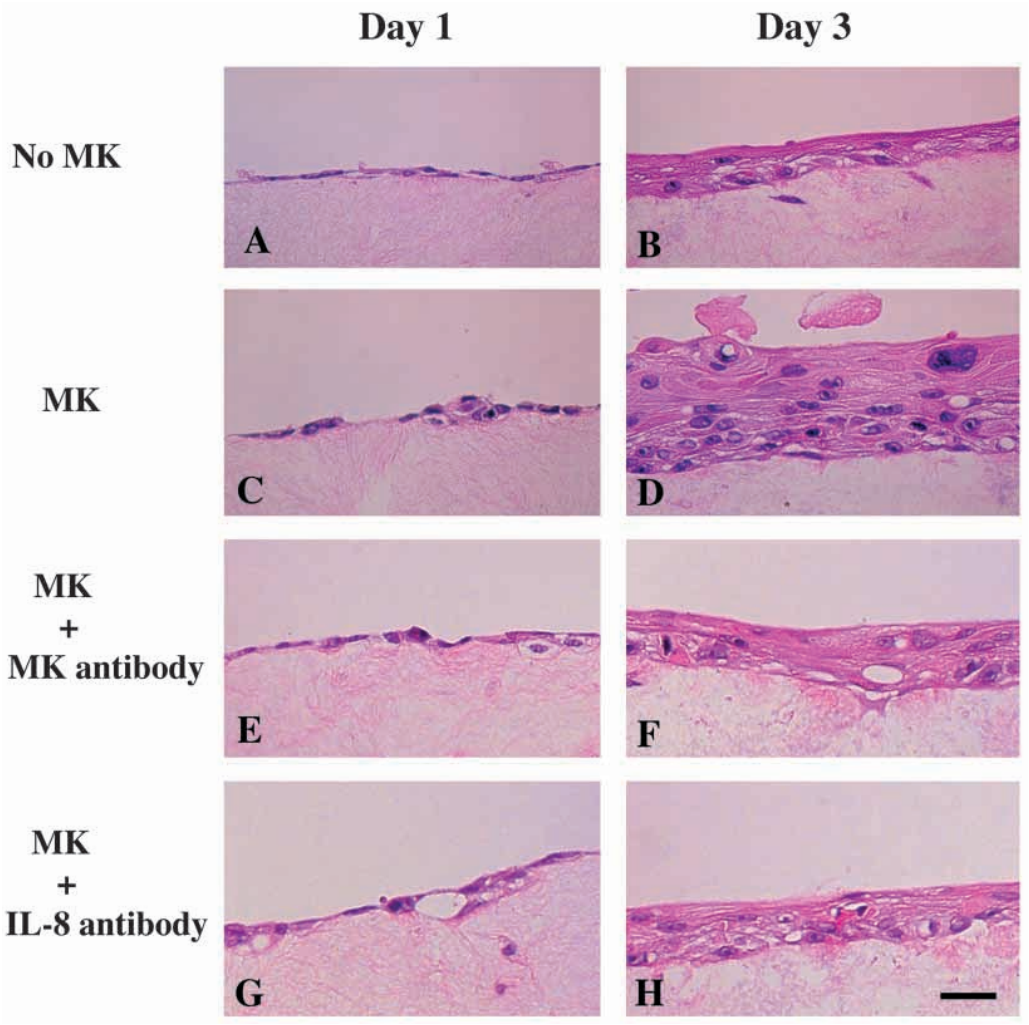

Fig. 1. Morphologies of BVMs cultured with or without MK. BVMs were cultured in DMEM-FCS for 1 day (24 hours) (A,C,E,G) or 3 days (72 hours) (B,D,F,H) with (C-H) or without (A,B) 100 ng/ml of MK. For antibody inhibition studies, anti-MK antibody (90 μg/ml) (E,F) or anti-IL-8 antibody (30 μg/ml) (G,H) was added. Then, BVMs were embedded in paraffin, cut into 5 μm sections and stained with hematoxylin-eosin. (A,C) HUVECs were attached and formed a monolayer on the HASMC-populated collagen gel on Day 1. (B) HUVECs were attached and formed a monolayer and secreted extracellular matrix on the HASMC-populated collagen gel on Day 3. (D) HUVECs were stratificated on the HASMC-populated collagen gel on Day 3. The extracellular matrix was secreted in large quantities. Bar, 100 μm.

Table 1. Effects of MK on the number of HUVEC in BVMs

	Left section	Center section	Right section	Average
Control	67.2±2.87	42.0±4.30	77.8±3.73	62.3±4.47
MK	130.4±8.13	92.2±15.79	123.4±10.43	115.3±9.41
MK+MK Ab	79.5±2.75	60.3±5.34	76.0±3.56	71.9±8.72
MK+IL-8 Ab	79.8±2.75	59.0±6.98	76.8±2.50	71.8±9.59

The number of HUVEC in BVMs was counted horizontally in 1 mm in hematoxylin-eosin-stained sections. BVMs were cultured for 3 days with MK (100 ng/ml), MK (100 ng/ml)+MK antibody (MKAb; 90 μg/ml), MK (100 ng/ml)+IL-8 antibody (IL-8Ab; 30 μg/ml) and without it (Control). The number of samples was five. **P*<0.05, ***P*<0.001.

