ORIGINAL RESEARCH

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IL-8 reduces VCAM-1 secretion of smooth muscle cells by increasing p-ERK expression when 3-D co-cultured with vascular endothelial cells

Abstract

Objective: The goal of this study was to investigate the crosstalk between vascular endothelial cells (ECs) and smooth muscle cells (SMCs) using a three-dimensional (3-D) co-culture model. In addition, the role of IL-8 in this crosstalk was investigated.

Methods: A 3-D co-culture model was constructed using a Transwell chamber system and type I collagen gel. Human umbilical artery smooth muscle cells (HUASMCs) were suspended in the gel and added to the upper compartment of the Transwell. Human umbilical vein endothelial cells (HUVECs) were then grown on the surface of the gel. The growth of HUASMCs was tested with a CFDA SE cell proliferation kit. IL-8 and other bioactive substances were investigated by ELISA and real-time PCR. The alteration of p-ERK expression related to the change in IL-8 levels was also examined by Western blot analysis.

Results: The proliferation rate of HUASMCs in the 3-D co-culture model was 0.679 ± 0.057 . Secretion and transcription of VEGF, t-PA, NO and VCAM-1 in the 3-D co-culture model were different than in single (2-D) culture. When 3-D co-cultured, IL-8 released by HUVECs was significantly increased (2.35 ± 0.16 fold) ($P < 0.05$) and the expression of VCAM-1 from HUASMCs was reduced accordingly (0.55 ± 0.09 fold). In addition, increasing or decreasing the level of IL-8 changed the level of p-ERK and VCAM-1 expression. The reduction of VCAM-1, resulting from increased IL-8, could be blocked by the MEK inhibitor, PD98059.

Conclusion: Crosstalk between HUVECs and HUASMCs occurred and was probably mediated by IL-8 in this 3-D co-culture model.

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Cardiovascular diseases, such as hypertension and coronary heart disease, have been associated with vascular inflammation and remodeling [1]. Our previous work reported that the vascular remodeling associated with hypertension was accompanied by inflammation [2]. These pathological processes were primarily due to dysfunction of the vascular endothelial cells (ECs) and vascular smooth muscle cells (SMCs), which are the two main components of blood vessels [3]. Most *in vitro* research has focused on either endothelial cells or vascular smooth muscle cells. Study of the vasculature as a whole, with the cell types cultured together, remains challenging. Although some researchers have utilized a co-culture model of vascular ECs and SMCs, they still failed to mimic normal vessels [4-6]. To study the interaction between the two types of vascular cells in a condition similar to *in vivo*, we designed a novel three-dimensional (3-D) co-culture blood vessel model which allows both 3-D growth of cells and cell-cell contact.

It was previously reported that IL-8 is involved in vascular inflammation [7]. Recently, Hastings and colleagues found that the level of IL-8 declined when vascular ECs were exposed to atheroprone flow, thereby increasing the expression of VCAM-1 by vascular smooth muscle cells [8,9]. Characterization of IL-8 as one of the pro-inflammatory or anti-inflammatory cytokines remains controversial [10]. Because a mouse homolog of IL-8 in humans has not yet been discovered, human-derived cells are more appropriate than are mouse-derived cells for this kind of study [11]. Compared with the levels of IL-6, IL-1 β and other cytokines, the level of IL-8 are expected to vary when vascular ECs and SMCs are co-cultured; therefore, IL-8 was assumed to play an important role in the interaction between vascular ECs and SMCs, and a 3-D blood vessel model might be more suitable for this research than a single-culture model. The characteristics of the signal transduction pathway of IL-8 involved in this process were also investigated.

Methods

Culture of HUVECs and HUASMCs

HUVECs and HUASMCs were acquired from the umbilical cords of healthy women following delivery. This study was authorized by the Ethics Committee of Shanghai Jiao Tong University. A 20 cm sample of each umbilical cord was obtained under sterile conditions and placed in a 250 ml flask filled with PBS at 4°C. Penicillin and streptomycin were added as necessary. The fetal membrane of the cord was cut, and two arteries and one vein were isolated. The umbilical vein lumen was rinsed two or three times with PBS (GIBCO) before 0.1% type I collagenase (Sigma) was infused and maintained at 37°C

for 8-10 minutes. Then the vein lumen was rinsed two or three times with medium. The fluid collected was centrifuged at 4°C, 800g $\times$ 5 min. The pellet was suspended with 1640 medium with 20% FBS (GIBCO) as well as LSGS (Low Serum Growth Supplement, GIBCO) and then transferred to an incubator. The medium was renewed after 6 h and then every 48 hours. Cells were passaged every 5-7 days [12].

