Inhibition of P38 MAPK Reduces Tumor Conditioned Medium-Induced Angiogenesis in Co-Cultured Human Umbilical Vein Endothelial Cells and Fibroblasts

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Tumor conditioned medium (CM) has been widely used to stimulate endothelial cells to form capillary-like structures in in vitro angiogenesis models. We report herein the effect of HT1080 and A549 CM after they were mixed with microvascular endothelial cells medium-2 (EGM-2) on angiogenesis in human umbilical vein endothelial cells (HUVECs). Both HT1080 and A549 CM decreased HUVEC proliferation, to different extents. While A549 CM significantly increased capillary-like structure formation in a co-culture system, no effect of HT1080 was apparent. Inhibition of p38 mitogen-activated protein kinase (MAPK) blocked both basal and A549 CM induced capillary-like structure formation, but inhibition of extracellular signal-regulated kinases (ERK) and that of c-Jun N-terminal protein kinases (JNK) MAPK had no such effect. Activation of ERK MAPK was inhibited by both CMs, whereas p38 MAPK was inactivated by HT1080 and activated by A549 CM and a control. Neither CM had an effect on JNK MAPK. The results suggest that p38 MAPK played a critical role in capillary-like structure formation in the co-culture, partly via promotion of apoptosis in HUVECs.

Key words: conditioned medium; p38 mitogen-activated protein kinase (MAPK); apoptosis; co-culture

Tumor angiogenesis is the formation of a network of blood vessels that penetrate into tumor tissues to supply nutrients and oxygen and remove waste products. It is a tightly regulated process, that involves proteolytic digestion of the extracellular matrix (ECM), proliferation and migration of endothelial cells, and formation of functional capillaries. In vivo tumor angiogenesis begins when tumor cells release angiogenic molecules, such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), which in turn send signals to preexisting blood vessels and thereby initiate the process of angiogenesis. Tumor cell CMs are commonly used in in vitro angiogenesis assays to mimic in vivo events during tumor angiogenesis. To date, in vitro angiogenic studies often focus on one individual step in the angiogenesis process, such as proliferation, migration, or differentiation of endothelial cells. Because blood vessels in humans consist of three major components, endothelial cells, vascular smooth muscle cells, and the ECM, cell-cell interactions play an important role in angiogenesis. Numerous studies have confirmed the importance of ECM in forming and maintaining the tubular structure of the vascular system. The ECM not only regulates angiogenesis by providing scaffold support and playing signaling roles, but also serves as a reservoir and modulator for growth factors. Fibroblasts, a type of connective tissue cells, are able to produce an ECM that is rich in collagen, fibronectin, elastin, and other matrix molecules, including basement membrane components. Most of these molecules can act as a scaffold for tubule formation. As a result, mixing endothelial cell with fibroblast is thought to be much closer to the in vivo situation and can be used to model the whole angiogenesis process. Although the co-cultured assay for angiogenesis takes a longer time to perform than other assays, such as using fibrin, 3-dimensional collagen, or matrigel assays, because it involves the formation of ECM by fibroblasts and subsequent migration, proliferation, and differentiation of endothelial cells into tubules, all the steps of angiogenesis are present in the co-cultured assay.

In mammalian cells, three major classes of MAP kinases, including extracellular signal-regulated kinases
such as proliferation, motility, and differentiation. Studies have shown that MAPKs are involved in p38 MAP kinases (p38), have been identified. Recent ERKs, c-Jun N-terminal protein kinases (JNKs), and MAPK was closely associated with capillary-like structure formation in the co-culture was significantly inhibited by p38 MAPK inhibitor SB-203580.

Materials and Methods

Cell culture and treatment. Human lung adenocarcinoma A549 cells and fibrosacoma HT1080 cells were purchased from ATCC (Manassas, VA), and human diploid embryonic lung fibroblasts TIG-1 from the Japanese Collection of Research Bioresources (Tokyo) were cultured in minimum Eagle’s medium (MEM) (Sanko, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) from Biowest (Nuaille, France). Human endothelial vascular cells (HUVECs) were purchased from Cambrex (Walkersville, MD) and cultured in Cambrex microvascular endothelial cells medium-2 (EGM-2). Propidium iodide (PI) was from Sigma (St. Louis, MO), SB203580, PD98059, and JNK inhibitor were from Calbiochem (San Diego, CA). WST-1 reagent was purchased from Cellworks (Botolph Claydon, Buckingham, UK). The phospho-p38, ERK, and JNK Mitogen-activated protein kinase (MAPK) antibodies were from Cell Signaling Technology (Danvers, MA).

