Co-Culture of Endothelial Cells and Smooth Muscle Cells Affects Gene Expression of Angiogenic Factors

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Abstract   Endothelial cells (EC) are in contact with the underlying smooth muscle cells (SMC). The interactions between EC and SMC in the vessel wall are considered to be involved in the control of growth and function of blood vessels. A co-culture system of EC and SMC and a method for separation of these cells was developed in order to investigate whether the presence of physical contact between EC and SMC affected the gene expression of angiogenic factors. Human EC and SMC were prepared from the great saphenous veins. Autologous EC were added on top of the confluent layer of SMC. After 72 h in co-culture, the EC were magnetically separated from SMC with the use of superparamagnetic beads. RT-PCR products for bFGF, bFGFR, VEGF, PDGF-AA, PDGF-BB, TGF-β, and β-actin were analyzed to study the mRNA expressions. The protein level of selected factors was studied by ELISA technique. In *co-cultured* SMC there was a statistically significant higher gene expression of VEGF, PDGF-AA, PDGF-BB, and TGF-β and significant lower gene expression of bFGF and its receptor than in single cultured SMC. The protein level of PDGF-BB and TGF-β was also significantly higher in co-cultured SMC. In *co-cultured* EC there were no significant differences in gene expression of PDGF-AA, PDGF-BB, and TGF-β compared with single cultured EC. The gene expression and protein synthesis of VEGF was significantly higher in *co-cultured* EC. The findings from the present study suggest that cell-cell interactions of EC and SMC affect the gene and protein expression of angiogenic factors. J. Cell. Biochem. 89: 1250–1259, 2003. © 2003 Wiley-Liss, Inc.

Key words: cell–cell interaction; vascular cells; angiogenic factor; smooth muscle cell; endothelial cell; co-culture

Blood vessels are composed of three cell layers. The intima, the inner layer, is a monolayer of endothelial cells (ECs). The media is composed of smooth muscle cells (SMCs) and extracellular matrix in large vessels. The adventitia, the outer layer, contains fibroblasts, extracellular matrix, capillaries, and nerves [Jones, 1979; Niklason and Langer, 1997; Carmeliet and Collen, 1998; Hungerford and Little, 1999; Niklason et al., 1999; Conway et al., 2001].

Communication between cells is an essential process in embryological development and is important for the maintenance of normal tissue physiology [Davies, 1986]. It is also necessary for a number of pathophysiological responses. In vascular tissue, structural and metabolic interactions occur between endothelium and smooth muscle cells. During vasculogenesis, growth factors released from EC induce migration of undifferentiated mesenchymal cells towards EC and upon contact with EC the mesenchymal cells differentiate into SMC [Hirschi et al., 1998]. The communication between EC and SMC in vascular wall occurs through synthesis and release of mediators into the surrounding medium, or through direct cell-to-cell contact via junctions formed at the point

of contacts between the cells [Davies, 1986]. The maintenance of normal arterial structure is dependent on interactions between the endothelium and SMC. EC and SMC act as a coupled system for the transmission of signals from receptors localized on the endothelium surface to the arterial wall and vice versa, from SMC to the endothelium [LG Spagnoli et al., 1980]. The interaction between EC and SMC in the vessel wall is considered to be an important factor in the control of blood vessel growth and function [Jones, 1979; Merrilees and Scott, 1981; Larson and Sheridan, 1982; Spagnoli et al., 1982; Davies et al., 1985b, 1988; Davies, 1986; Hajjar et al., 1987; Sato et al., 1990; Xu et al., 1990; Folkman and D'Amore, 1996; Vernon et al., 1997].

ECs regulate vascular tone through the synthesis of vasoactive molecules that are released into the underlying SMC [Vanhoutte, 1989]. Vasorelaxing molecules, such as nitric oxide and prostacyclin, produced by EC are known to have a growth-inhibitory action on cultured SMC [Akopov, 1988; KR Thomae et al., 1995], while vasoconstrictor molecules, such as endothelin and PDGF, have a stimulatory effect on SMC growth in culture [Janakidevi et al., 1992; Hiroaki Yoshida et al., 1996]. Soluble mediators such as TGF-β appear to have an important role in EC interactions with SMC. EC and SMC secrete TGF-β in a biologically inactive form that can be activated only when both cell types make close contact to each other [Nunes and Happel, 1996; Fillinger et al., 1997].