HUVEC were dispersed with 0.05% trypsin and 0.02% EDTA in PBS(–), and the cell density was adjusted to 1.0×10^6 cells/ml with DMEM supplemented with 5% FCS (DMEM-FCS), then seeded on HASMC gels, which were cultured for 7 days with DMEM-FCS at 1 ml/gel (1.0×10^6 cells). This complex was used as the BVM and was cultured at the air-liquid interface. The BVM was cultured with DMEM-FCS supplemented with MK (0, 10, 50, 100 and 200 ng/ml). The model was also cultured in DMEM supplemented with MK (100 ng/ml) or IL-8 (100, 500, 1000 ng/ml).

Cell proliferation assay

HUVEC and HASMC were maintained in the respective specific medium containing no supplemental factors except FCS. HASMC were seeded onto 96-well plates at a density of 0.5×10^4 cells per well, and HUVEC were seeded onto 96-well plates at a density of 1.0×10^4 cells per well.

To examine whether HASMC-derived soluble factors affect HUVEC proliferation, we employed a co-culture system with polycarbonate membrane (pore size, 0.4 μm) (Transwell 24-well culture plate, Corning Inc. NY). HASMC were seeded onto culture plates at a density of 5.0×10^4 cells per well and cultured for 24 hours with DMEM supplemented with 5% FCS. Then, HUVEC were seeded onto culture inserts at a density of 1.0×10^5 cells per well and cultured with DMEM supplemented with 5% FCS.