Preparation of tumor CM. HT1080 and A549 (1 x 10^6 cells) were seeded in a 100-mm dish with MEM containing 10% FBS overnight. The medium was replaced with serum-free MEM and incubated for 72 h. The CM was collected and filtered with a 0.2-μm filter. The aliquots were stored at −80°C in a freezer.

Cell proliferation assay. After 2 x 10^4 cells/well of HUVEC and TIG-1 cells were cultured in 24-well plates (n = 3) with EGM-2 and MEM (10% FBS) respectively at 37°C under an atmosphere of 95% O_2/5% CO_2 overnight, the culture medium was removed and washed twice with PBS. A mixture of tumor CM and EGM-2 at a ratio of 2:1 (v/v) was added to each well (1 ml/well). After the cells were incubated for 48 h, the medium was removed and washed twice with PBS. WST-1 reagent (500μl) was added to the wells and incubated at 37°C for 2 h. The reaction solution (100μl) was transferred to a new 96-well plate in sextuplicate, and the absorbance of samples was measured using a Tecan Spectra microplate reader (MTX Lab Systems, Wako, Osaka, Japan) at 450 nm.

Apoptosis assay. Apoptosis in the HUVECs was measured by the method of Nicoletti. Briefly, after treatment with HT1080 or A549 CM for 48 h, floating HUVECs in the supernatant were collected and harvested by trypsinization, and then resuspended with the supernatant. After centrifugation, the cells were fixed with 70% ice-cold ethanol for more than 2 h and incubated with a freshly prepared staining buffer (0.1% Triton X-100 in PBS, 200µg/ml RNase, and 20µg/ml PI) for 15 min at 37°C. DNA fragment analysis was performed by EPICS XL system II flow cytometry (Beckman Coulter, Miami, FL). About 10,000 cells from each group were measured. Data were analyzed using WinMDI 2.8 software (Scripps Institute, La Jolla, CA).

Tubules formation assay. HUVECs were mixed with TIG-1 cells at a ratio of 1:40, seeded in a 24-well plate, and cultured in EGM-2 medium overnight. The medium was removed and replaced with a mixture of tumor cells CM and EGM-2 (2:1, v/v). The mixed medium was changed every other day. The formed tubes were detected with HUVEC-specific marker von Willebrand Factor. Briefly, on day 11, the medium was completely removed, and a co-culture plate was fixed for 30 min with 70% cold ethanol solution. After it was washed with PBS containing 1% BSA, the co-culture plate was incubated with sheep anti-human von Willebrand Factor antibody (1:200) for 60 min at 37°C, followed by another 60 min incubation with secondary donkey anti-sheep IgG antibody conjugated with horseradish peroxidase (1:400). Both antibodies were from the tube staining kit. After the culture plate was washed, DAB substrate (1:10 in substrate buffer) was added until the tubules developed a dark brown color. Then the co-cultures were carefully washed three times with distilled H_2O and dried. Tubule formations in the co-culture system were visualized under a phase-contrast microscope, and photomicrographs were documented with an Olympus digital camera (Tokyo). Recorded images were analyzed by AngioSys 1.0 software for angiogenesis quantification (TCS Cellworks, Botolph Claydon, Buck-
Ingham, UK). First, the images were skeletonised. Layers of pixels were progressively stripped off until each tubule was rendered as a line of single pixels. The single pixel lines were detected and measured. Six random fields per well were pictured for tubule formation assessment.

Western blot. The cells were washed with PBS (pH 7.4), incubated with extraction buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM PMSF, 1% NP-40, 0.1% SDS, 10 µg/ml Aprotinin, and 10 mM EDTA) on ice, and collected with a scraper. The lysates were then centrifuged at 12,000 x g for 5 min. Thirty micrograms of protein samples were boiled at a ratio of 3:1 with sample buffer (250 mM Tris–HCl, pH 6.8, 40% glycerol, 20% β-mercaptoethanol, 8% SDS, and 0.04% bromophenol blue), and separated by SDS–PAGE. Following electrophoresis, the proteins on the gels were transferred onto Hybond-ECL membranes (Amersham Bioscience, Pollards Wood, UK), which were then blocked with 0.05% Tween 20-PBS (TBS) containing 10% skim milk powder (Wako, Osaka, Japan). After washing, the membranes were incubated with primary and secondary antibodies coupled with peroxidase. After it was washed three times with TBS, the bound antibody was developed using an ECL plus Western Blotting Detection System (Amersham Bioscience, Pollards Wood, UK).

