Research Paper

Tumor Necrosis Factor-α, Interleukin-8 and Interleukin-6 Are Involved in Vascular Endothelial Cell Capillary Tube and Network Formation Induced by Tumor-Associated Macrophages

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AIM: **The goal of this study is to investigate the involvement of inflammatory cytokines produced by tumor-associated macrophages in promoting tumor angiogenesis.**

METHODS: **To study the angiogenic effect of tumor-associated macrophages (TAMs), we overlaid human umbilical vein endothelial cells on top of Matrigel containing MCF-7 breast cancer cells with or without macrophages and investigated the outcome of endothelial cell capillary tube and network formation. We also determined the levels of interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) in the media of MCF-7 breast cancer cells co-cultivated with or without macrophages. Furthermore, anti-IL-8 receptor antagonizing antibody, IL-6 or TNF-α soluble receptor, and inhibitors against NF-κB, MEK, $p38^{MAPK}$, and JNK, respectively, were used to determine which signal transduction pathways are involved in TAMs-induced angiogenic activity.**

RESULTS: **The Matrigel mixed with MCF-7 cells and macrophages was more efficient than 100 ng/ml of VEGF to induce vascular endothelial cell tube and network formation. The expression of IL-6, IL-8 and TNF-α were significantly enhanced by co-cultivation of MCF-7 cells with macrophages. The promotion of capillary tube and network formation by TAMs was inhibited either with anti-IL-8 receptor antagonizing antibody or with IL-6 or TNF-α soluble receptor, suggesting that IL-8, TNF-α and IL-6 indeed participated in TAMs-induced angiogenesis. In addition, TAMs-induced angiogenic activity could also be attenuated by the presence of inhibitors against NF-κB, ERK, and $p38^{MAPK}$ signaling pathways.**

CONCLUSION: **IL-8, TNF-α and IL-6 were involved in TAMs-associated angiogenesis via NF-κB, ERK, and $p38^{MAPK}$-dependent signaling pathways.**

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Keywords:

tumor-associated macrophages

angiogenesis

TNF-α

interleukin-8

interleukin-6

VEGF

Introduction

Tumor angiogenesis is a prerequisite process for continuous growth and spreading of solid tumors. It can be induced and regulated not only by tumor cells but also by other cells in the stroma of tumor [1]. Tumor-associated macrophages (TAMs[2]) are one type of major stromal cells that provide aids for tumor angiogenesis [1,2]. Macrophages are thought to be part of the host immune system used to defend the tumor cells, however, after the influence of tumor cells and the microenvironment, the macrophages residing in the tumor, so called TAMs, switch their tumoricidal activity to tumor-promoting effects [2]. TAMs have displayed a different gene expression profile from inflammatory macrophages [3,4]. For example, the levels of carboxypeptidase M and prolidase expression are suppressed while CD14, CD16 and Toll-like receptor-related molecules MD-1 and RP105 are up-regulated in TAMs [3].

Macrophages produce many growth factors, cytokines and tissue matrix modulating factors that could be associated with angiogenesis [5]. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are potent mitogens to endothelial cells [6]. Cytokines like tumor necrosis factor-α (TNF-α) and interleukin (IL)-8 have been demonstrated to participate in macrophage-induced angiogenesis [7,8]. Tissue matrix modulating factors are also important for regulating angiogenesis [2]. Tissue matrix is a supporting medium for vessels but a barrier for protruding or migrating endothelial cells. Tissue matrix is also a reser-

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[2]**Abbreviation:** TAMs, tumor-associated macrophages; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; TNF-α, tumor necrosis factor-α; IL, interleukin; TGF-β, transforming growth factor-β; MMPs, matrix metalloproteinases; PBMCs, peripheral blood mononuclear cells; HUVECs, human umbilical vein endothelial cells; MCP-1, monocyte chemotactic protein-1; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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voir of angiogenic factors. Many angiogenic factors, such as VEGF, bFGF and transforming growth factor-β (TGF-β), have an affinity for heparin and thus are sequestered at cell surface or in the tissue matrix. Tissue matrix modulating factors are responsible for tissue matrix remodeling, and are capable of regulating the release of angiogenic factors by cleaving off their binding to cell surface or tissue matrix. It has been known that TAMs are enhanced by tumor cells to produce VEGF, bFGF, TNF-α, IL-8, and several tissue matrix proteinases including matrix metalloproteinases (MMPs)-2, 7, 9, and 12 [9]. Whether or not these growth factors, cytokines or proteinases are involved in TAM-associated tumor angiogenesis needs to be carefully evaluated with more studies.