Medial films scraped out of the two arteries were placed in stainless steel dishes and cut into 1 to 2 mm rings that were subsequently transferred and plated, 3-5 mm apart, onto 60 mm culture dishes and dried for 20 min. F12K with 20% FBS and SMGS (Smooth Muscle Growth Supplement, GIBCO) was used as the culture medium and renewed every 3-4 days. Cells grew to confluence and were passaged after 2 weeks [13].

Establishment of 3-D co-culture model

HUVECs at the 2th-4th passage and HUASMCs at the 2th-8th passage were used for this step. The 3-D co-culture model consisted of HUASMCs in the gel and HUVECs on the gel surface, just like their normal arterial distribution. It is called a 3-D culture because the cells grow in a support gel. Fig 1A shows a diagram of the model. The number of HUASMCs used was 10^6 - 10^7 per well, while the number of HUVECs used was $3-5 \times 10^5$ per well because excessive numbers of cells tended to mass together and are thus too dense to suspend. Type I collagen (GIBCO), 0.1 mM NaOH and 10 $\times$ DMEM (high glucose, GIBCO) were prepared in ice water. First, 200 μ l of Type I collagen (5 mg/ml) was added to 12 μ l 0.1 mM NaOH with immediate stirring, then 23 μ l of 10 $\times$ DMEM was added, resulting in a final pH near 7. Second, HUASMCs were re-suspended immediately in the 100 μ l collagen above. Third, the mixture was transferred into the upper compartment of the Transwell (3495 Corning). After incubation at 37°C for 20 minutes, the gel solidifies. Finally, HUVECs were re-suspended in 200 μ l of F12K (GIBCO) and transferred to the surface of the gel. An additional 1000 μ l of F12K was added into the lower compartment of the Transwell for continuous culture. The medium was renewed every 12 hours for the first 2 days.

Microscopy, immunohistochemistry and immunofluorescence

An inverted microscope was used to observe cells directly. Immunostaining of HUVECs and HUASMCs was performed with primary antibodies against CD34 (1:100), VEGF (1:50), factor VIII (1:50) and α -SMA (smooth muscle α -actin) (1:200). A CFDA SE cell proliferation kit (Invitrogen) was used to measure the growth rate of HUASMCs in the 3-D gel. After fixation with 4% paraformaldehyde and dehydration

FIGURE 1. 3-D co-culture model and cells. (A) Diagram of construction of the 3-D co-culture model. (B,C) HUVECs grown on the surface of the gel. (D,E) HUASMCs grown in the gel. Bars=10 μ m.

with 15% sucrose, 0.1 M glycine/1 $\times$ PBS, the gel was finally embedded in OCT (optimum cutting temperature) and sectioned. Primary antibodies against α -SMA, CD34, collagen (1:100) and VCAM-1 (1:200) were used for the immunofluorescence.

ELISA

SECTION 1: Three groups of HUVECs were cultured in the upper compartment of a Transwell for 24 hours (about 4×10^5 cells/well). The groups were comprised of 1) a single-culture group (HUVECs alone), 2) a 3-D single-culture group (HUVECs cultured on the gel surface) and 3) a 3-D co-culture group (HUVECs cultured on the surface of gel containing HUASMCs). Three groups of HUASMCs were also cultured in the upper compartment of a Transwell (approximately 10^7 cells/well) for 24 hours. The groups were: (1) single-culture (HUASMCs alone), (2) 3-D single-culture (HUASMCs in gel), and (3) 3-D co-culture (HUASMCs in gel coupled with VECs on gel surface). The protein levels of t-PA, VEGF and NO of HUVECs and VCAM-1 of HUASMCs were determined by ELISA, using the supernatant in the upper and lower compartments, as appropriate.

SECTION 2: HUVECs and HUASMCs were cultured in 3-D single-culture and 3-D co-culture for 24 hours. The supernatant from both the upper and lower compartment was collected and analyzed using an IL-8 ELISA kit (Peprotech).