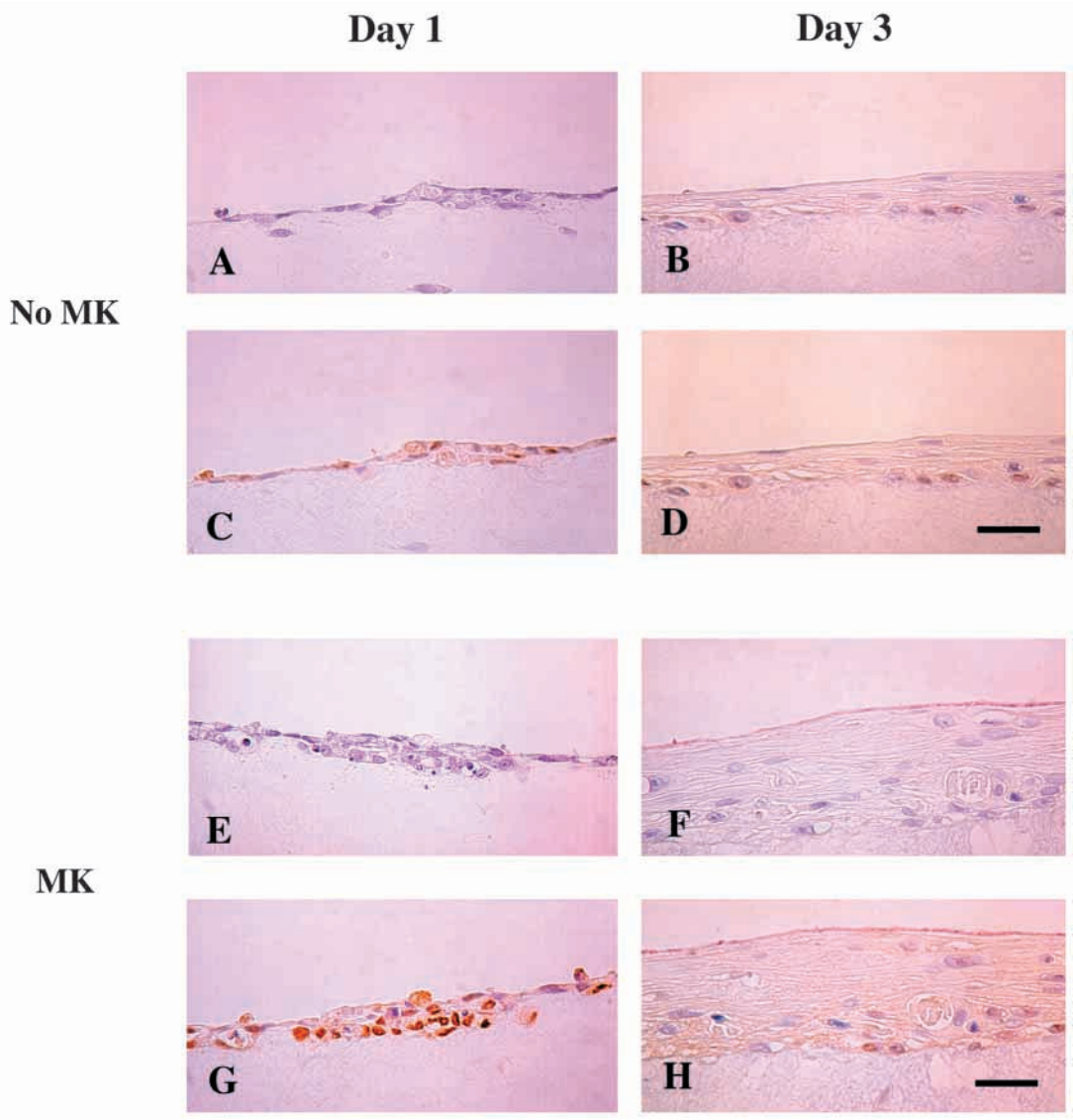

Fig. 2. Anti-PCNA immunostaining of BVMs. (A,B,E,F) Control staining without the first antibody on Day 1 (A,E) and on Day 3 (B,F). (C,D,G,H) Anti-PCNA immunostaining on Day 1 (C,G) and Day 3 (D,H). BVMS were either untreated (A,B,C,D) or cultured with 100 ng/ml of MK (E,F,G,H) as in Fig. 1.

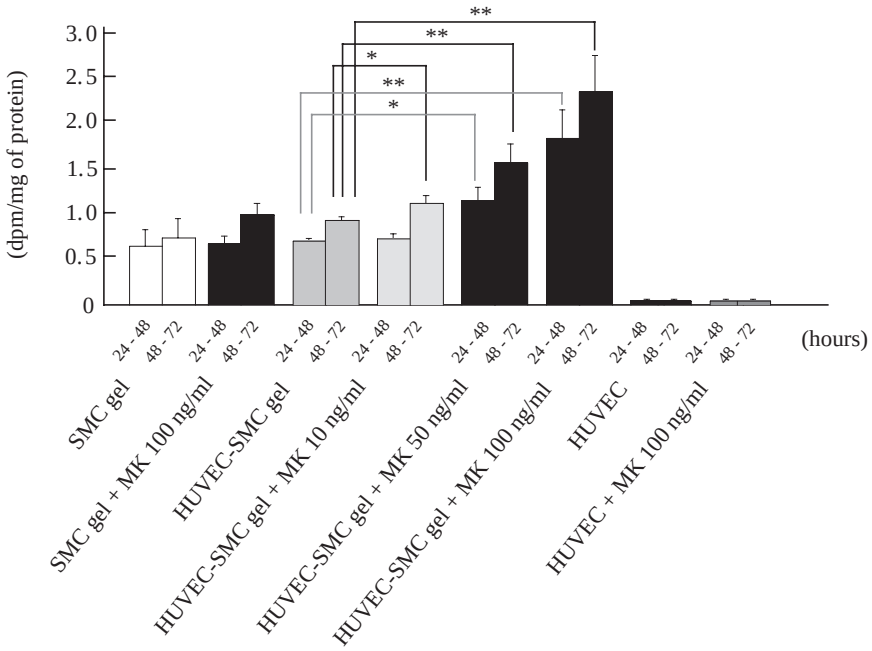

Fig. 3. [^{35}S]-labeled glycosaminoglycan synthesis by HUVEC treated with or without MK. Measurement of glycosaminoglycan synthesis was performed 24-48 hours and 48-72 hours after the start of culture. The number of samples in each experiment was six. *P<0.05; **P<0.01.

Cell numbers were assessed using a WST-1 cell counting kit (Wako Chemical Co., Tokyo, Japan), which is based on conversion of the tetrazolium compound to the formazan product by the cells, and numbers were determined at the indicated time points.

Preparation of HASMC-conditioned medium

Confluent HASMC in 10 cm dishes were incubated with 6 ml of DMEM for 48 hours at 37°C. The HASMC-conditioned medium was collected, centrifuged to remove dead cells and stored at –80°C.

Morphological observations

BVMs were cultured for 24 and 72 hours in DMEM supplemented with 5% FCS with or without MK (100 ng/ml). Then they were cultured for 24, 48 or 72 hours in serum-free DMEM with MK (0, 100 ng/ml) or IL-8 (0, 100, 500, 1,000 ng/ml) and fixed with 4% paraformaldehyde in PBS, pH 7.4. The BVMs were embedded in paraffin and cut into sections 5 μm thick vertically. Serial sections were mounted on slides, dried overnight and stored in an airtight box. Sections were stained with hematoxylin-eosin (H-E) or with a kit to detect proliferating cell nuclear antigen (PCNA) (DAKO, Kyoto, Japan).