Endothelial cells can be induced to display the morphology of capillary tubes and further network formation on Matrigel, which can serve as an *in vitro* angiogenic activity assay [10]. To demonstrate TAM's angiogenic activity, we in this study mixed human breast cancer MCF-7 cells and macrophages in Matrigel and found that this mixture could provide sufficient factors for promoting vascular endothelial cell capillary tube and network formation. The efficacy was even superior to VEGF alone, a typical potent inducer in angiogenesis. The levels of TNF-α, IL-8, and IL-6 were significantly elevated in the co-culture medium of MCF-7 cells and macrophages, and therefore, inhibitors against receptors of TNF-α, IL-8, and IL-6 were respectively added to endothelial cells to investigate the effects on the angiogenic activity of cancer cells-macrophages interaction. Our data suggest that TNF-α, IL-8, and IL-6 are involved in TAMs-induced angiogenesis.

Materials and Methods

Cancer cell culture

Human breast cancer MCF-7 cells were cultivated under 5% CO_2 in a humidified 37°C incubator with MEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin.

Preparation of human macrophages

Peripheral blood mononuclear cells (PBMCs) from healthy donors (obtained from the Chinese Blood Foundation, Taipei, Taiwan, Republic of China) were enriched by density-gradient centrifugation with Ficoll-Hypaque (Amersham Pharmacia Biotech., Uppsala, Sweden). Cells were collected, washed and suspended in serum-free RPMI-1640 medium and then plated into plastic cell culture dishes (25×10^6 cells/dish) and incubated at 37°C for 2 h. Non-adherent cells were removed after gentle rinsing of the dishes with RPMI-1640 medium. Adherent mononuclear cells were cultured with RPMI-1640 medium with 10% FBS in the presence of 500 unit/ml granulocyte-macrophage colony-stimulating factor (Strathmann Biotec AG, Hannover, Germany). After 4 days, non-adherent cells were removed and adherent cells were collected and used as the source of macrophages in the subsequent experiments. These cells were $CD14^+$ and $CD68^+$, and capable of ingesting fluorescence-labeled Latex beads (Sigma Chemical Co., St. Louis, MO, USA).

Preparation of vascular endothelial cells

Human umbilical cords from normal deliveries were collected. In a tissue culture hood, each cord was excised at 1 cm from both ends with a sterile scalpel to expose the sterile surface. The umbilical veins were perfused with PBS and then 0.2% collagenase in PBS until the vein was distended. The two ends of the cords were sealed with sterile clamps and incubated for 30 min at 37°C. Then, the cords were gently massaged to facilitate human umbilical vein endothelial cell (HUVEC) detachment from the vessel wall. The solution was flushed out and the lumen of the vein was washed with M199 medium (Gibco, Invitrogen Co., Grand Island, NY, USA) plus 100 U/ml of penicillin and 100 μg/ml of streptomycin. Cell suspension was collected and centrifuged at 1000 rpm for 5 min at 4°C. The cell pellet was resuspended in M199 medium supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin, and plated on 10-cm culture dishes and incubated in a humidified 37°C incubator with 5% CO_2. After attaching overnight, HUVECs were washed with PBS and cultivated in M199 medium supplemented with 20% FBS and 30 μg/ml of Endothelial Cell Growth Supplement (Upstate Inc., Lake Placid, NY, USA). HUVECs were further subjected to subculture or storage in liquid nitrogen. HUVECs between passages 3 and 7 were used for the subsequent experiments.

