

# Angiogenic changes in co-cultures of mast cells and myocardial microvascular endothelial cells under hyperglycemic conditions

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**Abstract.** The aim of the present study was to determine the correlation between angiogenesis and the differential expression of growth factors and their receptors when myocardial microvascular endothelial cells (MMVECs) were co-cultured with mast cell granules (MCGs) under hyperglycemic conditions. MMVECs and mast cells (MCs) were isolated from Wistar rats. An *in vitro* angiogenesis assay was used to observe any differences when MMVECs were co-cultured with MCGs in normal or hyperglycemic medium. The mRNA and protein expression of growth factors and their receptors were analyzed by real-time reverse transcription (RT)-PCR and western blot analysis. Real-time RT-PCR analysis demonstrated the upregulated mRNA and protein expression of vascular endothelial growth factor (VEGF) in the MMVECs; however, the expression of its receptor, fms-like tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1), decreased significantly, and the angiogenic ability of the MMVECs decreased under hyperglycemic conditions. The angiogenic ability of the MMVECs cultured under hyperglycemic conditions (even after the addition of MCGs) was inferior to that of the MMVECs cultured under normal glucose conditions. The specific inhibitor of tryptase, N-tosyl-L-lysine chloromethyl ketone (TLCK), suppressed angiogenesis regardless of the glucose concentration, and the specific inhibitor of chymase, N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), was not as effective as TLCK, which was mainly detected under hyperglycemic conditions. High glucose levels have a profound effect on angiogenesis; this effect may be more pronounced than the effects of MCGs on angiogenesis.

## Introduction

Mast cell (MC) functions have classically been associated with allergic responses (1), with previous studies indicating that these cells contribute to other common diseases, such as atherosclerosis, aortic aneurysm and cancer (2-4). Liu *et al* (5) found that MCs also contribute to obesity and diabetes. Mechanism studies have demonstrated that MCs contribute to white adipose tissue (WAT) and muscle angiogenesis. Aside from supplying WAT with nutrients, microvessels also provide a path for leukocyte infiltration followed by adipokine release (6,7). Reduced angiogenesis should limit nutrient supply, thereby impairing cell viability (8,9), and the inhibition of angiogenesis blocks adipose tissue development in mice.

Angiogenesis is a regulated balance between stimulatory and inhibitory factors. This process is regulated by several pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), angiopoietin-1 (Ang-1), fibroblast growth factor-2 [(FGF-2), also known as basic fibroblast growth factor (bFGF)], as well as anti-angiogenic factors, such as thrombospondin-1, angiostatin and Ang-2. Among various angiogenic factors, angiogenesis is mainly regulated by the interplay between VEGF and angiopoietins (10,11). A number of studies have shown that VEGF and angiopoietins are associated with diabetes in angiogenesis (12,13). However, angiogenesis in diabetes, which is associated with the dysregulation of neovascularization, has also been recognized. The molecular defects underlying these angiogenic abnormalities have generated much interest but remain elusive.

Microvascular endothelial cells are commonly used to study the mechanisms of diabetic complications, since they play an essential role in the abnormal angiogenesis process of several diseases, including diabetes mellitus. In the present study, we established a co-culture system to investigate the correlation between angiogenesis and various growth factors *in vitro*.

## Materials and methods

**Reagents.** All cell culture reagents, alamarBlue<sup>®</sup> cell viability reagent, TRIzol reagent, the SuperScript<sup>™</sup> III First-Strand Synthesis System for reverse transcription (RT)-PCR and Platinum SYBR-Green qPCR SuperMix were purchased from

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Invitrogen Life Technologies, Inc. (Grand Island, NY). Primary antibodies against VEGF, fms-like tyrosine kinase-1 (Flt-1), fetal liver kinase-1 (Flk-1),  $\beta$ -actin and secondary antibodies against either rabbit or mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL). All other reagents were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

*Cell culture of myocardial microvascular endothelial cells (MMVECs).* Wistar male rats [8-9 weeks of age; Shanghai Laboratory Animal Center (SLAC), Shanghai Institutes for Biological Science, Shanghai, China] were used for primary MMVEC isolation. The Animal Care Committee of Shanghai Jiaotong University approved the animal protocols. MMVECs were isolated as previously described (14). Briefly, the rats were anesthetized with sodium pentobarbital (60 mg/kg). After thoracotomy, the hearts were rapidly removed and washed in phosphate-buffered saline (PBS). The atria, visible connective tissue, valvular tissue, the right ventricle, and the epicardial and endocardial surfaces of the left ventricle were carefully removed, and the remaining myocardial tissue was cut into sections (1 mm<sup>3</sup>). Myocardial tissues were then seeded on culture plates pre-coated with rat-tail tendon gelatin and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. After a 40-min attachment period, the tissues were cultured in DMEM (25 mmol/l D-glucose) supplemented with 20% fetal bovine serum (FBS), 50 U/ml heparin, 100 U/ml penicillin and 100 g/ml streptomycin. The tissue pieces were discarded and the medium was changed after approximately 70 h. MMVECs were identified by their typical 'cobblestone' appearance with positive CD31 and CD34 immunostaining. Only MMVECs at second passage were used for experiments; cells were allowed to grow to 80-90% confluence followed by serum-starvation for 24 h before the experiments commenced.