Endothelial-SMC contact can be mimicked in vitro by plating EC and SMC together in tissue culture. Fillinger et al. [1997] and Saunders and D'Amore [1992] have demonstrated that in a co-culture system, using a porous polyethylene terephthalate (PET) membrane, SMC send out cytoplasmic projections through the membrane pores into EC, probably in order to make contact with EC.

Studies in vivo show that EC and SMC in the vessel wall, produce different stimulatory and inhibitory angiogenic factors.

In vitro studies are usually based on single cell cultures, which unfortunately exclude the interactions between different cell types, which might influence the biological observations.

Cell–cell interactions in vitro have been investigated in different ways: microcarrier techniques [Davies and Kerr, 1982; Davies et al., 1985b; Davies, 1986]; transferral of conditioned media [Powell et al., 1996; Fillinger et al., 1997]; co-cultures in which EC and SMC were plated on opposite sides of a porous membrane [van Buul-Wortelboer et al., 1986; Saunders and D'Amore, 1992; Fillinger et al., 1993, 1997; Powell et al., 1996]; direct co-culture of equal number of both cell types [Jones, 1979; Merrilees and Scott, 1981; Hirschi et al., 1998], and; three-dimensional co-culture systems consisting of a collagen gel, SMC and confluent monolayer of EC on the top surface [van Buul-Wortelboer et al., 1986; Ziegler et al., 1995].

The present experiment was designed to investigate the hypothesis that the presence of physical cell contact between EC and SMC in a co-culture system affects the gene expression of angiogenic factors. In order to test this hypothesis we developed a co-culturing system of EC and SMC and, of equal importance, a method for separation of the cells after co-culture.

MATERIALS AND METHODS

Cell Culture

Human endothelial cells and smooth muscle cells were prepared from the macroscopically healthy part of the great saphenous vein from 7 donors, subjected to surgery for varicose veins.

Endothelial Cells (EC)

The veins were rinsed with Phosphate Buffer Saline (PBS) until the solution was clear and free from blood. The veins were filled with 0.1% collagenase type I (Sigma, Germany) in Hanks Balanced Salt Solution (HBSS) for 20 min at 37°C. Thereafter the veins were washed with pre-warmed EC-culture medium, E 199 (Biowittaker), supplemented with penicillin/streptomycin 3 U/ml (Sigma), L-glutamine (Sigma), fetal bovine serum (FBS) 20% (Biowittaker), heparin 1.3 U/ml, endothelial cell growth factor (ECGF) 0.15 mg/ml. The effluent was collected, centrifuged (1,000 rpm), resuspended in pre-warmed EC-culture medium. The cells were plated onto polystyrene culture flasks and incubated at 37°C in a humidified atmosphere (95% air and 5% CO_2). The cells were grown to subconfluency and then expanded by passing with a 1:3 split (detached with Trypsin (200 mg/L)/ EDTA (500 mg/L)). The culture media was changed every 48 h.

Smooth Muscle Cells (SMC)

SMC were isolated from the medial layer of the great saphenous vein by using explant technique. The vessel was cut longitudinally and the endothelial lining was removed by scraping. The medial layer of the vessel wall was dissected free from the adventitial layer. The medial layer was cut in to small pieces (1 mm^2), which were placed in 6-well polystyrene tissue culture plates. The pieces were covered by cover slips and the wells were filled with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, penicillin/streptomycin (100 U/ml), Na-pyruvate (1 mM), non-essential amino acids (0.1 mM; Gibco, Sweden) and L-glutamine (2 mM). The culture media was changed every 72 h. The cells were grown until confluence and they were expanded by passing a 1:3 split (detached with Trypsin (200 mg/L)/EDTA (500 mg/L)). For all experiments, SMC were used at passage 4.

Both EC and SMC were frozen at the second passage to ensure identical cell passage number in all experiments. For all experiments, the cells were used at passage 4.