Vascular endothelial cell capillary tube and network formation

Matrigel from BD Bioscience (San Jose, CA, USA) was thawed and diluted with the equal volume of serum-free MEM. Five hundred μl of diluted Matrigel was added into a 30-mm culture dish and incubated at 37°C for 30 min. After the matrigel was solidified, HUVECs (4×10^5) in 1 ml of M199 medium were added in each dish and kept incubating at 37°C. To determine the angiogenic activity of TAMs, the Matrigel was mixed with 2×10^5 of MCF-7 cells in the absence or presence of 1×10^5 of macrophages (Figure 1). As the positive control, the Matrigel was mixed with 100 ng/ml of VEGF (R&D Systems, Minneapolis, MN, USA). Time-lapse photography was performed to monitor the process of vascular endothelial cell tube and network formation in the incubator with CCM-330F monitoring system (Astec Co., Tokyo, Japan).

To analyze what cytokine(s) and signal transduction pathway(s) is involved in TAMs-induced angiogenic activity, 200 μl of diluted Matrigel that mixed with MCF-7 cells (4×10^4) and macrophages (2×10^4) was added into each well of 24-well plate and incubated at 37°C for 30 min. After the matrigel was solidified, HUVECs (7×10^4) alone or mixed with inhibitors in 200 μl of M199 medium were added in each well and kept incubating at 37°C for 3 h. The inhibitors used were recombinant Human VEGF R2 (R&D Systems), VEGF receptor inhibitor SU5416 (Calbiochem, EMD Biosciences, San Diego, CA, USA), IL-8 receptor antagonizing antibody (IL-8 RA, R&D Systems), IL-6 soluble receptor (IL-6 sR, R&D Systems), TNF-α soluble receptor (sTNF-R1, R&D Systems), NF-κB activator inhibitor 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline (Calbiochem), JNK inhibitor SP600125 (Calbiochem), MEK inhibitor PD98059 (Calbiochem), and

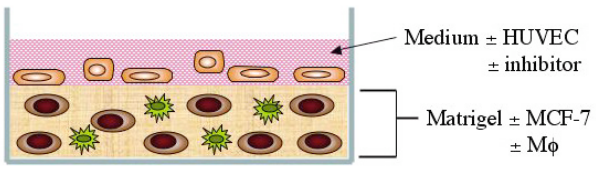

Figure 1: Schematic experimental design for assay of TAMs-induced vascular endothelial cell tube and network formation. Matrigel was mixed with MCF-7 breast cancer cells in the absence or presence of macrophages. After the matrigel was solidified, HUVECs in M199 medium were added and kept incubating at 37°C. As the positive control, the Matrigel was mixed with 100 ng/ml of VEGF. In addition, inhibitors against cytokines and signaling pathways were added to HUVEC suspension to study which cytokines and signaling pathways involved in TAMs-induced vascular endothelial cell tube and network formation. Mφ, macrophage.

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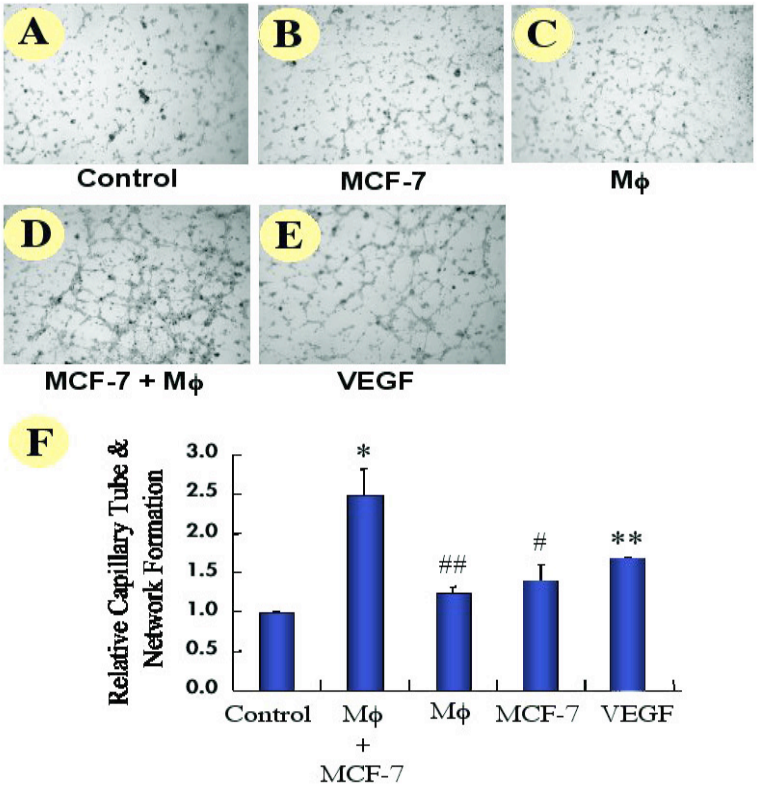