*Collection and isolation of MCs.* MCs were isolated as previously described, with minor modifications to the procedure (15). Briefly, MCs from the peritoneal cavities of male Wistar rats (14-16 weeks of age; SLAC, Shanghai Institutes for Biological Science) were collected by lavage with 15 ml of RPMI-1640 (1% FBS). After a 1-h attachment period in the incubator, non-adherent cells, mainly MCs, were separated by using percoll density gradient centrifugation at 2,500 rpm for 15 min at 4°C. Cells that remained at the percoll interface were aspirated and re-suspended in PBS. MCs were washed in PBS twice and cultured in RPMI-1640, supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 mg/ml streptomycin. MCs isolated by this procedure exceeded 90% purity based on staining with 0.05% toluidine blue O.

*Preparation of MC granules (MCGs).* The standard incubation was conducted in 200  $\mu$ l of RPMI-1640 containing 5.0x10<sup>6</sup> cell/ml MCs. After the MCs were pre-incubated at 37°C for 15 min, compound 48/80 (5  $\mu$ g/ml), a non-cytotoxic MC-specific stimulator, was added to stimulate the MCs for 1 min. The MCs were centrifuged at 800 x g for 5 min and the supernatant containing the material released from the stimulated MCs was stored at -80°C for further experiments (16). The tryptase and chymase activity of the MCGs was measured before each experiment,

using the same method as previously described (17). In principle, tryptase and chymase activities were quantified using the chromogenic substrates, *N* $\alpha$ -benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) and *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SAAPP), respectively. Tryptase activity was determined by its ability to cleave a synthetic substrate, BAPNA (2 mmol/l) in Tris-HCl (0.1 mol/l; pH 8.0) and glycerol (1 mol/l) at 410 nm, while chymase activity was determined spectrophotometrically by the rate of hydrolysis of SAAPP (0.7 mmol/l) in NaCl (1.5 mol/l) and Tris (0.3 mol/l; pH 8.0) at 405 nm. Protease activity was expressed in milliunits/millilitre (mU/ml), in which 1 unit of enzyme activity was defined as the 1  $\mu$ mol of degraded substrate/min at 25°C. The tryptase activity of the MCGs used in the present study was 9.46 mU/ml, and the chymase activity was 3.57 mU/ml.

*MMVEC-MC co-culture.* MMVECs (1x10<sup>5</sup> cells/well) and MCGs obtained from 1x10<sup>5</sup> activated MCs were co-cultured in DMEM with low (5.56 mmol/l) and high glucose levels (25 mmol/l). The inhibitors of tryptase [N-tosyl-L-lysine chloromethyl ketone (TLCK)] and chymase [N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)] were used in further experiments when MMVECs were grown to 85-90% confluence before being quiesced in DMEM with 1% FBS for 24 h and treated with MCGs.

*Cell proliferation assay.* The cell proliferation rate was assessed by cell counting using alamarBlue<sup>®</sup> cell viability reagent. Second passage MMVECs were seeded onto 96-well cell culture plates at an initial density of 5x10<sup>3</sup> cells/well (100  $\mu$ l) in DMEM with 5% FBS in the presence or absence of MCGs. Following overnight incubation, 10  $\mu$ l of alamarBlue<sup>®</sup> cell solution were added and further incubated for 2 h. Cell growth rate was quantified by the difference in absorbance at 570 and 600 nm using a microplate reader (Tecan Group Ltd., Männedorf, Switzerland), and the absorbance reading was repeated for 5 consecutive days to generate a cell growth curve.

*Cell migration assay.* Cell migration was evaluated using a transwell chamber assay (Corning Inc., Corning, NY). In brief, second passage MMVECs were trypsinized, resuspended in 200  $\mu$ l serum-free DMEM (4x10<sup>4</sup> cells/well) and were then placed onto the upper chamber, while another 800  $\mu$ l of DMEM with a different glucose concentration and 2% FBS were placed in the lower compartment of the transwell chamber. MCGs, TLCK or TPCK were also added to examine their effects on cell migration activity, as described above. Following incubation for 12 h at 37°C, allowing time for the cells to migrate from the upper to the lower chamber, cells that migrated to the lower side of the transwell inserts were washed, fixed with methanol for 10 min and then stained with crystal violet dye for cell counting. Cell migration ability was quantified by an average of cells in 5 random microscopic fields (x200 magnification) at the lower side of the transwell insert.

*Cell scratch wound healing assay.* Wound healing assay was performed as described previously (18). Once second passage MMVECs reached approximately 90% confluence in 24-well plates, a wound was made with a 200  $\mu$ l pipette tip. The cells were washed with PBS and further incubated in DMEM with a

different glucose concentration and 1% FBS in the presence or absence of MCGs. The effects of TLCK and TPCK on MCGs were also examined. After 24 h of incubation, cultured cells were photographed and cell migration activity was quantified with ImageJ software (version 1.37) by measuring the area of the cells that moved beyond a reference line.

**Capillary-like tube formation assay on Matrigel.** Matrigel (BD Biosciences, Bedford, MA) was used for the capillary-like tube formation assay. Briefly, 100  $\mu$ l/well of this matrix solution were added to a 24-well culture plate and 30 min were allowed for gelation at 37°C. MMVECs obtained as described above, which had been resuspended in DMEM with a different glucose concentration and 1% FBS, were added to the top of the Matrigel ( $5 \times 10^4$  cells/well) and incubated for 18 h at 37°C. Tube formation was carefully observed either in the presence or absence of MCGs, and the effects of TLCK and TPCK were also examined. Once cell culture images were captured, tube structures were quantified by counting all branches in 3 random fields from each well (19). Each experiment was repeated 3 times.