EC identity was confirmed by cobblestone appearance and by positive staining for von Willebrand's factor (Dako, Sweden). The identity of SMC was confirmed by their "hill and valley" appearance and by positive staining for smooth muscle α-actin (Dako).

The cells were also tested for mycoplasma contamination before use in the experiment.

Co-Culture

The cells (EC, SMC), from the same donor were separately grown until confluence. The EC were thawed and passaged once. EC at the passage 3 were detached by Trypsin/EDTA, gathered and centrifuged (1,000 rpm). The pellet was resuspended in 5 ml DMEM and then spread on the top of the confluent SMC (0.5×10^6–0.7×10^6 cells per T-25 flask). The reason for putting EC on top of the SMC was to mimic the layering in native vessel and have EC in close contact into the medium. The EC density was higher than SMC (1.5×10^6–2.1×10^6 cells per T-25 flask) (higher number of EC in a confluent T-25 flask compared to a confluent flask of SMC was due to the size of these cells. EC are, in general, smaller than SMC. So, the number of EC in an almost confluent 25-cm^2 flask was about three times

more that SMC in 25-flask. An almost confluent 25-flask EC was used in order to have a layer of EC on SMC). After totally 72 h co-culturing, the EC and SMC were separated (see below). RNA and cell lysate was prepared from the separated cells. Single cultures of EC and SMC were used as controls.

Co-cultures were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde for electron microscopy.

Separation of EC from SMC

The medium was removed and EC and SMC were detached by incubating in Trypsin (200 mg/L)/ EDTA (1mM) for 5 min at 37°C. A solution of PBS/5% FCS was added to the cell suspension to neutralise Trypsin/EDTA. After centrifugation at 1,000 rpm for 8 min, the pellet was resuspended in 1ml PBS/0.1% BSA. Then 25 μl washed superparamagnetic beads (Dynal, ASA, Norway) per ml cell were added to the cell suspension according to the protocol given by the manufacturer's.

The mixture of EC, SMC and washed beads was incubated during 20 min at room temperature with gentle tilting and rotation and was then placed in MPC for 2 min. The EC and beads were drawn towards the MPC and formed a pellet. The supernatant, SMC, was transferred in to a separate tube. The pellet was gently resuspended in fresh PBS/0.1% BSA and placed in the MPC for another 2 min. The supernatant was added to the SMC. This step was repeated four times to optimally separate the two cell types. The same protocol was followed with the control groups treated by beads. The separated EC and SMC after totally 72 h co-culture were used for RNA extraction and preparation of cell lysate.

The purity and accurate separation of the two cell types were confirmed by culturing for another 3–4 passages, followed by immunostaining in order to check any presence of the other cell type. EC stained with smooth muscle specific α-actin and SMC stained with EC specific von Willebrand's factor.

RNA Isolation and Quantitation

RNA extraction was performed with an RNeasy Mini Kit (Qiagen, Sweden) according to the protocol given by the manufacturer's.

The concentration and purity of RNA was determined by measuring the absorbance (A) at 260 nm and 280 nm in a spectrophotometer.

RNA with an A260/A280 ratio 1.8–2 was considered pure and accepted for further analysis.

The integrity of the total RNA was checked by running the RNA on a 2% agarose gel electrophoresis with ethidium bromide staining.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Primers for basic fibroblast growth factor and its receptor (bFGF, bFGFR), vascular endothelial growth factor (VEGF), platelet-derived growth factor AA and BB (PDGF-AA, PDGF-BB), transforming growth factor-β (TGF-β) and β-actin were selected and designed from the human sequence of each gene for use in RT-PCR reactions. An optimal number of cycles were chosen in the exponential phase of PCR reaction before plateau phase to avoid the accumulation of non-specific products.

One step RT-PCR was carried out with a 25 µl total volume containing 17.5 µl reaction mix (Life Technologies, Sweden), 0.5 µl RT/Taq Mix (Life Technologies), 3 µl template (30 ng/µl), 4 µl primers (2.5 µM) (antisense 2 µl + sense 2 µl) (DNA Technology A/S, Denmark).

The RT-step consisted of 30 min at 45°C. During the PCR step DNA was denatured at 94°C for 2 min, annealed with the primers at annealing-temperatures for 2 min (Table I) and extended at 72°C for 7 min. Different numbers of cycles were performed to ensure that the PCR reaction was in the linear range (Table I and Fig. 4).