Figure 2: TAMs-induced vascular endothelial cell tube and network formation. To determine the angiogenic activity of TAMs, HUVECs were added to the Matrigel that was kept alone (A) or mixed with macrophages (C) or MCF-7 cells in the absence (B) or presence (D) of macrophages. As the positive control, the Matrigel was mixed with 100 ng/ml of VEGF (E). The relative levels of capillary tube and network formation were quantitated by scaling the total length of capillary tubes and normalized to the level of control (F). The data represent mean ± SD of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ if compared with control by *t*-test. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ if compared with MCF-7 plus macrophages by *t*-test. Mϕ, macrophage.

p38^{MAPK} inhibitor SB202190 (Calbiochem). The formation of vascular endothelial cell capillary tube and network was recorded by microscopic photography, and quantitated by manually scaling the total length of the capillary tubes in the network of endothelial cells.

Detection of VEGF and cytokine production

The levels of IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α in the co-culture media were detected by the Human Inflammation Cytometric Bead Array Kit (BD Bioscience). Briefly, beads of different FL-3 fluorescent intensities were pre-conjugated with monoclonal antibodies against various cytokines by the manufacturer. These beads were incubated with 20 μl of sample media (or cytokine standards) and phycoerythrin (PE)-labeled monoclonal antibodies against various cytokines at room temperature for 3 h. Beads were then subjected to flow cytometric analysis. The amount of a given cytokine in the sample media was calculated by converting the intensity of PE fluorescence to concentration after comparing to results obtained with the cytokine standards. The production of VEGF in the culture supernatant was detected by a sandwich-ELISA using a human VEGF CytoSet^{TM} set (Biosource, Camarillo, CA, USA).

Results

Angiogenic activity induced by the interaction between MCF-7 cells and macrophages

To explore the role of TAMs in tumor angiogenesis, we first determined the angiogenic activity of TAMs by *in vitro* vascular endothelial cell tube and network formation assay. As shown in Figure 2A, endothelial cells could not efficiently form capillary tube and network when they were laid over the Matrigel without VEGF and FBS. Similar results were observed if the Matrigel was mixed only with MCF-7 cells or macrophages (Figure 2B and 2C). However, the Matrigel combined with MCF-7 cells plus macrophages was capable of inducing efficient vascular endothelial cell capillary tube and network formation (Figure 2D). The induction level was superior to that with Matrigel mixing with 100 ng/ml of VEGF (Figure 2D, 2E and 2F).

Time-lapse photography was performed to monitor the whole process of vascular endothelial cell capillary tube and network formation induced by MCF-7 cells plus macrophages. It was observed that capillary tube and immediately subsequent network formation began at ~1.5 h and soon peaked at ~3.5 h. After 6 h, the network gradually broke down and the cells finally aggregated (see supplementary movie online). The represent images in sequence from 0 to 9 h at 0.5-h intervals were shown in Figure 3. This kinetics was parallel with that of VEGF (data not shown).