**Real-time RT-PCR.** Total RNA was extracted from MMVECs using TRIzol Reagent according to the manufacturer's instructions. RNA (2  $\mu$ g) was then reverse-transcribed into cDNA using the SuperScript™ III First-strand Synthesis System for RT-PCR and was further amplified by SYBR-Green RT-PCR amplification with specific oligonucleotides as follows: VEGF sense, 5'-GCA CTG GAC CCT GGC TTT AC-3' and antisense primer, 5'-CTG CAG GAA GCT CAT CTC TC-3'; Flk-1 sense, 5'-ACA GTT CCC AGA GTG GTT GG-3' and antisense primer, 5'-GTC ACT GAC AGA GGC GAT GA-3'; Flt-1 sense, 5'-CCA CTT CTG TCT TGC CAC ACA-3' and antisense primer, 5'-CCA ACC AAT TAA GAC CTT CTG-3'; and  $\beta$ -actin sense, 5'-CAC CCG CGA GTA CAA CCT TC-3' and antisense primer, 5'-CCC ATA CCC ACC ATC ACA CC-3'. The SYBR-Green RT-PCR amplification was carried out in a 25  $\mu$ l reaction volume that contained 12.5  $\mu$ l 2X Platinum SYBR-Green qPCR SuperMix, 200 nM each of the forward and reverse primer, and 2  $\mu$ l of diluted cDNA using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad, Richmond, CA). The thermal profile for SYBR-Green real-time RT-PCR was conducted for 40 cycles under the following conditions: denaturation at 94°C for 30 sec, annealing at optimal temperature for each primer pair for 30 sec, and extension at 72°C for 45 sec. Final extension was at 72°C for 10 min. A single amplification product was confirmed by running a melting curve for all PCRs. Each sample was assayed in triplicate.

**Western blot analysis for VEGF, Flt-1 and Flk-1.** MMVECs were lysed in lysis buffer, and the protein content was measured using the BCA assay (both from Beyotime, China). Equal amounts of protein were loaded and separated onto an 8 to 12% Tris-glycine gel and transferred onto PVDF membranes. Primary antibodies against VEGF (1:500 dilution), Flt-1 (1:500 dilution), Flk-1 (1:500 dilution) and  $\beta$ -actin (1:500 dilution) were added to the membranes, incubated overnight at 4°C, followed by reaction with appropriate secondary antibodies (1:4,000 dilution, goat anti-rabbit IgG and goat anti-mouse IgG-HRP linked; Cell Signaling Technology) for 2 h. ECL

reagent was then used to determine the immunoreaction, which was measured by densitometry on X-ray film, using GIS software (Bio-Tanon, Shanghai, China). All western blot analysis experiments were repeated at least 3 times with different cell preparations.

**Statistical analysis.** Data are presented as the means  $\pm$  SD. Statistical significance was assessed by one-way ANOVA followed by post hoc analysis using the Student-Newman-Keuls test. A P-value <0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL).

## Results

**Impaired angiogenesis of MMVECs in high glucose.** MMVEC proliferation, migration and capillary-like tube formation are important components of the process of angiogenesis. The proliferation, migration and capillary-like tube formation ability of the MMVECs co-cultured with MCGs increased significantly in spite of high or low glucose. However, the MMVECs cultured under low glucose conditions showed a higher proliferation rate compared with those co-cultured with MCGs under hyperglycemic conditions from day 3 to 5 (Fig. 1) (P<0.05). This phenomenon was also observed in both cell scratch wound healing assays and the capillary-like tube formation of MMVECs cultured under low glucose conditions compared to those co-cultured with MCGs under hyperglycemic conditions (Fig. 1). The effect of MCGs on MMVECs was significantly inhibited by the addition of TLCK and TPCK, resulting in even lower migration ability and capillary-like tube formation (P<0.05, respectively). There was no significant difference observed between low and high glucose conditions when TLCK was added to the MMVECs co-cultured with MCGs. When TPCK was added to the MMVECs co-cultured with MCGs under low glucose conditions, there was a significant increase in migration ability and capillary-like tube formation compared to the MMVECs co-cultured with MCGs under high glucose conditions (Fig. 2) (P<0.05).

**Analysis of the mRNA expression changes by qRT-PCR.** VEGF mRNA expression increased in a time-dependent manner in the MMVECs co-cultured with MCGs within 2 h, with the peak occurring after 4 h of culture in hyperglycemic medium and after 8 h of culture in low-glucose medium (P<0.05). VEGF mRNA expression in the MMVECs cultured under hyperglycemic conditions was significantly higher compared to that in MMVECs cultured under low glucose conditions, in spite of the MCGs, and this increase continued for up to 24 h (Fig. 3A). Flt-1 mRNA expression in the MMVECs decreased in a time-dependent manner, with a peak present after 4 h of culture under hyperglycemic conditions and after 8 h of culture under low glucose conditions (P<0.05). Flt-1 mRNA expression in the MMVECs co-cultured with MCGs was significantly higher than that in MMVECs cultured in different concentrations of glucose. Flt-1 mRNA expression in the MMVECs co-cultured with MCGs under hyperglycemic conditions was significantly lower compared to that in the MMVECs cultured under low glucose conditions and this