Determination of sulfated glycosaminoglycan synthesis

Synthesis of sulfated glycosaminoglycans by BVMs was measured by [^{35}S] incorporation as described previously (Ohta et al., 1999). Briefly, we measured glycosaminoglycan synthesis at 24-48 hours and 48-72 hours after the start of culture. For the measurement of glycosaminoglycan synthesis at 24-48 hours, BVMs were cultured for 24 hours with DMEM supplemented with 5% dialyzed FCS and then incubated in medium supplemented with 0.5% dialyzed FCS containing 45 mCi/ml of [^{35}S] sulfate (Dupont NEN Research Products, Boston, MA) for 24 hours. In the measurement of glycosaminoglycan synthesis at 48-72 hours, BVMs were cultured for 24 hours with DMEM supplemented with 5% dialyzed FCS and incubated in medium supplemented with 0.5% dialyzed FCS for 24 hours. Then, these were incubated in medium supplemented with 0.5% dialyzed FCS containing 45 mCi/ml of [^{35}S] sulfate for 24 hours. After removal of the medium and washing with PBS five times, BVMs were incubated with 0.5 ml of 0.25% trypsin and 0.1% collagenase at 37°C for 30 minutes, and the resultant cell suspension was collected. The dishes were washed with 0.5 ml of PBS. The cell suspension and the washing solution were combined, and the cells were removed by centrifugation. Aliquots of 0.4 ml of supernatant from each BVM were digested with 1 mg of pronase (Wako Chemical Co.) for 3 hours at 37°C. The digests were mixed with 0.1 ml of 0.2 M NaCl containing 2 mg of chondroitin 4-sulfate as a carrier and 0.5 ml of 1.0% cetylpyridinium chloride (Wako Chemical Co.). Radioactivity in the precipitated cetylpyridinium-glycosaminoglycan complex was measured by liquid scintillation counting as described previously (Ohta et al., 1999). To determine incorporation into chondroitin sulfates or heparan sulfate, the pronase digests were boiled for 10 minutes and digested with 0.35 units of chondroitinase ABC or a mixture of 30 milliunits each of heparitinase I and II. The reduced radioactivity recovered in the cetylpyridinium-glycosaminoglycan complex owing to enzymatic digestion was assigned to be in the glycosaminoglycan.

Estimation of MK receptors and PG-M/versican by western blotting analysis or by reverse transcription polymerase chain reaction

HUVEC or HASMC were plated at a density of 1.0×10^6 cells in a 10 cm diameter dishes with each growth medium. On the following day, these were incubated with serum-free DMEM with or without MK (100 ng/ml) for 24 hours at 37°C. Cells were washed twice with PBS and were collected by scraping. BVMs cultured for 3 days with or without MK was also used as samples.

Proteins were separated by 7% SDS-PAGE, and PTPζ and LRP were detected by western blotting as described previously (Muramatsu et al., 1993) with anti-human PTPζ antibody or anti-mouse LRP antibody. The immunoreactive bands were revealed by an enhanced chemiluminescence kit (Amersham Life Science, Buchinghamshire, England). For quantitative estimation, bands were scanned by Chemi Doc (Bio-Rad, Tokyo, Japan).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) of PG-M/versican was performed by using the one step RT-PCR kit (QIAGEN Gmbh., Hilden, Germany). For these reactions, 1.0×10^5 cells were used. The oligonucleotides used for amplification of human PG-M/versican cDNA fragment (nucleotide number 5377-6061, GenBank accession number X15998) were as follows: forward, CATCCCTGCCAATTCCTC, and reverse, TCTGTGGGAGAAGCTTCC. They were heated at 37°C for 30 minutes and at 94°C for 3 minutes, and then subjected to 35 cycles of denaturation (94°C for 30 seconds), annealing (55°C for 30 seconds) and extension (72°C for 30 seconds). Amplification products were subjected to electrophoresis in 1.5% agarose gel and stained with ethidium bromide. For quantitative estimation, bands were scanned by Chemi Doc.