TNF-α, IL-6, and IL-8 induced by the interaction between MCF-7 cells and macrophages

Many inflammatory cytokines are produced by macrophages and may play roles in the angiogenesis of wound healing. Therefore, we explored what protein factors were induced by the interaction between MCF-7 cells and macrophages. We measured the levels of VEGF, IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α from the culture media of MCF-7 cells, macrophages, and MCF-7 co-cultivated with macrophages, respectively. The data are shown in Table 1. We found that the amounts of VEGF, TNF-α, IL-6, and IL-8 were all elevated in the co-culture medium of MCF-7 cells plus macrophages rather than the medium of MCF-7 cells or macrophages alone. Specifically, the levels of TNF-α, IL-6 and IL-8 were significantly induced during the interaction between MCF-7 cells and macrophages ($P < 0.05$ if compare with MCF-7 cells or macrophages alone). The comparable levels of TNF-α, IL-6 and IL-8 could be measured from the macrophages treated with the conditioned medium from MCF-7 cells (data not shown), confirming that these three cytokines were mainly expressed by macrophages in the co-culture with MCF-7 cells. Other cytokines, such as IL-12p70,

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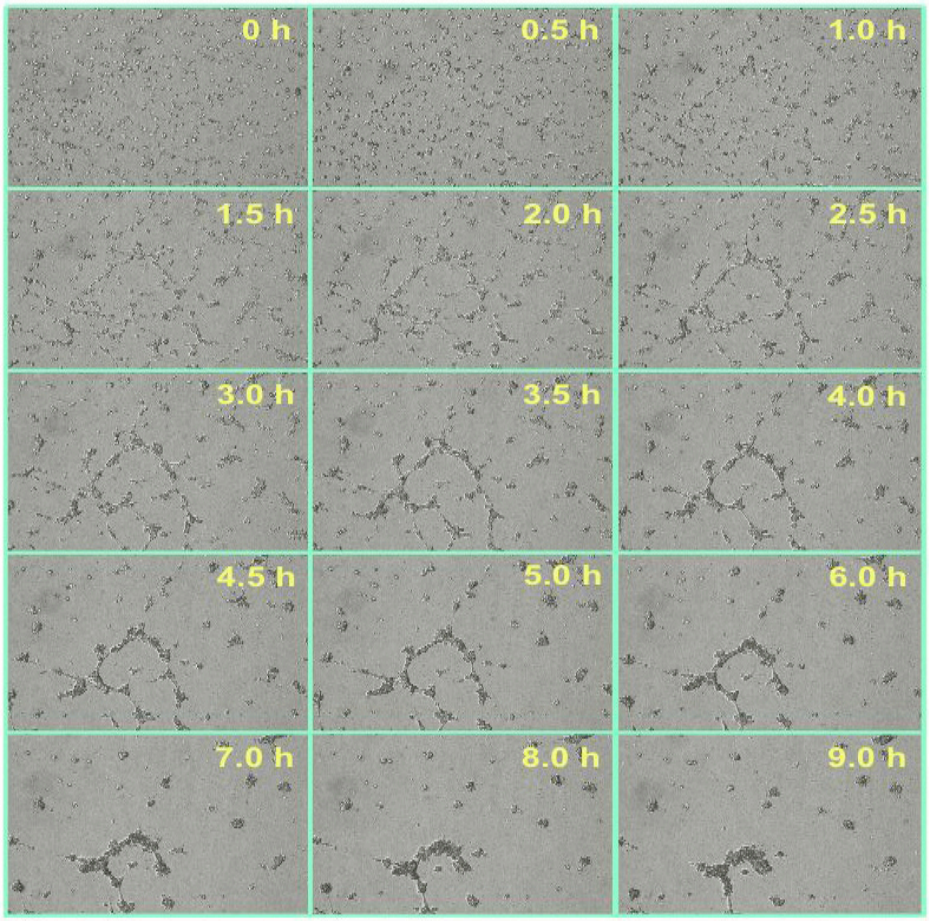

Figure 3: Representative images from monitoring of TAMs-induced vascular endothelial cell capillary tube and network formation by time-lapse photography. HUVECs were laid over the Matrigel that was mixed with MCF-7 cells plus macrophages. It was noted that capillary tube and network formation began to appear at ~1.5 h and soon peaked at ~3.5 h, and after 6 h the network gradually broke down and the cells finally aggregated.