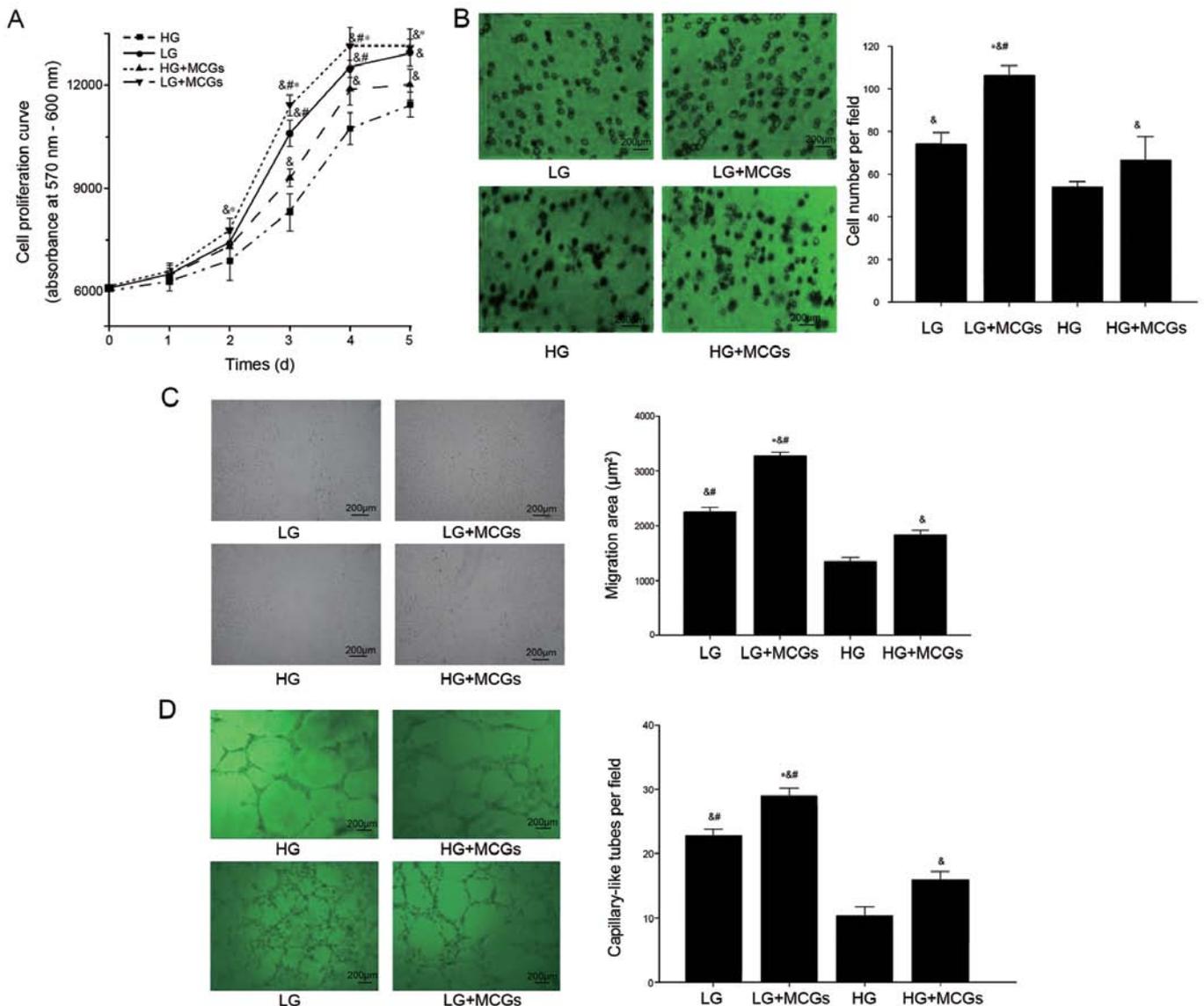


Figure 1. Angiogenic changes under different conditions *in vitro*. (A) Cell proliferation curve was obtained by alamarBlue<sup>®</sup> cell viability reagent. (B-D) Representative images of migration across the Transwell membrane, scratch wound healing and capillary-like tube formation, captured under a contrast phase microscope (x200). Values are presented as the means  $\pm$  SE (n=3). <sup>&</sup>P<0.05 vs. HG; <sup>#</sup>P<0.05 vs. HG + MCGs; and <sup>\*</sup>P<0.05 vs. LG. HG, high glucose; LG, low glucose; MCGs, mast cell granules.

tendency continued for up to 48 h (Fig. 3B). Flk-1 mRNA expression in the MMVECs decreased in a time-dependent manner. Flk-1 mRNA expression in the MMVECs co-cultured with MCGs was significantly higher than that in MMVECs cultured in different concentrations of glucose. Flk-1 mRNA expression in the MMVECs co-cultured with MCGs under hyperglycemic conditions was significantly lower compared to that in MMVECs cultured under low glucose conditions and the tendency continued for up to 48 h (Fig. 3C).

The addition of either TLCK or TPCK to the co-culture system abolished the effects of MCGs on VEGF, Flt-1 and Flk-1 mRNA expression in the MMVECs cultured in different concentrations of glucose; the effects of TLCK however, were more significant. However, the VEGF, Flt-1 and Flk-1 mRNA expression levels in the MMVECs co-cultured with MCGs following the addition of TLCK were much lower in the

absence of MCGs. When the MMVECs were co-cultured with MCGs under hyperglycemic conditions, following the addition of TLCK, VEGF and Flt-1 expression significantly decreased (Fig. 4).