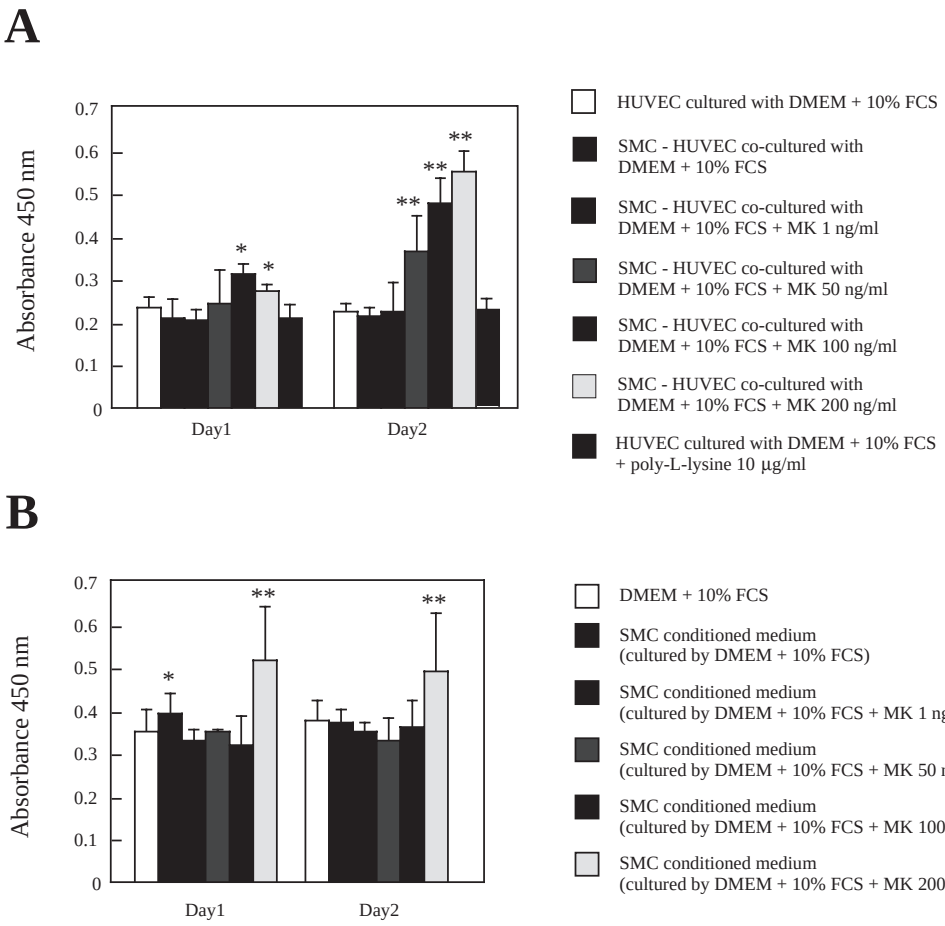

Fig. 4. HUVEC proliferation assay. (A) HUVEC proliferation assay in the co-culture system (Transwell 24-well culture plate). (B) HUVEC proliferation assay with HASMC-conditioned medium treated with or without MK. The number of samples in both A and B was five, and the experiments were repeated twice with reproducible results. **P*<0.05; ***P*<0.001.

ELISA

The amounts of IL-8, HGF, bFGF and VEGF were determined by ELISA with commercial kits according to the manufacturer's instructions, whereas that of MK was determined as described previously (Muramatsu et al., 1996).

Statistical analysis

The values are expressed as means±s.d. Group means were compared using the two-tailed Student's *t*-test. Differences were analyzed statistically by ANOVA. Values with $P<0.05$ were considered significant.

Results

Effects of MK on BVMs

When human MK was added to BVMs at a dose of 100 ng/ml, HUVEC became stratified and also appeared to secrete large amounts of extracellular matrices (Fig. 1D). MK at 200 ng/ml showed similar effects, whereas MK was less effective at 10 ng/ml or 50 ng/ml (data not shown). Although MK is a basic protein, poly-L-lysine at the concentration of 100 ng/ml showed no effects (data not shown). Affinity-purified anti-MK antibody inhibited the action of MK (Fig. 1F). The number of HUVEC in BVMs increased by 1.8-fold 3 days after the addition of 100 ng/ml MK as compared with BVMs cultured without MK (Table 1).

To determine whether the increase in the number of HUVEC in BVMs by MK was caused by an increased cell proliferatin, we stained the BVM sections with anti-PCNA antibody, which stains the nuclei of dividing cells. On Day 1, the numbers of intensely stained HUVEC were much higher in BVMs treated with MK as compared with control BVMs (Fig. 2). On Day 3, numbers of proliferating HUVEC decreased both in control BVMs and in BVMs treated with MK.

In relation to secretion of extracellular matrix, we assayed glycosaminoglycan synthesis by determining incorporation of $[^{35}S]O_4$ into glycosaminoglycans. When MK was added to HASMC gel and HUVEC cultured separately, we observed no enhancement of glycosaminoglycan synthesis. In BVMs, MK enhanced glycosaminoglycan synthesis by 1.68-fold at the dose of 50 ng/ml and 2.52-fold at 100 ng/ml, but was less effective at 10 ng/ml (Fig. 3). The ratio of radioactive sulfate incorporation into chondroitin sulfate and heparan sulfate in BVMs was 1: 0.6, and the value was not changed after MK treatment; MK stimulated the synthesis of both chondroitin sulfate and heparan sulfate.

To examine the effects of increased synthesis of glycosaminoglycans, we also added chondroitin-4 sulfate, chondroitin sulfate E, dermatan sulfate and heparin at the concentration of 10 μg/ml to the culture of BVMs together with 100 ng/ml of MK. However, the action of MK was not significantly affected (data not shown).

Role of HASMC in the MK-dependent increase in proliferation of HUVEC

When MK (100 ng/ml) was added to HUVEC or HASMC

Table 2. Effects of MK on IL-8 production by BVMs

MK (ng/ml)	0	1	10	100
IL-8 (ng/ml)	8.59	10.97	14.62	20.57

HUVEC were cultured in the presence or absence of MK for 24 hours, and the amount of IL-8 in the medium was assayed by ELISA. The number of samples was four.

cultured separately, no increase in cell proliferation was observed (Fig. 4A). However, when HUVEC were co-cultured with HASMC at a ration of 1:10, respectively, MK significantly increased the cell proliferation (Fig. 4A). Poly-L-lysine showed no effects. When conditioned medium of HASMC cultured with 100 ng/ml MK was added, proliferation of HUVEC was increased; conditioned medium of HASMC cultured without MK was slightly active on Day 1, but was not active on Day 2 (Fig. 4B). Thus, co-operation with HASMC is required for stimulation of HUVEC proliferation by MK.