Table 1: Measurement of the levels of VEGF and some inflammatory cytokines in the culture media of MCF-7 cells, macrophages, and MCF-7 cells co-cultivated with macrophages*

	A: MCF-7	B: macrophages	C: MCF-7 + macrophages	*P* value
VEGF	181.1 ± 80.8	< 31.3	284.5 ± 162.8	$P < 0.05$ if B *vs.* C
TNF-α	< 7.8	10.3 ± 5.7	745.0 ± 555.8	$P < 0.05$ if A *vs.* C; $P < 0.05$ if B *vs.* C
IL-1β	< 8.9	< 8.9	15.5 ± 0.4	
IL-6	< 9.4	< 9.4	621.7 ± 376.3	$P < 0.05$ if A *vs.* C; $P < 0.05$ if B *vs.* C
IL-8	< 3.1	293.8 ± 5.0	46196.5 ± 8756.1	$P < 0.01$ if A *vs.* C; $P < 0.001$ if B *vs.* C
IL-10	< 1.5	< 1.5	10.1 ± 6.2	
IL-12p70	< 4.6	< 4.6	< 4.6	

*The data represent mean ± SD of three independent experiments. The unit is pg/ml.

IL-10 and IL-1β, were not induced by co-culture of MCF-7 cells with macrophages (Table 1).

TNF-α, IL-6, and IL-8 involved in TAMs-induced angiogenic activity

We further studied whether VEGF, IL-8, IL-6, TNF-α were involved in the endothelial cell capillary tube and network formation induced by TAMs. The inhibitors against receptors of VEGF (VEGF R2 and SU5416), IL-8 (IL-8 RA), IL-6 (IL-6 sR), and TNF-α (sTNF-R1) were individually added to the vascular endothelial cells that were laid on the Matrigel mixed with MCF-7 cells and macrophages. As shown in Figure 4, the level of endothelial cell capillary tube and network formation was induced with the Matrigel containing MCF-7 cells plus macrophages; however, this induction was significantly inhibited by all the inhibitors if they were respectively present in the vascular endothelial cell suspension. These results suggest that IL-8, IL-6, and TNF-α were all involved in TAMs-induced angiogenic activity.

Angiogenic activity induced by TAMs via NF-κB, ERK and p38^{MAPK}-dependent pathways

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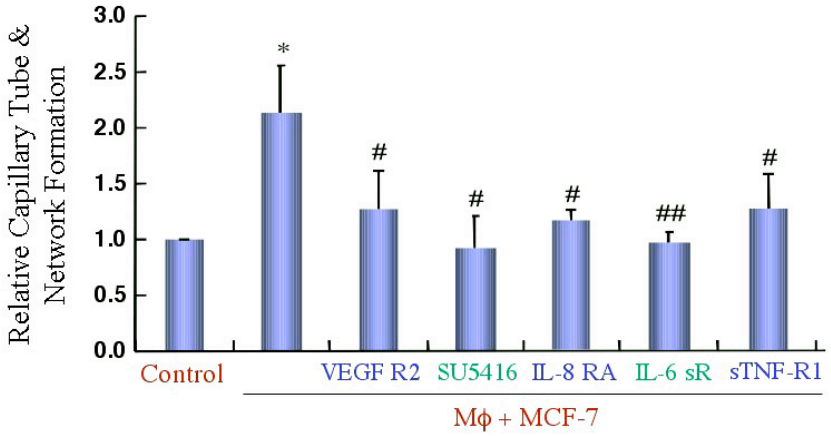

Figure 4: TNF-α, IL-6, and IL-8 involved in TAMs-induced angiogenic activity. The inhibitors against VEGF receptor (10 ng/ml of VEGF R2 and 2 μM of SU5416), IL-8 receptor (500 ng/ml of IL-8 RA), IL-6 receptor (15 ng/ml of IL-6 sR), and TNF-α receptor (50 ng/ml of sTNF-R1) were individually added to HUVECs that were laid on the Matrigel mixed with MCF-7 cells and macrophages. The relative levels of capillary tube and network formation were quantitated by scaling the total length of capillary tubes and normalized to the level of control. The data represent mean ± SD of three independent experiments. $^{*}P < 0.05$ if compared with control by *t*-test. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ if compared with MCF-7 plus macrophages by *t*-test. Mϕ, macrophage.