Results

HT1080 and A549 CM decrease HUVEC and TIG-1 cell proliferation

Stimulation of the proliferation of vascular endothelial cells by tumor cell-derived angiogenic factors is an essential step in angiogenesis. Hence, we first examined the effect of HT1080 and A549 CM on HUVEC proliferation. Tumor cell CM was prepared by culture of tumor cells in serum-free MEM, followed by mixing the CM with EGM-2 at a ratio of 2 to 1 (v/v). A control was prepared by mixing cell-free fresh MEM with EGM-2 at the same ratio. In the mixtures, one source of growth factors presented was released from tumor cells and the others were presented in EGM-2. After HUVECs were challenged with the mixtures for 48 h, cell proliferation was evaluated by WST-1 assay. This assay is widely used in the determination of cellular proliferation and cytotoxicity due to its simplicity, low cost, high accuracy, and sensitivity.26) The principle of the assay is based on the cleavage of a water-soluble WST-1 tetrazolium salt (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by the mitochondrial dehydrogenases of viable cells to form a yellow-orange, water-soluble formazan. HUVEC proliferation was decreased significantly at 25% and 26% by HT1080 and A549 CM respectively, as compared to the control values. In the case of TIG-1, however, neither CM had any effect on cell proliferation (Fig. 1).

Detection of tumor CM induced apoptosis in HUVECs

When HUVECs were cultured in HT1080, A549, or control CM, apoptosis-like cells floating in the supernatant were observed in all three treatment groups, presumably as a result of serum deprivation. To confirm the occurrence of apoptosis, the breakdown of DNA fragments was measured by flow cytometry. A subG1 peak, which is characteristic of apoptotic cells, was detected in the samples from all three groups. A549 CM induced apoptosis in 25.8% of HUVECs when a total of 10,000 cells were counted. While similar induction (25.5%) was observed in the control group, HT1080 CM induced the least apoptosis (11.3%) in HUVECs (Fig. 2).

Effects of MAPK inhibitors on capillary-like structure formation in co-culture

To determine the role of MAPK in capillary-like structure formation in the co-culture model, specific inhibitors of MAPK at non-cytotoxic concentrations were added to the culture medium, which was prepared by mixing EGM-2 with fresh serum-free MEM (1/2, v/v). The capillary-like structure formation was ex-
pressed as the measured total length of capillary-like structure. The results indicate that SB203580 (an inhibitor of p38, 10 μM) significantly inhibited capillary-like structure formation, while PD98059 (an inhibitor of MEK, an upstream kinase in the ERK pathway, 10 μM) and JNKi (a JNK inhibitor, 1 μM) showed no apparent effect (Fig. 3E). SB203580 also induced different morphological changes from PD98059 and JNKi treatment of HUVECs, as evidenced by shorter tubule lengths and denser branch points (Fig. 3B). These data indicate that p38 MAPK is essential for capillary-like structure formation of HUVECs in the co-culture model.

**SB203580 inhibited A549 CM enhanced capillary-like structure**

The effect of tumor CM on capillary-like structure formation was further evaluated in the study. As shown in Fig. 4, HT1080 and A549 CM had different induction potentials on the formation of capillary-like structures in the co-culture. With respect to the total length of capillary-like structures, no significant changes were observed as between HT1080 CM and the control group, but there was a 50% increase in the A549 CM treatment group (Fig. 4E). The elevated capillary-like structure formation is most likely mediated by p38 activation, since enhanced formation was drastically reduced in the presence of 10 μM SB203580 (Fig. 4E), suggesting a critical role of p38 in A549 CM induced capillary-like structure formation.

**HT1080 and A549 CM inhibited activation of p38 and ERK without affecting JNK**

The importance of MAPKs on the regulation of cell proliferation, induction of apoptosis, and initiation of angiogenesis in endothelial cells is well recognized. In the present study, we investigated the effect of HT1080 and A549 CM on the activation of MAPKs. After HUVECs were exposed to tumor CM for 48 h, the cells were lysed and the expression of MAPKs was analyzed by Western blotting. As shown in Fig. 5, treatment of HT1080 CM resulted in significant decreases in both p38 and ERK MAPK in HUVECs. A549 CM led to a smaller decrease in ERK MAPK and had no effect on the activation of p38 MAPK. Neither HT1080 nor A549 CM showed a detectable effect on JNK MAPK or α-tubulin activity.