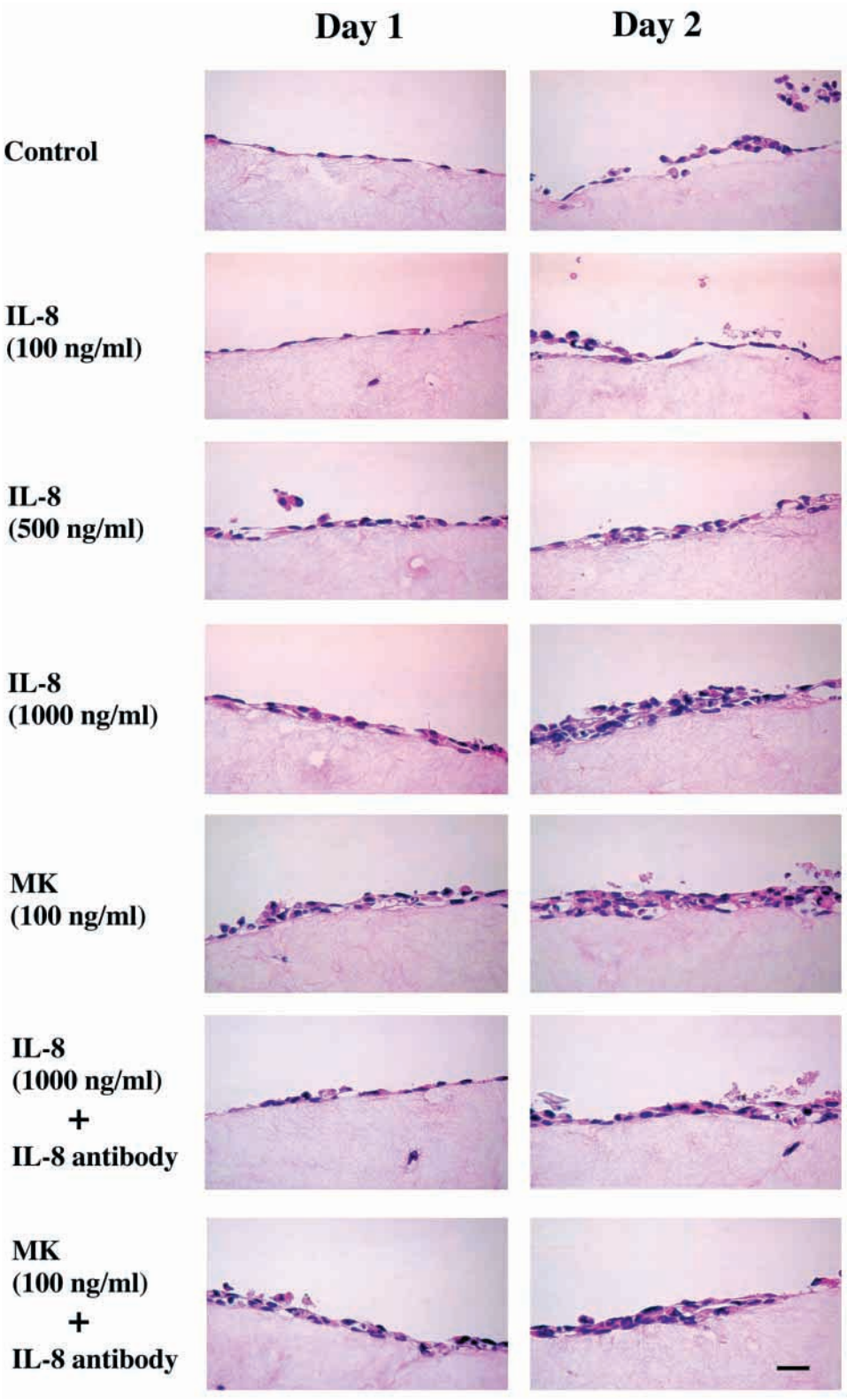

Fig. 5. Morphologies of BVMs cultured in DMEM with or without IL-8 and MK. BVMs were cultured for 1 or 2 days, with IL-8 (100, 500, 1000 ng/ml), MK (100 ng/ml) or without factors (Control). For antibody inhibition, 30 μg/ml of anti-IL-8 antibody was added. Bar, 100 μm.

Identification of IL-8 as a factor secreted by HASMC

The effectiveness of HASMC-conditioned medium suggested that a factor secreted by HASMC in the presence of MK promoted proliferation of HUVEC. To identify such a factor in the conditioned medium of HASMC, we performed an ELISA for three human growth factors, that is, VEGF, HGF and bFGF, as wells as the cytokine IL-8. The level of IL-8 was significantly altered by treatment with MK for 24 hours; 100 ng/ml of MK caused a 2.39-fold increase of IL-8 level in the conditioned medium (Table 2). The levels of other factors were not significantly altered by MK.