**VEGF, Flt-1 and Flk-1 protein expression.** VEGF protein expression increased in the MMVECs co-cultured with MCGs with the increase commencing at 2 h and reaching a peak at 8 h (Fig. 5) (P<0.05). Flt-1 protein expression decreased in a time-dependent manner in the MMVECs. Flt-1 protein expression in the MMVECs co-cultured with MCGs was significantly higher than that in the MMVECs exposed to different concentrations of glucose. Flt-1 protein expression in the MMVECs cultured under hyperglycemic conditions was significantly lower than that in MMVECs exposed to low glucose levels (Fig. 5B). Flk-1 protein expression decreased in a time-dependent manner

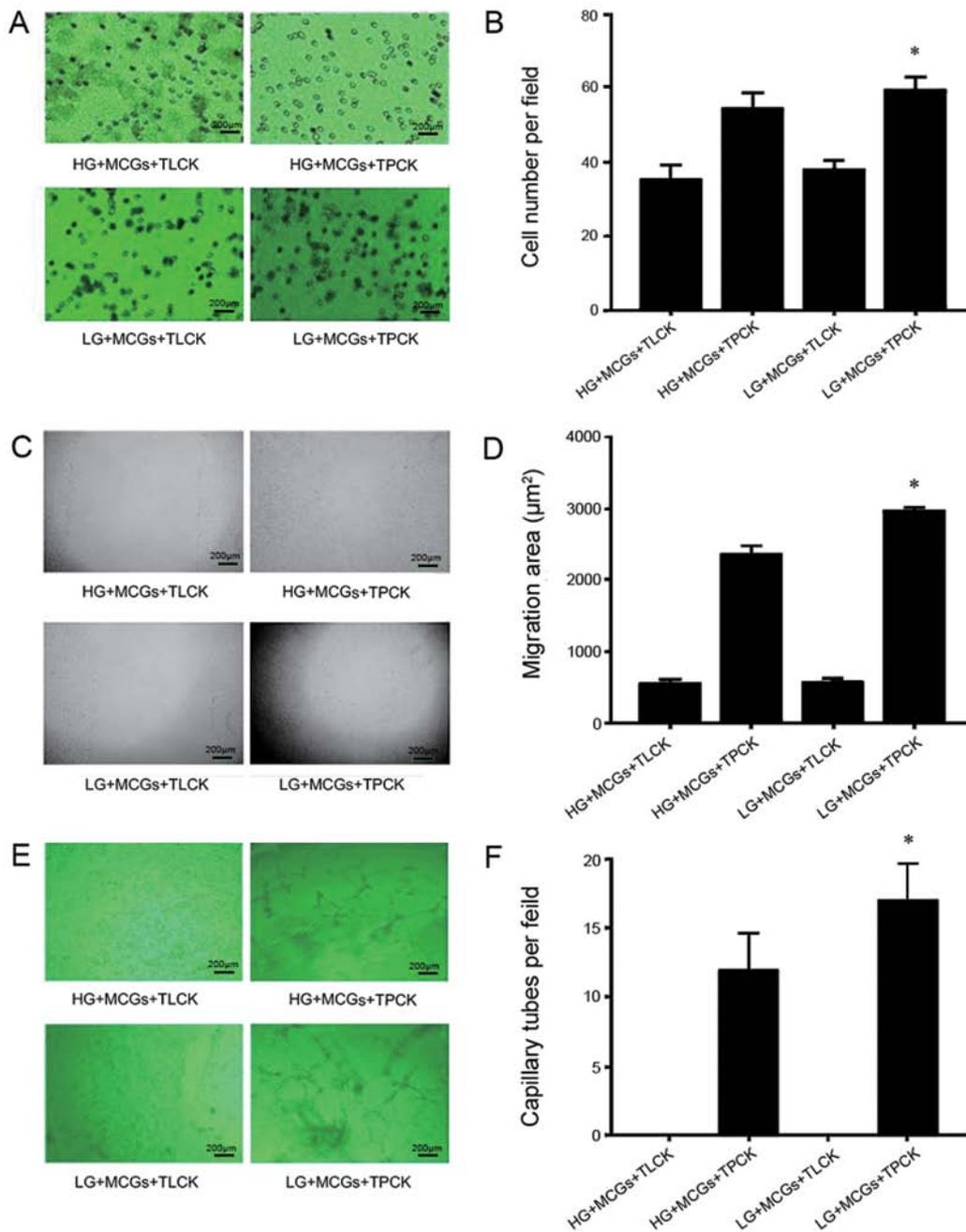


Figure 2. Effect of TLCK or TPCK on proliferation, scratch wound healing and tube formation of myocardial microvascular endothelial cells (MMVECs) cultured in normal and high-glucose medium. Shown are representative (A, C and E) images and (B, D and F) values obtained for cell number, migration area and capillary tube formation, respectively in the cells treated with TLCK or TPCK. (\* $P < 0.05$  vs. HG + MCGs + TPCK). TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; HG, high glucose; LG, low glucose; MCGs, mast cell granules.

in the MMVECs. The Flk-1 protein expression levels in the MMVECs co-cultured with MCGs was significantly higher than that in MMVECs exposed to different glucose concentrations. Flk-1 protein expression in the MMVECs co-cultured with MCGs under hyperglycemic conditions was significantly lower compared to that in MMVECs exposed to low glucose and this tendency continued for up to 48 h (Fig. 5C).

The addition of either TLCK or TPCK to the co-culture system abolished the effects of MCGs on VEGF, Flt-1 and Flk-1 protein expression in the MMVECs exposed to different concen-

trations of glucose; the effects of TLCK though were more significant. The protein expression of VEGF, Flt-1 and Flk-1 following the addition of TLCK to the MMVECs co-cultured with MCGs was much lower than in the MMVECs cultured without MCGs. The protein expression of Flt-1 and Flk-1 in the MMVECs co-cultured with MCGs, after the addition of TLCK under low glucose conditions, was significantly higher than that in the MMVECs cultured without MCGs. However, it is noteworthy that this phenomenon was not observed in the MMVECs cultured under hyperglycemic conditions (Fig. 6).