SECTION 3: 3-D co-culture models of HUVECs and HUASMCs were divided into three groups, to which IL-8 antibody (100 ng/ml, Peprotech), IL-8 (100 ng/ml, Peprotech) or normal medium was added for an additional 24 hours. The supernatant of the upper compartment was collected and analyzed using a t-PA ELISA kit (Sigma) and VEGF ELISA kit (Becton Dickinson). The supernatant of the lower compartment was analyzed using an sVCAM-1 ELISA kit (Bender MedSystem).

SECTION 4: The three groups of 3-D co-culture models were cultured with IL-8 for 24 hours, then PD98059 (100 μ M), SB202190 (100 μ M) or the blank medium were added for an additional 24 hours. The supernatant of the lower compartment was analyzed using an sVCAM-1 ELISA kit.

TABLE 1. RT-PCR primers designed for indicated genes; t-PA, tissue-plasminogen activator; SMA, smooth muscle α -actin; MYO, myocardin.

primers	sense primer	antisense primer
β -actin	5'-TGGCAGCCAGCACAATGAA-3'	5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'
GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGG-3'
IL-8	5'-ACACTGCGCCAACACAGAAATTA-3'	5'-TTTGCTTGAAGTTTCACTGGCATC-3'
t-PA	5'-TCGAGACTCAAAGCCCTGGTG-3'	5'-AGGCTGACCCATTCCCAAAGTAG-3'
VEGF	5'-GAGCCTTGCTTGCTGCTCTAC-3'	5'-CACCAGGGTCTCGATTGGATG-3'
VCAM-1	5'-CGAAAGGCCAGTTGAAGGA-3'	5'-GAGCACGAGAAGCTCAGGAGAAA-3'
SMA	5'-ATTGCCGACCGAATGCAGA-3'	5'-ATGGAGCCACCGATCCAGAC-3'
MYO	5'-GATCTTTGAGGCTATGACCCAGGA-3'	5'-CGGTTTGTGGAGACTGTAGAAGCA-3'

Real time RT-PCR

Primers were designed and provided by Takara: t-PA (tissue-type Plasminogen Activator), VEGF, SMA, MYO (myocardin), VCAM-1 and IL-8 (Table 1). A PrimeScript® RT reagent Kit (Takara DRR037A) was employed for the reverse transcription reaction, using a PTC-200 PCR instrument (BIO-RAD). An FQ Kit with SYBR® PrimeScript® RT-PCR Kit II (Takara DRR083A) was employed for quantitative RT-PCR with PTC-200, CFD-3200 (BIO-RAD).

The traditional co-culture model was used (HUVECs were plated in the upper compartment of the Transwell while HUASMCs were plated in the lower part) as a comparative model. After 24 hours, the HUASMCs were collected for RT-PCR. Total RNA of HUASMCs in the normal umbilical artery and the 3-D co-culture model was also collected for RT-PCR. The mRNA ratio of VCAM-1: SMA was compared by quantitative real-time PCR using the method of $2^{-\Delta\Delta C_t}$.

As detailed in SECTION 1, HUVECs were digested with 0.125% trypsin / EDTA (GIBCO) and centrifuged (including HUVECs grown on top of the gel). Total RNA was extracted by TRIzol (Invitrogen). Total RNA of HUASMCs was extracted from the whole mixed gel with TRIzol. The mRNA levels of t-PA, VEGF, VCAM-1, SMA and MYO were evaluated.

As detailed in SECTION 2, the mRNA level of IL-8 was evaluated.

As detailed in SECTION 3, the mRNA levels of t-PA, VEGF and VCAM-1 were evaluated.

Western blotting

HUASMCs were prepared and collected as described and were lysed in RIPA buffer. The total protein was then resolved with a 7.5% SDS-PAGE gel and blotted on a polyvinyl derivative membrane. The primary antibody [p-ERK (CST, 1:1000)] was incubated with the blot at 4°C overnight. The blot was incubated with a horseradish peroxidase-conjugated secondary antibody [goat anti-mouse IgG (CST, 1:5000)] for 1h at room temperature and then antibody detection was performed using ECL reagents (GE Healthcare, USA).

Statistical analysis

Data were analyzed by one-way ANOVA and a Student's t-test using SPSS 13.0. The results are given as the mean $\pm$ SE from six independent experiments. $P < 0.05$ was considered a statistically significant difference.