The PCR products of bFGF, bFGFR, VEGF, PDGF-AA, PDGF-BB, TGF-β, and β-actin were semi-quantified using the fluorescent labeling method (ABI Prism 377, GeneScan,[TM] Perkin Elmer). The labelled strand was detected on a polyacrylamide gel. The ratio of the amplified gene/β-actin was used for comparative analysis.

The sequences of the observed PCR-products matched the gene sequences (90–99%) in the gene bank (www.ncbi.nlm.nih.gov).

Enzyme-Linked Immunosorbent Assay (ELISA)

Protein level was measured by ELISA technique according to the manufacturer's instructions (R&D systems). The factors with significant changes in gene expression were selected for protein analysis. For practical reason, these factors were selected and this may imply a certain limitation of the experiment.

The conditioned media, from the co-cultures and the controls, were collected at the end of each experiment to analyze the secreted protein level.

The cell lysates, from co-cultures and the controls, were prepared in order to analyse the intracellular protein levels. EC and SMC were washed with PBS and separated from the co-culture, as described before. The cells were washed again with PBS and span at 1000 rpm, for 8 min. The cell pellet resuspended in cold lysis buffer containing PBS, 0.05% Tween-20 (Merck, Germany) and protease inhibitor cocktail (Boeringer Mannheim, Germany). The cell mixture was kept at −20°C overnight. The cell lysate was thawed and homogenized by ultrasonic disintegrator for 5 min. The supernatant carefully collected after 10 min spinning at 4°C, and transferred to a clean tube.

Both the conditioned media and the cell lysates kept frozen at −80°C.

Western Blotting

The influence of the cell separation technique, using magnetic beads, on three central molecules, i.e., phosphorylate c-Jun NH$_2$-terminal kinase (JNK), extracellular signal-regulated kinases (ERKs) and p60Src, was tested as

TABLE I. Sense and Anti-Sense Sequences of Primers Used in RT-PCR

Gene	Primer sequence (sense; antisense)	Tm (°C)	Cycle nr.
bFGF	GGAGAAGAGCGACCCTCACATCAAG; CCAGTTCGTTTCAGTGCCACATACCAA	59	21
bFGFR	AAGAAGTGCATACACCGAGACCTG; CACATCACTCTGGTGGGTGTAGAT	65	21
β-actin	TGGGTCAGAAGGATTCCTATGT; CAGCCTGGATAGCAACGTACA	64	17
PDGF-AA	TGGGCCACCTGGACGCTGCG; CCTGCCCATTCGGAGGAAGAG	64	21
PDGF-BB	TTTCTCACCTGGACAGGTCG; GAAGGAGCCTGGGTTCCCTG	64	20
VEGF	GCACCCATGGCAGAAGGAGG; CCTTGGTGAGGTTTGATCCGCATA	60	20
TGF-β	CACCTGCAAGACTATCGACAT; TCGGAGCTCTGATGTGTTGAA	58	22

follows. It was reasoned that no change in these factors would make it unlikely that beads had any influence on the results.

Confluent EC in 10-cm culture plates washed with PBS and detached by Trypsin/ EDTA, gathered, centrifuged (1,000 rpm) and incubated with magnetic beads for 5, 20, 30, 40, and 60 min. Cell lysates were obtained by adding lysis buffer containing 20 mM Tris, 1% Triton X-100, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, 150 mM NaCl (Sigma). This step was followed centrifugation at $4°C$. The supernatant was transferred to a new tube. The protein concentrations were determined by using a standard curve of BSA. Equal amounts of protein, from each time point, were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) on 12% polyacrylamide resolving gel and 5% stacking gel and blotted onto to a nitrocellulose membrane. Phosphorylated JNK, ERKs, and p60Src were detected by Enhanced ChemiLuminescence (ECL, Santa Cruz Biotechnology, California, USA) with primary polyclonal antibodies (rabbit polyclonal IgG, 1:2000, Santa Cruz Biotechnology) to pSrc; perk, and pJNK and horseradish peroxidase (HRP) conjugated secondary antibodies (goat anti-rabbit IgG-HRP, 1:2000, Santa Cruz Biotechnology). The blots were exposed against Kodak film.