To determine whether IL-8 is a mediator of the actions of MK, we added anti-IL-8 antibody to the culture of BVMs and found that the effect of MK was suppressed on Day 3 (Fig. 1H, Table 1). Various concentrations of IL-8 were also added to BVMs. In this experiment, BVMs were observed only on day 1 and day 2, since culture in DMEM without serum was required to examine the effects of IL-8, and this caused damage to BVMs on day 3. On day 2, IL-8 at 1000 ng/ml induced stratification of endothelial cells to a degree similar to that induced by 100 ng/ml MK. Lower concentrations of IL-8 were less

effective (Fig. 5). In the serum-free system, anti-IL-8 antibody also inhibited MK action (Fig. 5).

Expression of MK and MK receptors in HUVEC and HASMC

To understand the basis of MK-dependent interactions between HUVEC and HASMC, we investigated whether MK and MK receptors are expressed in these cells. ELISA revealed that the HUVEC-conditioned medium, which was collected 20 hours after initiation of culture, had 0.9 ng/ml of MK, whereas no MK (less than 0.05 ng/ml) was detected in the conditioned medium of HASMC.

Previous studies have indicated that the MK receptor is a molecular complex containing LRP (Muramatsu et al., 2000) and proteoglycans such as PTPζ (Maeda et al., 1999). PTPζ was detected both in HUVEC and HASMC, and its amount increased in both cells and also in BVMs after MK treatment (Fig. 6A). The amount of LRP was below detection in cells before MK treatment, whereas it became detectable after MK treatment in HASMC but not in HUVEC (Fig. 6A). The level of expression of PG-M/versican, which is a pericellular chondroitin sulfate proteoglycan and binds to MK (Zou et al., 2000), was increased less significantly after treatment with MK (Fig. 6B). The result with the induction of LRP in HASMC is consistent with the view that HASMC are the target of MK.

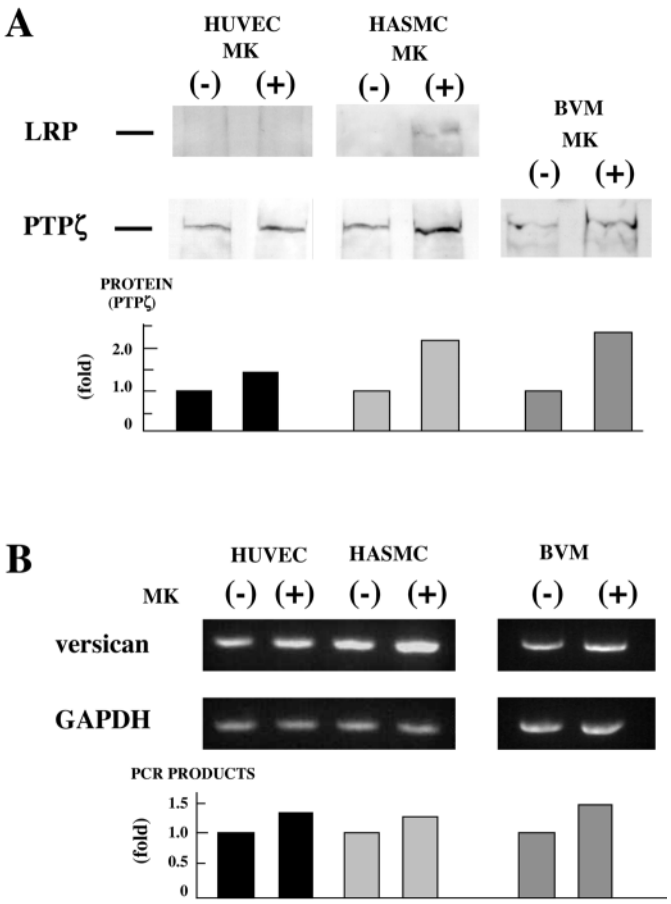

Fig. 6. Effects of treatment with 100 ng/ml MK for 24 hours on expression of PTPζ, LRP and PG-M/versican. (A) Proteins were separated by SDS-PAGE, and PTPζ and LRP were detected by western blotting with the respective antibody. The intensity of bands was normalized to that of actin, and each band was shown as the ratio of the value in the treated cells to that in the non-treated cells. (B) Expression of versican revealed by RT-PCR. The intensity of bands was normalized to that of GAPDH and was shown as the ratio of the value in the treated cells to that in the non-treated cells.

Discussion

We devised an artificial BVM, in which interaction of the endothelial cell layer and smooth muscle cells takes place as in blood vessels. When MK was added to the BVM, stratification of HUVEC occurred and proliferation of HUVEC and production of extracellular matrix synthesis increased. These activities of MK were not observed when it was applied to cultures of HUVEC. Although endothelial cells form a monolayer in blood vessels, HUVEC in the BVM formed a multi-cell-layer upon MK stimulation. This may have been because of the lack of liquid flow force, which may force proliferating endothelial cells to form a thin layer in blood vessels in vivo.