Results

Characteristics and identification of HUVECs and HUASMCs

Between 10^4 and 10^5 HUVECs were acquired from each approximately 20 cm long umbilical cord. HUVECs are a cobblestone-like or leaf-like shape and are distributed as islands. Cells from the 2nd-8th passages were used in this experiment.

Primary passages of HUASMCs grew radially and were perpendicular to the edge of the tissue pieces with yields of 4×10^5 cells per dish. The earlier passages of the HUASMCs were spindle-shaped, long, and striped, with a typical "hill and val-

FIGURE 2. Histology of the 3-D co-culture model and normal artery. (A) HE stained section from the 3-D co-culture model. (B) HE stained section of normal umbilical artery. (C) Positive staining by FITC conjugated mouse anti-human α -SMA. (D) Positive staining by TRITC conjugated mouse anti-human CD34. (E,F) Results of C and D merged together, F was the enlarged selected part of E. Bars=10 μ m.

ley" formation. Cells in the 2th-8th generations were suitable for these experiments.

Immunohistochemistry of the HUVECs indicated that the cells were CD34 (+), VEGF (+), factor VIII (+) and α -SMA (-). Immunohistochemistry of the HUASMCs indicated that the cells were CD34 (-) and α -SMA (+).

The behavior of the 3-D co-culture model was similar to normal vascular structure

HUVECs in the 3-D co-culture model formed a vascular structure in the gel (Fig 1B, C). HUASMCs growing in 3-D culture, as compared with the single-culture (Fig 1D, E), were hooked with each other in the gel.

Hematoxylin and Eosin (H&E) and immunofluorescence staining of the 3-D co-culture model showed a structure similar to that of the umbilical artery (Fig 2A-F). Collagen type II and VCAM-1 were detected in the 3-D co-culture model (data not shown).

A HUASMC proliferation curve indicated that the average proliferation ratio of HUASMCs in the 3-D co-culture model to the single-culture model was 0.679 ± 0.057 (Fig 3A-E), within a week.

The mRNA ratio of VCAM-1:MYO in HUASMCs was 238.95 ± 22.67 , 167.96 ± 14.49 , and 155.99 ± 9.39 in traditional co-culture, 3-D co-culture or normal artery, respectively. There was no statistically significant difference between the 3-D co-culture and the normal umbilical artery ($P > 0.05$), whereas a statistically significant difference was found between the traditional co-culture and normal umbilical artery ($P < 0.01$) (Fig 4A).

The levels of biologically active substances were variable among the single-culture (2-D), 3-D single-culture and 3-D co-culture models

The level of VEGF expressed by HUVECs was higher in the 3-D single-culture than in the single-culture (2-D) or 3-D co-culture ($P < 0.05$), and the level of t-PA was lower in the 3-D single-culture ($P < 0.05$) (Fig 4B) (Table 2). These results were consistent with the RT-PCR results (Fig 4C). The level of NO was higher in the single-culture (2-D) than in the 3-D single-culture and 3-D co-culture ($P < 0.05$) (Fig 4B).

The level of VCAM-1 expressed by HUAMCs decreased across the single-culture (2-D), the 3-D single-culture and the 3-D co-culture models (Fig 4B) ($P < 0.05$). These results were consistent with the RT-PCR results. The mRNA level of MYO and α -SMA increased across the single-culture (2-D), the 3-D

FIGURE 3. *Growth of HUASMCs in different culture conditions.* Quantitative immunofluorescence demonstrated a decline of intensity from A to D with the elapse of time (A=1st day, B=3rd day, C=5th day, D=7th day). E shows the different growth rates among single-culture (2-D), 3-D culture and quiescent culture (absence of FBS in medium induced cells into quiescence, and there was no fluorescence decay in the single-culture and 3-D culture), and compared to single-culture, the average growth ratio of HUASMCs in 3-D culture to single-culture within a week was 0.679 ± 0.057 . Values were means $\pm$ SE; n= 6.

single-culture and the 3-D co-culture models (Fig 4C) (Table 3) ($P < 0.05$).

The level of IL-8 was different between the 3-D single-culture and the 3-D co-culture.

HUVEC IL-8 expression was higher than HUASMC IL-8 expression ($P < 0.05$) in both 3-D single-culture and 3-D co-culture (Fig 5A). The expression and transcription of IL-8 by HUVECs was higher in 3-D co-culture than in 3-D single-culture ($P < 0.05$) (Fig 5B).