Electron Microscopy

Cell cultures were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M Na cacodylate, pH 7.2. They were further prepared for scanning electron microscopy with the OTOTO method [Friedman and Ellisman, 1981] followed by dehydration with ethanol and hexamethyldisilazane [Braet et al., 1997]. Dried specimens were mounted on aluminum stubs and sputter coated with chromium in an Edwards Xenosput 2000 unit before examination in a Zeiss 982 Gemini scanning electron microscope.

Statistical Analysis

All analyses were performed in duplicate for each of the observations. For comparisons between the two groups of data, the Student's t-test was used ($n = 12$ for gene expression analysis and $n = 7$ for protein analysis). A value of $P < 0.05$ was considered statistically significant. Data were expressed as mean $\pm$ SEM.

RESULTS

Morphology

EC in the single cultures (controls) grew with the characteristic "cobblestone" morphology. SMC grew in a "hill and valley" growth pattern characteristic of confluent SMC in a single culture. Both EC and SMC (controls) were identified immunocytochemically.

SMC in the co-culture system were longer and thinner than in single cultures (controls) and had a more spindle-shaped appearance. EC established a monolayer on the SMC layer during the 72 h of co-culture.

Double immunostaining of EC and SMC for von Willebrand's factor and α-actin gave evidence of the presence of both cell types in the co-culture (Fig. 1).

The isolated EC and SMC from co-cultures were also cultured separately for further 3–4 passages in order to confirm their purity and their accurate separation (the purity of EC was identified by staining for smooth muscle α-actin and the purity of SMC by staining for von Willebrand's factor). No EC contamination in SMC cultures and no SMC contamination in EC cultures were observed. EC and SMC displayed the characteristic growth pattern and they were also identified immunocytochemically. EC with superparamagnetic beads grew normally and adhered normally to the culture flask. After
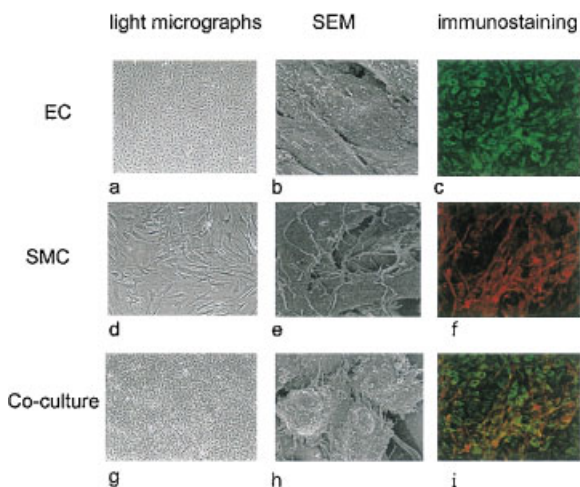

Fig. 1. Scanning electron micrograph (1,000×), light micrographs and double immunostaining of EC, SMC and co-cultures, cell-cell contacts through a large number of small projections. **a–c**: EC identified by staining for von Willebrand's factor in the co-culture, (**d–f**) SMC identified by staining for smooth muscle α-actin in the co-culture, (**g–i**) EC and SMC together in the co-culture. [Color figure can be viewed in the online issue, which is avaliable at www.interscience.wiley.com.]

3–4 passages, almost all the beads were gone from EC because of the regular change of the culture medium and of the passage of the cells.

In order to exclude any effects of the beads on gene/protein expression of angiogenic factors results from experiments with beads were compared to those without beads. There were no differences (data not shown). As another control the effect of the beads on signaling molecules such as pSrc, pJNK, and pERK were studied by incubating EC with the beads for 5, 20, 30, 40, 50, and 60 min. No differences were observed (data not shown). The signaling molecules were not studied in relation to single/co-culture.

Expression of Angiogenic Factors

There were no significant differences in expression of bFGF, bFGFR, PDGF-AA, PDGF-BB, and TGF-β between the co-cultured EC and the control groups. The expression of VEGF in co-cultured EC, however, was significantly higher in the co-cultured EC than in the control groups.