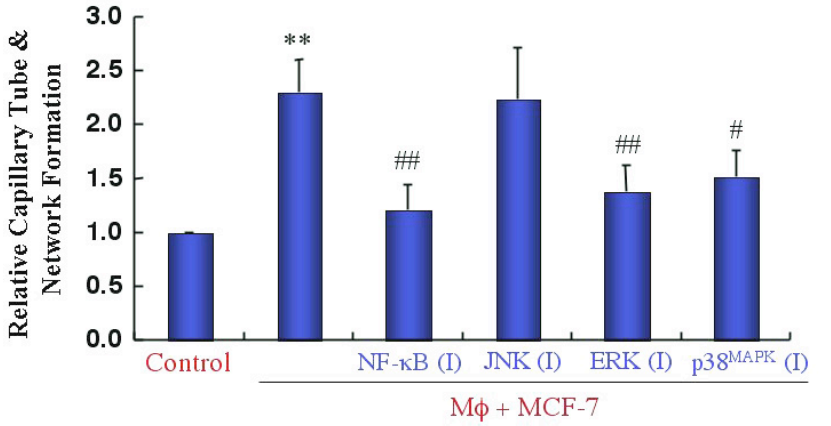

Figure 5: NF-κB, ERK and $p38^{MAPK}$-mediated signaling pathways involved in TAMs-induced angiogenic activity. The NF-κB inhibitor (100 nM), JNK inhibitor (SP600125, 5 μM), MEK inhibitor (PD98059, 5 μM), and p38 inhibitor (SB202190, 5 μM) were individually added to HUVECs that were laid on the Matrigel mixed with MCF-7 cells and macrophages. The relative levels of capillary tube and network formation were quantitated by scaling the total length of capillary tubes and normalized to the level of control. The data represent mean ± SD of three independent experiments. $^{**}P < 0.01$ if compared with control by *t*-test. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ if compared with MCF-7 plus macrophages by *t*-test. Mϕ, macrophage.

Cytokines such as IL-6, IL-8 and TNF-α may induce a broad spectrum of intracellular signal transduction pathways and regulate a series of cellular activities. Therefore, we further investigated which pathways may be involved in the endothelial cell capillary tube and network formation induced by TAMs. We checked NF-κB, JNK, ERK, and $p38^{MAPK}$ signaling pathways by incubating vascular endothelial cells with the specific inhibitors including 6-Amino-4-(4-phenoxyphenylethylamino)quinazoline (NFκB activator inhibitor), SP600125 (JNK inhibitor), PD98059 (MEK inhibitor), and SB202190 ($p38^{MAPK}$ inhibitor). In Figure 5, we found that the inhibitors against NF-κB, ERK and $p38^{MAPK}$ signaling pathways were able to significantly inhibit the vascular endothelial cell tube and network formation induced by the communication between MCF-7 cells and macrophages. However, the JNK inhibitor did not inhibit this TAMs-induced angiogenic activity. Hence, TAMs-induced vascular endothelial cell tube and network formation can be mediated through the activation of NF-κB, ERK and $p38^{MAPK}$ signaling pathways in vascular endothelial cells following exposure to IL-6, IL-8 and TNF-α.