FIGURE 4. Characteristics of single culture, 3-D single culture, 3-D co-culture and normal artery. (A) mRNA ratios of VCAM-1: SMA in HUASMCs (one of the indexes of phenotypic switching). The results were 238.95 ± 22.67 , 167.96 ± 14.49 and 155.99 ± 9.39 from conditioned culture, 3-D co-culture and normal artery, respectively. (B) Proteins were secreted by HUVECs (VEGF, t-PA, NO) and HUASMCs (VCAM-1) (Table 2). (C) Gene transcription of HUVECs (VEGF, t-PA) and HUASMCs (VCAM-1, MYO, SMA) in different culture conditions (Table 3). Values were means $\pm$ SE; $n = 6$. * $P < 0.05$, † $P < 0.05$.

TABLE 2. Proteins secreted by HUVECs (VEGF, t-PA, NO) and HUASMCs (VCAM-1)

	single culture	3-D single culture	3-D co-culture
VEGF(μ g/ml)	156.29 $\pm$ 6.27	215.64 $\pm$ 10.50	165.23 $\pm$ 5.43
t-PA (μ g/ml)	9.57 $\pm$ 0.13	4.88 $\pm$ 0.05	8.40 $\pm$ 0.15
NO(μ g/ml)	71.54 $\pm$ 3.67	49.19 $\pm$ 0.95	53.89 $\pm$ 1.79
VCAM-1 (μ g/ml)	51.08 $\pm$ 1. 16	33.02 $\pm$ 1.32	25.80 $\pm$ 0.34

TABLE 3. Gene transcription of HUVECs (VEGF, t-PA) and HUASMCs (VCAM-1, MYO, SMA) in different culture conditions.

mRNA ratio	single culture	3-D single culture	3-D co-culture
VEGF	1.00	1.73 $\pm$ 0.09	1.35 $\pm$ 0.04
t-PA	1.00	0.48 $\pm$ 0.07	0.80 $\pm$ 0.05
VCAM-1	1.00	0.52 $\pm$ 0.08	0.28 $\pm$ 0.03
MYO	1.00	2.27 $\pm$ 0.11	3.11 $\pm$ 0.12
SMA	1.00	1.73 $\pm$ 0.14	2.67 $\pm$ 0.11

TABLE 4. Gene transcription of HUVECs (VEGF, t-PA) and HUASMCs (VCAM-1) in 3-D co-cultures treated with IL-8, anti-IL-8, or blank medium.

mRNA ratio	IL-8	Anti-IL-8	Blank
VEGF	1.02 $\pm$ 0.03	1.01 $\pm$ 0.04	1.00
t-PA	1.01 $\pm$ 0.03	0.99 $\pm$ 0.03	1.00
VCAM-1	0.55 $\pm$ 0.09	1.51 $\pm$ 0.12	1.00

IL-8 treatment had no significant effect on the secretion and transcription of t-PA or VEGF by HUVECs ($P > 0.05$), while secretion and transcription of VCAM-1 by HUASMCs decreased in the IL-8 treated group ($P < 0.05$) and increased in the IL-8 antibody-treated group when compared with the control group ($P < 0.05$) (Figure 5C) (Table 4).

Western blot analysis showed that the expression of p-ERK by HUASMCs increased in the IL-8 treated group ($P < 0.05$) and decreased in the IL-8 antibody-treated group, compared with the control group ($P < 0.05$) (Fig 6). The decrease of VCAM-1 secretion by HUASMCs, which was induced by IL-8, could be blocked by PD98059 (one of the MEK inhibitors) but not by SB202190 (P38 MAPK inhibitor) (data not shown).