In co-cultured SMC there was statistically significant higher gene expression of VEGF, PDGF-AA, PDGF-BB, and TGF-β and lower gene expression of bFGF and its receptor than in the control groups (Fig. 2).

The expression of β-actin was not influenced by co-culturing (data not shown).

Protein Analysis

In co-cultured EC the protein level of PDGF-BB and TGF-β was significantly lower and the protein level of VEGF was significantly higher than the control groups. In co-cultured SMC the protein level of PDGF-BB and TGF-β was significantly higher and protein level of VEGF was significantly lower than the control groups (Fig. 3).

The protein level of bFGF, secreted to the culture media, was significantly lower in the single cultured SMC and the co-culture compare to the single cultured EC. There were no detectable levels of TGF-β in the conditioned media of EC, SMC cultured alone or co-cultures
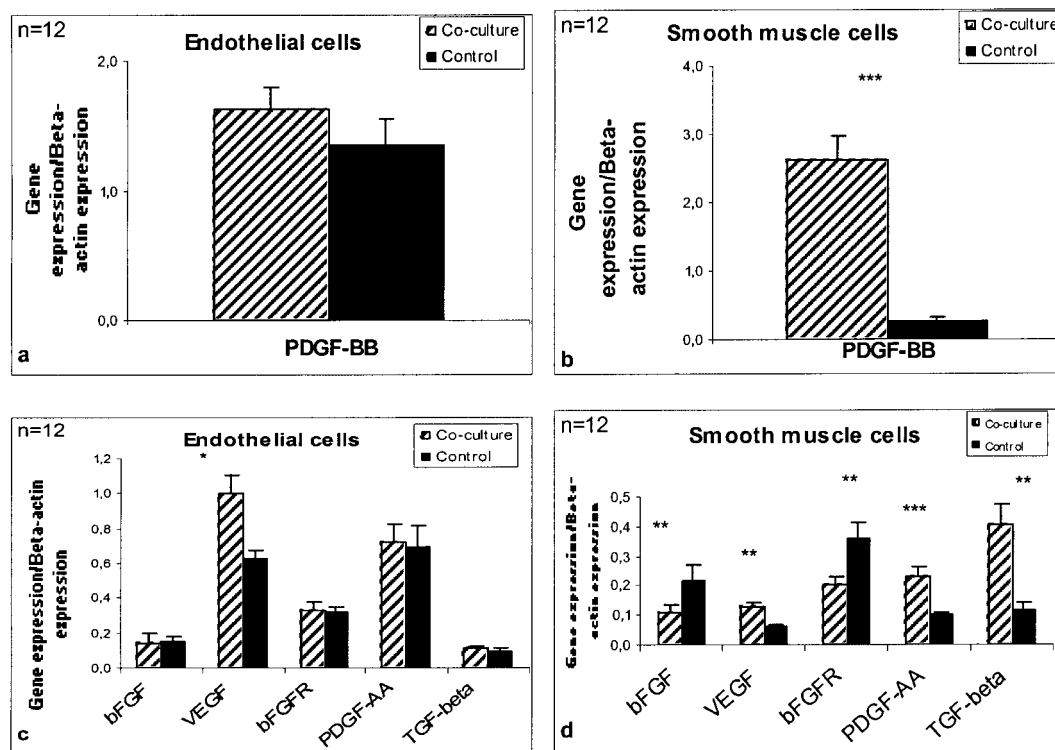