MK was identified as an angiogenic factor, on the basis of its in vivo angiogenic activity of cells transfected with MK cDNA (Choundhuri et al., 1997). However, our repeated attempts to demonstrate in vitro angiogenic activity of MK failed (Y.S., H.M., Y.T., K.-I.H. et al., unpublished). Proliferation of endothelial cells is an important step in angiogenesis. The present results provided in vitro evidence for the reported in vivo angiogenic activity of MK for the first time. Furthermore, the requirement of the presence of smooth muscle cells for the effect of MK on HUVEC explains the failure to observe direct MK activity on endothelial cells. MK is overexpressed in a number of human carcinomas, namely, gastric, colon and hepatocellular carcinomas (Tsutsui et al., 1993; Aridome et al., 1995; Ye et al., 1999), breast carcinoma (Garver et al., 1994), lung carcinoma (Garver et al., 1993), urinary bladder carcinoma (O'Brien et al., 1996) and neuroblastoma (Nakagawara et al., 1995). This enhanced expression of MK implies that it is beneficial to tumor growth. MK enhances cell growth (Muramatsu and Muramatsu, 1991) and cell migration (Takada et al., 1996; Maeda et al., 1999; Horiba et al., 2000; Qi et al., 2001) and suppresses apoptosis (Owada et al., 1999; Qi et al., 2000). Together with the above-mentioned activities, which will help tumor growth and spread, the angiogenic activity of MK will significantly contribute to tumor progression. Means to suppress MK expression or MK activity in tumor tissue should be explored with the aim of development of new treatment methods for malignancy.

Although proteoglycans play essential roles in MK signaling (Kaneda et al., 1996; Ahkter et al., 1998; Maeda et al., 1999; Ueoka et al., 2000), glycosaminoglycans including heparin did not inhibit MK activity to BVMs. Previously, we observed that heparin did not inhibit MK action on bovine aortic endothelial cells to enhance fibrinolytic activity, instead heparitinase digestion of these target cells abolished the MK activity (Ahkter et al., 1998). Most probably, endothelial cells have high-affinity binding sites for glycosaminoglycans and prevent the action of glycosaminoglycans to inhibit MK activity.

In enhancing HUVEC proliferation, the direct target of MK was identified as HASMC, which secreted factor(s) acting on HUVEC. Furthermore, LRP, a component of the MK receptor

(Muramatsu et al., 2000) was upregulated in HASMC. We also noticed that HUVEC produced MK, whereas HASMC did not. These findings place MK as a factor involved in epithelial-mesenchymal interactions, as has been suggested by earlier studies (Kadomatsu et al., 1990; Mitsiadis et al., 1995a; Mitsiadis et al., 1995b; Toriyama et al., 1996). In tissues undergoing epithelial-mesenshymal interactions, MK is generally expressed more strongly in epithelial cells (Kadomatsu et al., 1990; Mitsiadis et al., 1995b). However, MK often acts on mesenchyme-derived cells; it enhances growth of NIH3T3 cells (Muramatsu and Muramatsu, 1991), synthesis of extracellular matrix molecules by dermal fibroblasts (Yamada et al., 1997) and fibroblast-mediated collagen gel contraction (Sumi et al., 2000). HASMC studied here are also cells derived from the mesenchyme. Therefore, MK might play a significant role in the complex interplay of the epithelial and mesenchymal cell layers.

We found that MK increased IL-8 production by HASMC. IL-8 belongs to a family of small, structurally related cytokines similar to platelet factor 4 (Clark-Lewis et al., 1993). It is produced by phagocytes and mesenchymal cells exposed to inflammatory stimuli (interleukin-1 or tumor necrosis factor) and activates neutrophils, inducing chemotaxis and exocytosis. IL-8 promotes HUVEC growth and migration, which is similar to bFGF, and induces corneal neovascularization (Strieter et al., 1992). Thus, IL-8 was expected to be a mediator of the action of MK on endothelial cells. Indeed, anti-IL-8 antibody inhibited the action of MK on BVMs. Furthermore, addition of 1000 ng/ml IL-8 to BVMs resulted in stratification of HUVEC to the same degree as observed upon addition of 100 ng/ml MK. We noticed that the level of IL-8 in culture medium of MK-treated HASMC was only around 20 ng/ml. IL-8, which was added to the medium of BVMs at the level of 100 ng/ml, showed little effect. In BVMs treated with MK, IL-8 is delivered from the basal layer facing the collagen gel, and this difference in delivery may partly explain the ineffectiveness of exogenously added IL-8 at 100 ng/ml. However, the difference may be better explained by the presence of other factors that enhance the action of IL-8. It is even possible that upregulation of such a factor is another critical event.

Recently, we found that MK increases expression of MIP-2 in proximal tubular epithelial cells (Sato et al., 2001). MIP-2 is probably the mouse counterpart of IL-8. The increased MIP-2 level and the direct chemotactic activity of MK on neutrophils explain MK-dependent recruitment of neutrophils to injured renal tubules (Sato et al., 2001). The increased expression of IL-8/MIP-2 by MK in two entirely different systems suggested that IL-8/MIP-2 is an important mediator of MK action at the tissue level.

We thank H. Inoue, T. Adachi and T. Katoh for secretarial assistance. This work was supported in part by grants in aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan.

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