Discussion

Vascular endothelial cells (ECs) and vascular smooth muscle cells (SMCs), which perform the main functions of blood vessels, are also the two major cellular components of blood vessels. These cells work independently and cooperatively to ensure the functional integrity of blood vessels [14]. Traditional

FIGURE 5. (A) IL-8 secreted by different cells from different compartments. S-VE-up: 26.33 ± 1.17 ; S-VE-low: 1.71 ± 0.29 ; S-SMC-up: 4.93 ± 0.38 ; S-SMC-low: 5.23 ± 0.44 ; 3D-up: 51.53 ± 2.13 ; 3D-low: 6.76 ± 0.37 (ng/ml). 5th column: (1st + 3rd) column was significantly higher. (S-VE=single-culture of VEs; S-SMC=single-culture of SMCs; 3D=3D co-culture; up=upper compartment of Transwell; low= lower compartment of Transwell). (B) mRNA expression of IL-8 in HUVECs and HUASMCs in 3-D single-culture and 3-D co-culture. Compared to 3-D single-culture, the mRNA expression level of IL-8 in HUVECs and HUASMCs in 3-D co-culture were 2.35 ± 0.16 and 1.03 ± 0.05 , respectively. (C) Gene transcription of HUVECs (VEGF, t-PA) and HUASMCs (VCAM-1) in 3-D co-cultures treated with IL-8, anti-IL-8, or blank medium (Table 4). Values were means $\pm$ SE; n = 6. * $P < 0.05$, † $P < 0.05$.

methods of co-culture were not suitable to investigate the interaction of these vascular cells. For example, in traditional co-culture, the two types of cells interact only through the medium, so it is hard to observe communication between the two types of cells. In addition, the traditional flat culture does not reflect actual cell growth in three-dimensional space *in vivo* [4]. Here, we established a 3-D co-culture model, in which cell growth was similar to that in blood vessels. This serves as a more valid model to observe the interaction between vascular ECs and vascular SMCs (crosstalk). Homologous HUVECs and HUASMCs, which were derived from the same umbilical cord, were used instead of commercial cell lines, to ensure that the results of the study adhere closely to the true *in vivo* state. The cells in the 2nd-8th generations were used because cells at this stage are dynamically stable [9].

Because type I collagen is the most common fibrillar collagen, it was used for constructing the 3-D blood vessel model as a gel matrix [15]. HUVECs in the gel of the 3-D model and formed tubular structures different from those formed in the single-culture, whereas HUASMCs in the gel demonstrated a dendritic shape. When seeded in the gel, 3.5×10^5 of HUVECs and 1×10^6 - 10^7 of HUASMCs would lead to tubular structure formation. Excessive numbers of cells tended to mass together and are too dense to suspend, while fewer cells could not contact each other to form the desired tubular structure [16].

Because the proliferation rate of cells in the gel could not be measured in traditional ways, such as by MTT, a CFDA SE cell proliferation kit was utilized [17]. Cells were labeled using

the green fluorescent probe CFDA SE. When the cells divide, the fluorescence intensity per cell decreases by half. The fluorescence can be detected even after cells have split 8 times. Thus, the proliferation rate was measured by fluorescence microscopy according to the fluorescence intensity, which was inversely related to the number of cell divisions. It was found that HUASMCs in the 3-D model grew slowly, compared with those in flat culture, which showed that cells cultured in the 3-D model behaved differently than those in flat culture. Table 2 and Table 3 summarize the differences, between 2-D and 3-D cultures, for protein secretion and mRNA expression for some bioactive substances. These difference may be attributed to one or more of the following: firstly, cells in 3-D model contact each other from more directions than in 2-D culture; secondly, cells in 3-D model may have had less nutrition than in 2-D model, which would lead to slower growth in HUASMCs; and finally, the bioactivities of HUVECs and HUASMCs could also be affected by the collagen support, as *in vivo*. It is expected that this new 3-D co-culture model will allow for advances in research on the biological properties of blood vessels.

The mRNA ratios of VCAM-1: SMA and VCAM-1: MYO were usually related to phenotypic changes of the vascular SMCs [18-20]. The mRNA ratio of VCAM-1:SMA of HUSMCs in the 3-D co-culture model was similar to that in normal umbilical artery, which implies that the phenotype of HUSMCs in the 3-D co-culture model resembles normal blood vessels.

In this study, our results also indicate that the active substances secreted in different parts of the 3-D co-culture model were not consistent: the factors detected in the upper compartment were mainly secreted by HUVECs, whereas those detected in the lower compartment were mainly generated by HUASMCs. The levels of the active substances varied in different kinds of cultures. For example, the presence of HUASMCs decreased the level of VEGF and increased the levels of t-PA and NO in HUVECs. Similarly, the presence of HUVECs reduced the level of VCAM-1 and promoted the transcription of MYO and SMA in HUASMCs. These results suggest that HUVECs might exert an anti-inflammatory effect on HUASMCs and that interactions between the cells occur in this 3-D co-culture model.