Discussion

Tumor angiogenesis is an important process required for continuous growth and spreading of solid tumors. Beyond a certain size, a solid tumor exhibits multiple areas of hypoxia because of insufficient vascular supply of oxygen and nutrients to meet the metabolic demands. The tissue in times of hypoxic and necrotic stress will express a broad array of protein factors. Many factors, such as monocyte chemotactic protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF), are potent chemokines chemotactic toward the monocytes in nearby blood vessels [11,12]. Monocytes are thus continually recruited into tumors, differentiated into macrophages, and then accumulate in the hypoxic areas. After affected by cancer cells and hypoxic microenvironment, macrophages are induced to express several growth factors, cytokines and tissue matrix-modulating proteins that are thought to associate with promotion of tumor cell invasion and tumor angiogenesis [2]. The accumulating evidence has suggested that high TAM infiltration is advantageous to tumor spreading, most notably via enhancement of tumor angiogenesis and tumor cell migration and invasion [2,12,13]. This is consistent with the clinical findings that high levels of TAMs correlate with poor prognosis in various types of cancer [2]. However, the detailed mechanisms accounting for how TAMs facilitate tumor angiogenesis and tumor cell migration/invasion still need to be explored.

Many inflammatory cytokines are involved in macrophages-associated angiogenesis during the wound healing process [5,14,15]. In this study, we used *in vitro* assay of vascular endothelial cell tube and network formation to demonstrate the involvement of IL-6, IL-8 and TNF-α in TAMs-induced angiogenic activity. IL-8 was a growth-stimulating cytokine to vascular endothelial cells and induced angiogenesis in animal [16]. Koch *et al.* found that the culture medium of macrophages had the same effects, and this effect was markedly inhibited by adding anti-IL-8 antibody. This result suggests that IL-8 contained in the macrophage

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culture medium had a functional role in macrophage-induced angiogenesis [17]. TNF-α is also one of the major cytokines involved in macrophage-induced angiogenesis. TNF-α was a growth-inhibitory and even cytotoxic protein when it was overexpressed in endothelial cells [18,19]. However, exogenous application of TNF-α indeed promoted formation of new blood vessels in several *in vivo* models [20,21]. The ability of anti-TNF-α antibodies to completely neutralize the angiogenic activity in the culture medium of activated macrophages confirmed the role of TNF-α as an angiogenic molecule secreted by macrophages [22]. In addition, macrophages are an important source of IL-6. Some *in vitro* studies had reported that IL-6 had no effect or even growth-inhibitory effect on endothelial cells [23,24]. IL-6 can be synergistic with other cytokines to promote angiogenesis, or it can contribute to the vascular formation by modulating the tissue matrix via induction of target proteins like MMPs and VEGF. MMPs are capable of regulating the release of some angiogenic factors by cleaving off their binding to cell surface or tissue matrix. VEGF not only serves as a potent mitogen to vascular endothelial cells, but is also a vascular permeability factor that causes continual extravasation of fibrin and fibronectin to generate tissue matrix [1].

Our data have suggested that TAMs could induce tumor angiogenesis in a TNF-α, IL-8 and IL-6-dependent manner. Furthermore, we studied what intracellular signaling pathway(s) was required for TAMs-induced vascular endothelial cell tube and network formation, and found that the TAMs-induced angiogenic activity was dependent on NF-κB, ERK and $p38^{MAPK}$-mediated pathways. The detailed mechanisms accounting for how these pathways involved in angiogenic process remain to be investigated. TAMs have been reported to induce cancer cell invasion through TNF-α-dependent activation of NF-κB and JNK pathways in cancer cells [25]. Angiogenesis requires regulation of vascular endothelial cell migration and invasion. Our data suggest that different pathways were adopted by cancer cells and vascular endothelial cells to achieve cell migration and invasion.

In conclusion, we used the *in vitro* assay of capillary tube and network formation of vascular endothelial cells to confirm the angiogenic function of TAMs. With this assay, we provided evidence that TNF-α, IL-8 and IL-6 indeed participated in the TAMs-induced angiogenic activity. Our data also suggest that TAMs-induced tumor angiogenesis was via NF-κB, ERK and $p38^{MAPK}$-mediated pathways. MAPK has been reported to regulate cytoskeleton remodeling and $p38^{MAPK}$ inhibitors were shown to inhibit the migration of endothelial cells [26,27]. Further mechanistic studies regarding how these pathways involved in tumor angiogenesis need to be explored.

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