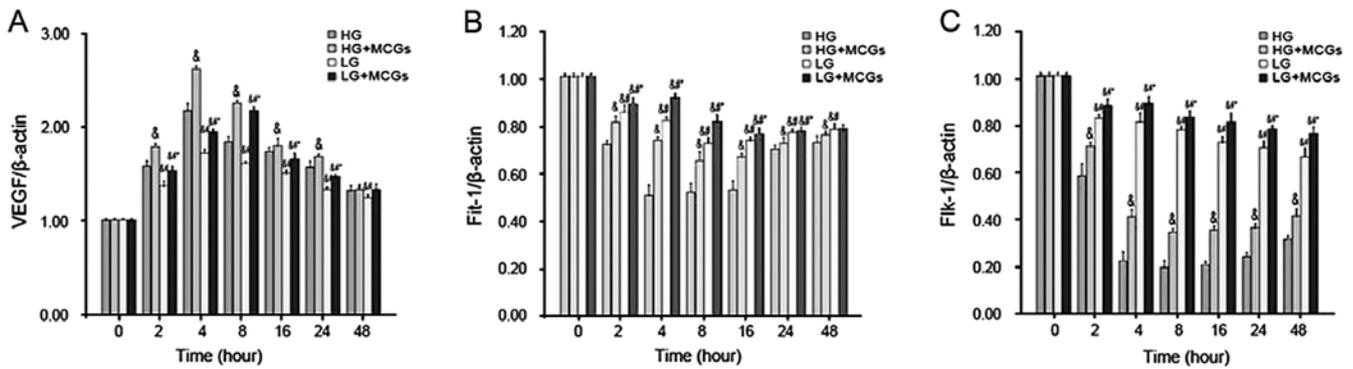


Figure 3. Vascular endothelial growth factor (VEGF), fms-like tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1) mRNA expression in myocardial microvascular endothelial cells (MMVECs). (A) VEGF expression increased significantly in MMVECs cultured with MCGs at 2 h, VEGF expression reached at its peak point at 4 h in the HG group, and at 8 h in the LG group. Even in the presence of MCGs, VEGF expression in the LG group was significantly lower than that in the HG group ( $P < 0.05$ ). (B) Flt-1 expression decreased significantly in the MMVECs at 2 h ( $P < 0.05$ ), and reached at its low point at 4 h in the HG group, and at 8 h in the LG group. (C) Flt-1 expression was significantly different between the HG and LG group ( $P < 0.05$ ). Flt-1 expression was significantly higher in the presence of MCGs. Flk-1 expression decreased significantly in the HG group at 2 h, and reached its low point at 8 h. Even in the presence of MCGs, Flk-1 expression in the HG group was significantly lower than that in the LG group ( $P < 0.05$ ).  $\delta P < 0.05$  vs. HG;  $\# P < 0.05$  vs. HG + MCGs; and  $\# P < 0.05$  vs. LG. HG, high glucose; LG, low glucose; MCGs, mast cell granules.

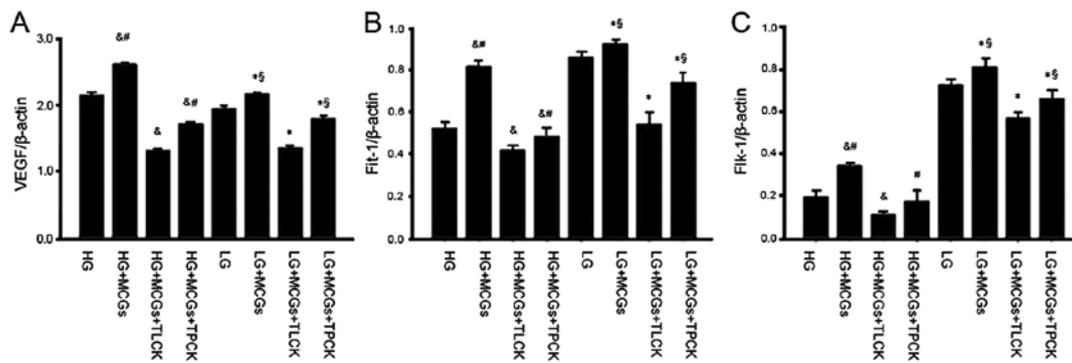


Figure 4. Vascular endothelial growth factor (VEGF), fms-like tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1) mRNA expression in myocardial microvascular endothelial cells (MMVECs) cultured in the presence of TLCK or TPCK. The mRNA expression of VEGF, Flt-1 and Flk-1 decreased significantly in the presence of TLCK or TPCK, particularly in the presence of TLCK, though the mRNA expression of VEGF in the LG group was not significantly different from that in the HG group; however, the expression of Flt-1 and FLK-1 in the LG group was significantly higher than that in the HG group ( $P < 0.05$ ).  $\delta P < 0.05$  vs. HG;  $\# P < 0.05$  vs. HG + MCGs + TLCK;  $* P < 0.05$  vs. LG; and  $\# P < 0.05$  vs. LG + MCGs + TLCK. TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; HG, high glucose; LG, low glucose; MCGs, mast cell granules.

## Discussion

Angiogenesis is tightly regulated by pro- and anti-angiogenic factors. In tumor models, MCs have been shown to play a decisive role in inducing the angiogenic switch that precedes malignant transformation. MCs are a rich source of several potent angiogenic factors, including VEGF, bFGF, transforming growth factor (TGF)- $\beta$ , tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-8 (20,21). In our study, we found that a close correlation exists between angiogenesis and various growth factors in MMVECs co-cultured with MCGs. In our co-culture system, MCGs increased the migration and lumen formation in MMVECs, as well as the expression of VEGF, Flt-1 and Flk-1.