Interestingly, HUVECs in the 3-D co-cultured model were found to have contributed to the increased secretion and transcription of IL-8, which consequently decreased the secretion and transcription of VCAM-1 in HUASMCs. This finding was consistent with those of Hastings *et al* [9], who found that VCAM-1 expression by smooth muscle cells was regulated by human IL-8 in response to endothelial cells exposed to atheroprone flow. Although the role of IL-8 as pro-inflammatory or anti-inflammatory factors remains controversial, this study shows that IL-8 reduced the level of VCAM-1. In this respect, these findings were consistent with those of Hasting *et al*. These experiments were different from Hasting's in a number of important ways. First, these experiments were performed using a 3-D model while Hasting merely placed VECs and SMCs on the opposite surfaces of the Transwell. Thus, the cellular organization in our model is closer to that found in an artery. Second, when cells were 3-D co-cultured, VECs released more IL-8 (but not other cytokines, such as IL-6 or IL-10) than single 3-D cultured, which suggests that co-culture attributed to the increase in IL-8 in our model and revealed that IL-8 might be involved in the crosstalk between VECs and SMCs. Finally, the 3-D co-culture alone increased the level of IL-8 without any additional interventions, as required by Hasting *et al*. These differences might provide clues to understand the conflicting results of previous studies using single culture models. In order to get uniform results, it was necessary to use 3-D co-culture model.

To confirm whether IL-8 is involved in the crosstalk between HUVECs and HUASMCs or exerts anti-inflammatory effects on the HUASMCs, IL-8 and IL-8 antibody were added to the 3-D co-cultured model. The results showed that IL-8 had no effects on the secretion and transcription of VEGF and t-PA in HUVECs, but decreased the secretion and transcription of VCAM-1 in HUASMCs, suggesting that IL-8 sup-

FIGURE 6. Western blot analysis of p-ERK in HUASMCs treated with IL-8 and anti-IL-8. The ratios of p-ERK/β-actin in the three groups were 0.21 ± 0.04 , 0.73 ± 0.03 , and 0.41 ± 0.04 , respectively. Values were means $\pm$ SE; n= 6. * P<0.05.

pressed inflammation in HUASMCs and that phenotypic modulation toward anti-inflammation might be triggered by IL-8 released by HUVECs.

The expression of p-ERK in HUASMCs increased when IL-8 was added, while the other phosphorylation products did not change (data not shown). This result suggest that IL-8 inhibited the expression of VCAM-1 through the ERK signaling pathway. To test this hypothesis, HUASMCs were cultured with the MEK inhibitor PD98059 or the p38MAPK inhibitor SB202190. The reduction of VCAM-1 by IL-8 was found to be blocked by PD98059, but not by SB202190.

In summary, the 3-D co-cultured blood vessel model, composed of HUVECs, HUASMCs and type I collagen, is a

novel model for studying the function of cells of the vasculature. This study demonstrated that the 3-D co-cultured blood vessel model was close to normal vessels in terms of cell morphology, growth and phenotype. The 3-D co-cultured model allows better understanding of the crosstalk between different cells. Using this model, co-cultured HUVECs were shown to secrete IL-8, which prevented HUASMCs from expressing VCAM-1. Suppression of VCAM-1 expression in HUASMC cells is mediated by the p-ERK signaling pathway [21,22].