**Discussion**

The use of tumor CM as an inducer to stimulate endothelial cells to form capillary-like structures, which mimic in vivo angiogenesis, is common in in vitro angiogenesis analysis. Proliferation of endothelial cells is a prerequisite for tumor angiogenesis. Tumor tissues not only secrete angiogenic factors such as VEGF and bFGF, which stimulate proliferation of endothelial cells, but also produce metabolic waste products that might function as anti-angiogenic factors. The initiation of in vivo tumor angiogenesis depends on a delicate balance between angiogenic and anti-angiogenic factors. Tumor
cell CM accumulates both angiogenic and anti-angiogenic factors in a time-dependent manner. In this study, the decreased HUVEC proliferation due to HT1080 and A549 CM was probably due to the mitogenic effects of angiogenic factors presented in the tumor cell CM which did not overcome the effects of anti-angiogenic factors. Contrary to our data, Ito et al., have reported that HT-1080 CM promoted the growth of HUVECs in their study, in which HT1080 CM was prepared by incubating HT1080 cells with serum-free DMEM for 24 h in the presence of ascorbate.27) By comparison, our co-culture system was generated by incubating HT1080 cells with serum-free DMEM for 72 h in the absence of ascorbate, and the final medium was obtained by diluting HT1080 CM with EGM-2 at a 2:1 ratio. Although it has been reported by Ito and Grant that HT1080 CM increased tube formation on a matrigel,27,28) no stimulatory effect on capillary-like structure formation was observed in our co-culture system. The discrepancies are thought to be caused by differences in the preparation of CM and the models used in tubule formation analysis.

In order to investigate the role of MAPK in tumor-induced angiogenesis in the co-culture model, MAPK specific inhibitors were applied. Although the culture medium was prepared by mixing fresh MEM and EGM-2 (2:1, v/v), the angiogenic factors contained in EGM-2 were sufficient to stimulate the formation of capillary-like structures. SB203580 (10 μM) markedly inhibited

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Fig. 3. Effect of MAPK Inhibitors on Capillary-Like Structure Formation in Co-Culture System.

The HUVEC/TIG-1 co-cultures were incubated with mixtures of EGM-2 and non-cell conditioned MEM at a ratio of 1:2 (v/v). Each co-culture contained control (A), 10 μM SB203580 (B), 10 μM PD98059 (C) or 1 μM JNKi (D). The medium was changed every 2 d. On day 11, tubule formations were detected with a tubule staining kit, and were visualized under a phase-contrast microscope. The photomicrographs were documented by a digital camera. Recorded images were analyzed by software for angiogenesis quantification (E). Six random fields per well were pictured for tubule formation assessment. Data are expressed as the means ± SD. Significant differences (*P < 0.05, n = 3) were determined by Student’s t-test. Bar = 100 μm.
the formation of capillary-like structures, while PD-98059 (10 μM) and JNKi (1 μM) showed no apparent effect. Our results are in agreement with those achieved by Jackson et al., who found that SB220025, another specific p38 MAPK inhibitor, inhibited angiogenesis in the murine air pouch granuloma model.29) Other report, by Yang et al., found that SB203580 at 20 μM inhibited HUVEC coalescence and thereby blocked the formation of tube structures in three-dimensional collagen matrices,22) and that PD98059 at 20 μM also blocked tube formation by inducing HUVEC apoptosis. Furthermore, Miura et al. have reported that inhibition of JNK blocked tube formation on matrigel.21) The discrepancies among these studies may be attributed to the different models used in the analysis of tube formation. In 3-dimensional collagen matrices and matrigel models, PD98059 and JNK inhibitors exert their inhibitory functions only on HUVECs, while in a coculture model, both HUVECs and fibroblasts are affected. JNK inhibitor and PD98059 are more likely to modulate tube formation in fibroblasts, which produce many potent angiogenic factors. In addition, the different doses of MAPK inhibitors used also might have contributed to the differences observed.

p38 MAPK is usually activated by extracellular or intracellular stresses. Although its activation is involved in many physiological activities, such as platelet aggregation and glucose transport, growth inhibition and apoptosis are often consequences of activation.30–33) Although apoptosis occurred in HUVECs when treated with tumor cell CM and control CM, all concomitant with the activation of p38 MAPK, HT1080 CM induced
less apoptosis and less activation of p38 MAPK in HUVEC than did A549 or control CM, suggesting that the proapoptotic effect of tumor CM is highly divergent and cell-type dependent. The fact that capillary-like structure formation in all groups indicates that apoptosis occurring in HUVECs does not prevent HUVECs from differentiating into capillary-like structures. Other reports have also suggested that the occurrence of apoptosis in the endothelium is required during the formation of capillary network structure, since A549 CM treatment induced more apoptosis and less activation of p38 MAPK in HUVEC than did A549 or control CM, suggesting that A549 CM has higher potential than HT1080 CM in the stimulation of capillary-like structure formation in our co-culture system.

In summary, the findings presented in this report indicate that p38 MAPK plays a critical role in endothelial cell and fibroblast based capillary-like structure formation in a co-culture system partly through the promotion of apoptosis in HUVECs. Future study will address the question how the extent of apoptosis and survival occurring in HUVECs affects capillary-like structure formation in co-culture systems.

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