VEGF, as a major regulator of angiogenesis, binds and activates 2 tyrosine kinase receptors, Flt-1 and Flk-1. These receptors regulate physiological, as well as pathological

angiogenesis. Flk-1 has strong tyrosine kinase activity, and transduces the major signals for angiogenesis. Flk-1 is a direct signal transducer for pathological angiogenesis, including cancer and diabetic retinopathy. Although the affinity of Flt-1 to VEGF is more than 10-fold that of Flk-1 (22), Flk-1 plays an important role in proliferation, migration and the survival process. Thus, Flk-1 and its signaling appear to be critical targets for the suppression of these diseases. It has been established that high glucose levels are the direct cause of capillary vessel damage in diabetes mellitus (23,24). Sasso *et al* (25) found that the expression of VEGF in individuals with chronic coronary artery disease and diabetes mellitus was significantly higher compared to individuals who did not suffer from diabetes mellitus; however, in our study, even in the presence of MCGs exposed to high glucose, this tendency still existed. The expression of VEGF in the MMVECs exposed to high glucose was significantly higher than that in the MMVECs

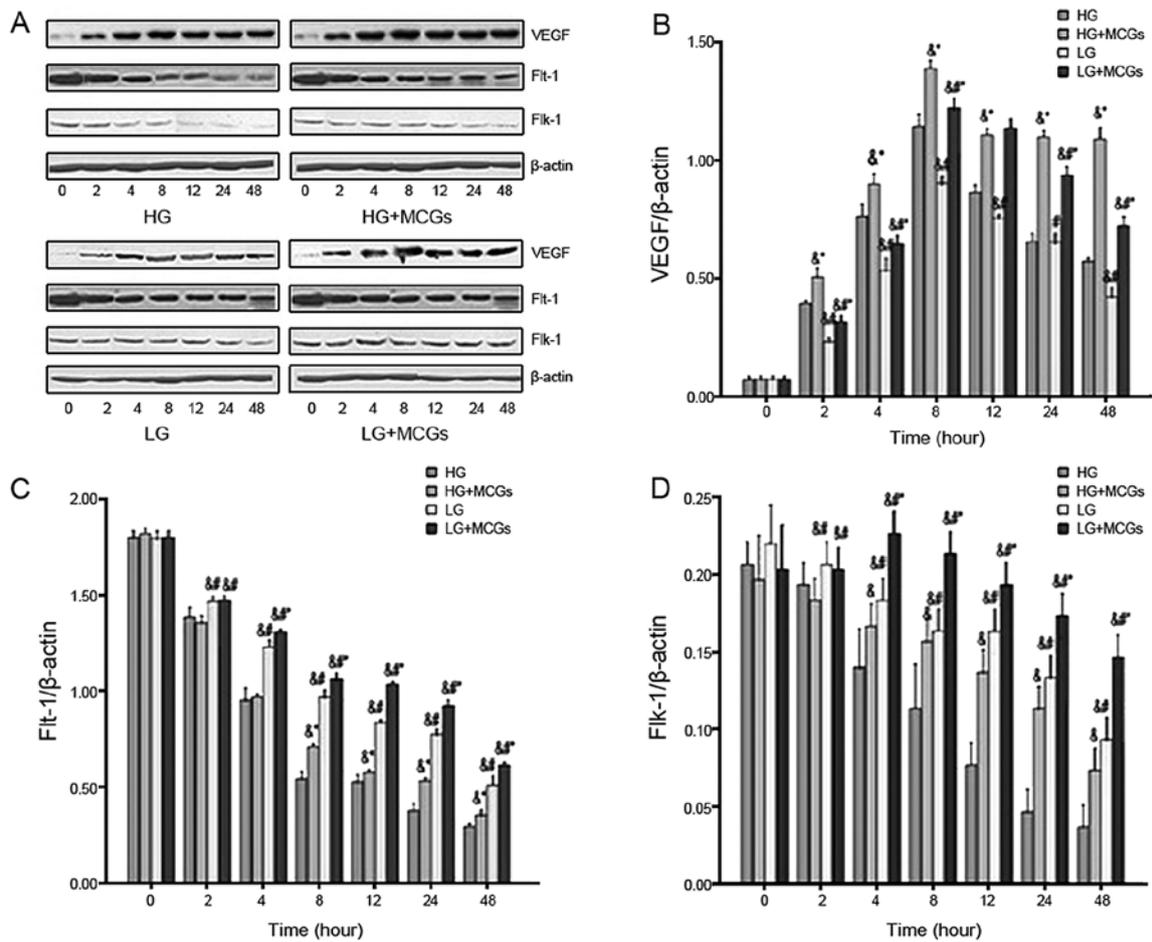


Figure 5. Vascular endothelial growth factor (VEGF), fms-like tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1) protein expression in myocardial microvascular endothelial cells (MMVECs) cultured under different conditions. (A) Western blot analysis of VEGF, Flt-1 and Flk-1 protein expression in MMVECs cultured under high or low glucose conditions without or with MCGs. (B) VEGF expression increased significantly in MMVECs cultured with MCGs at 2 h, and reached at its peak point at 8 h. In the presence of MCGs, VEGF expression was significantly higher than that in absence of MCGs ( $P < 0.05$ ). (C) Flt-1 expression decreased significantly in the MMVECs at 2 h. The expression of Flt-1 in the LG group was significantly high than that in the HG group and Flt-1 expression was significantly higher in presence of MCGs. (D) Flk-1 significantly decreased significantly at 2 h. Even in the presence of MCGs, Flk-1 significantly in the HG group was significantly lower than that in the LG group. (\* $P < 0.05$  vs. HG; # $P < 0.05$  vs. HG + MCGs; and \$ $P < 0.05$  vs. LG). HG, high glucose; LG, low glucose; MCGs, mast cell granules.