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References

1. Wang YJ, Wang JT, Fan QX, Geng JG. Andrographolide inhibits NF-kappaBeta activation and attenuates neointimal hyperplasia in arterial restenosis. *Cell Res* 2007;17(11):933-41.
2. Dai Q, Xu M, Yao M, Sun B. Angiotensin AT1 receptor antagonists exert anti-inflammatory effects in spontaneously hypertensive rats. *Br J Pharmacol* 2007;152(7):1042-8.
3. Tabak O, Gelisgen R, Uzun H, Kalender B, Balci H, Curgunlu A, et al. Hypertension and hemostatic/fibrinolytic balance disorders. *Clin Invest Med* 2009;32(6):E285.
4. Heydarkhan-Hagvall S, Helenius G, Johansson BR, Li JY, Mattsson E, Risberg B. Co-culture of endothelial cells and smooth muscle cells affects gene expression of angiogenic factors. *J Cell Biochem* 2003;89(6):1250-9.
5. Lavender MD, Pang Z, Wallace CS, Niklason LE, Truskey GA. A system for the direct co-culture of endothelium on smooth muscle cells. *Biomaterials* 2005;26(22):4642-53.
6. Fillinger MF, Sampson LN, Cronenwett JL, Powell RJ, Wagner RJ. Coculture of endothelial cells and smooth muscle cells in bilayer and conditioned media models. *J Surg Res* 1997;67(2):169-78.
7. Chung SW, Park JW, Lee SA, Eo SK, Kim K. Thrombin promotes proinflammatory phenotype in human vascular smooth muscle cell. *Biochem Biophys Res Commun* 2010;396(3):748-54.
8. Hastings NE, Feaver RE, Lee MY, Wamhoff BR, Blackman BR. Human IL-8 regulates smooth muscle cell VCAM-1 expression in response to endothelial cells exposed to atheroprone flow. *Arterioscler Thromb Vasc Biol* 2009;29(5):725-31.
9. Hastings NE, Simmers MB, McDonald OG, Wamhoff BR, Blackman BR. Atherosclerosis-prone hemodynamics differentially regulates endothelial and smooth muscle cell phenotypes and promotes pro-inflammatory priming. *Am J Physiol Cell Physiol* 2007;293(6):C1824-33.
10. Gimbrone MA Jr, Obin MS, Brock AF, Luis EA, Hass PE, Herbert CA, et al. Endothelial interleukin-8: a novel inhibitor of leukocyte-endothelial interactions. *Science* 1989;246(4937):1601-3.
11. Olson TS, Ley K. Chemokines and chemokine receptors in leukocyte trafficking. *Am J Physiol Regul Integr Comp Physiol* 2002;283(1):R7-28.
12. Blackman BR, Garcia-Cardena G, Gimbrone MA Jr. A new in vitro model to evaluate differential responses of endothelial cells to simulated arterial shear stress waveforms. *J Biomech Eng* 2002;124(4):397-407.
13. Thormodsson FR, Olafsson IH. Isolation and culturing of human vascular smooth muscle cells. *Methods Mol Biol* 2005;299:197-210.
14. Chiu JJ, Chen LJ, Chen CN, Lee PL, Lee CI. A model for studying the effect of shear stress on interactions between vascular endothelial cells and smooth muscle cells. *J Biomech* 2004;37(4):531-9.
15. Orgel JP, Irving TC, Miller A, Wess TJ. Microfibrillar structure of type I collagen in situ. *Proc Natl Acad Sci U S A* 2006;103(24):9001-5.
16. Montanez E, Casaroli-Marano RP, Vilaro S, Pagan R. Comparative study of tube assembly in three-dimensional collagen matrix and on Matrigel coats. *Angiogenesis* 2002;5(3):167-72.
17. Coupland LA, Cromer D, Davenport MP, Parish CR. A novel fluorescent-based assay reveals that thrombopoietin signaling and Bcl-X(L) influence, respectively, platelet and erythrocyte lifespans. *Exp Hematol* 2010;38(6):453-461.e1.
18. Petersen EJ, Miyoshi T, Yuan Z, Hirohata S, Li JZ, Shi W, et al. siRNA silencing reveals role of vascular cell adhesion molecule-1 in vascular smooth muscle cell migration. *Atherosclerosis* 2008;198(2):301-6.
19. Braun-Dullaues RC, Mann MJ, Sedding DG, Sherwood SW, von der Leyen HE, Dzau VJ. Cell cycle-dependent regulation of smooth muscle cell activation. *Arterioscler Thromb Vasc Biol* 2004;24(5):845-50.
20. Chen J, Kitchen CM, Streb JW, Miano JM. Myocardin: a component of a molecular switch for smooth muscle differentiation. *J Mol Cell Cardiol* 2002;34(10):1345-56.
21. Isakson BE, Duling BR. Heterocellular contact at the myoendothelial junction influences gap junction organization. *Circ Res* 2005;97(1):44-51.
22. Chiu JJ, Chen LJ, Lee CI, Lee PL, Lee DY, Tsai MC, et al. Mechanisms of induction of endothelial cell E-selectin expression by smooth muscle cells and its inhibition by shear stress. *Blood* 2007;110(2):519-28.