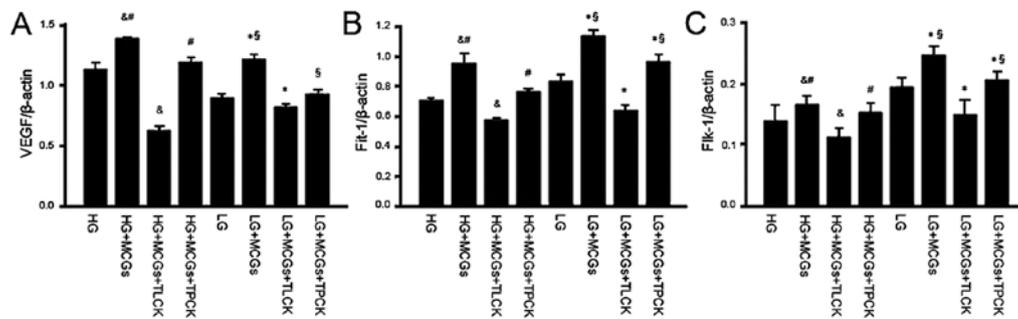


Figure 6. Vascular endothelial growth factor (VEGF), fms-like tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1) protein expression in myocardial microvascular endothelial cells (MMVECs) in the presence of TLCK or TPCK. The expression of VEGF, Flt-1 and Flk-1 decreased significantly in the presence of TLCK. The expression of VEGF, Flt-1 and Flk-1 in the LG group was significantly higher than that in the HG group ( $P < 0.05$ ). \* $P < 0.05$  vs. HG; # $P < 0.05$  vs. HG + MCGs + TLCK; \$ $P < 0.05$  vs. LG; and \$ $P < 0.05$  vs. LG + MCGs + TLCK. TLCK, N-tosyl-L-lysine chloromethyl ketone; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; HG, high glucose; LG, low glucose; MCGs, mast cell granules.

exposed to low glucose; the expression of Flt-1, Flk-1 and their downstream signals was significantly suppressed. Chou *et al* found that the expression of VEGF, Flt-1 and Flk-1 were signif-

icantly decreased in heart tissues but were increased 2-fold in the retina and kidney in diabetes mellitus patients (25). In the current study, we found that capillary-like tube formation

and the migration of MMVECs cultured under low glucose conditions increased compared to MMVECs cultured under high glucose conditions; however, Flt-1 and Flk-1 expression decreased, particularly Flk-1 expression. The reason that capillary-like tube formation and the migration of MMVECs cultured under low glucose conditions increased significantly, compared to the MMVECs cultured under high glucose conditions, even in the presence of MCGs, may be the fact that the expression of VEGF increased. However, the expression of the receptors decreased and the expression of Ang-2 increased, all these factors likely affecting the angiogenic ability of MMVECs in high glucose. These results demonstrate that high glucose levels are an important factor for the angiogenic ability of MMVECs *in vitro*. We also found that the duration of culture had a pronounced effect on MMVEC growth under high glucose conditions. Su found that the number of MMVECs cultured in high glucose for 24-96 h increased in the transcriptionally silent period and decreased in the DNA synthesis period (26). Abe found that the number of retina cells cultured in high glucose decreased by 78% after 10 days (27). We also found that the expression of VEGF and its receptors in MMVECs significantly decreased as the culture duration increased.

Tryptase and chymase are the predominant proteases present in MCGs; the amount of these proteases is 20-50% in MCs. Tryptase directly added to dermal microvascular endothelial cells caused a significant augmentation of capillary growth, which was suppressed by specific tryptase inhibitors. Tryptase also directly induced the cell proliferation of human dermal microvascular endothelial cells (HDMECs) in a concentration-dependent manner (28). MCs act at sites of new vessel formation by secreting tryptase, which then functions as a potent and previously unrecognized angiogenic factor (28). In our study, we found that a close correlation existed between MMVECs and MCGs and angiogenesis. In our co-culture system, MCGs increased migration and lumen formation in MMVECs, but the application of TLCK or TPCK significantly attenuated angiogenesis, in spite of the glucose concentration; TLCK was particularly effective in this respect. We also found that capillary-like tube formation and the migration of MMVECs co-cultured with MCGs, when exposed to TLCK or TPCK, significantly decreased. It seems that MCGs contain or secrete many of the angiogenic and anti-angiogenic factors. When the main mediators of angiogenesis, tryptase and chymase, were inhibited, anti-angiogenic factors in MCGs, such as endothelin-1 (ET-1), had a significant anti-angiogenic effect.

Liu *et al* found that MCs contribute to obesity and diabetes. Mechanism studies have revealed that IL-6 and interferon (IFN)- $\gamma$  in MCs contribute to WAT and muscle angiogenesis (5). However, this result was not so different from our findings. The reason may be that: i) our study focused on MMVECs co-cultured with MCGs *in vitro*; ii) diabetes mellitus is a chronic process, while cell culture studies are necessarily conducted over a much shorter time period; it may well be a completely different pathophysiological process; and iii) the angiogenic process of MCs isolated from animals is likely to be complex and perhaps differs from what occurs in other cell types *in vitro*.

In conclusion, our results demonstrate unequivocally that tryptase is the main angiogenic mediator in MCGs. High glucose levels have a profound effect on angiogenesis; this effect may be more pronounced than the effects of MCGs on angiogenesis.

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