Fig. 2. Gene expression of different angiogenic factors in co-culture of EC and SMC in 72 h. The ratio, expression of angiogenic factor/β-actin used for comparative analysis. **a**: No significant differences in expression of PDGF-BB in EC; (**b**) significantly higher gene expression of PDGF-BB in co-cultured SMC; (**c**) No significant differences in expression of bFGF and its receptor, PDGF-AA and TGF-β in co-cultured EC, significantly higher gene expression of VEGF in co-cultured EC compared with control groups; (**d**) significantly lower gene expression of bFGF and its receptor and significantly higher gene expression of VEGF, PDGF-AA and TGF-β in co-cultured SMC compared with control groups. *P < 0.05, **P < 0.01, ***P < 0.001, results are expressed as mean ± SEM.
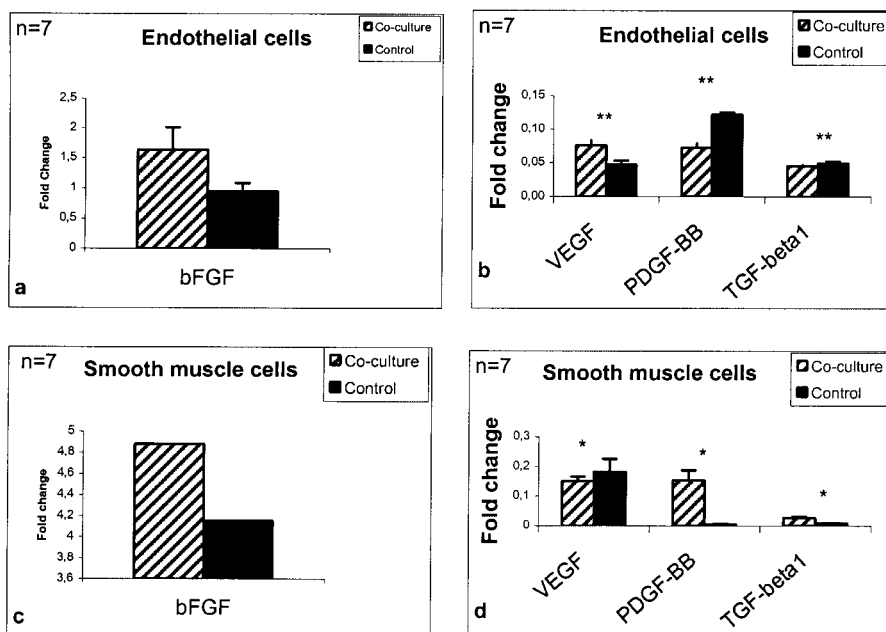
Fig. 3. Protein level of bFGF, VEGF, PDGF-BB, and TGF-β in co-culture of EC and SMC in 72 h (10^6 cells). No significant differences in protein level of bFGF (**a**) in EC (**c**) in SMC. **b**: significantly lower protein level of PDGF-BB and significantly higher protein level of VEGF in co-cultured EC compared with the control groups; (**d**) significantly higher protein level of PDGF-BB and TGF-β and significantly lower protein level of VEGF in co-cultured SMC compared with the control groups. *$P < 0.05$, **$P < 0.01$, results are expressed as mean ± SEM.
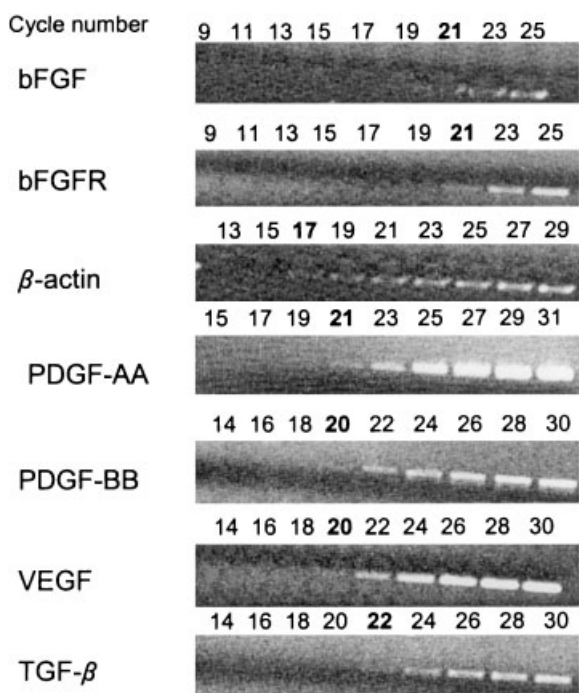

Fig. 4. PCR-products (one step RT-PCR) of different factors at different cycle on 2% agarose gel electrophoresis with ethidium bromide. An optimal number of cycles were chosen in the exponential phase of PCR reaction before plateau phase to avoid the accumulation of non-specific products.

when measured by ELISA at 72 h of culturing. The protein level of PDGF-BB, in the conditioned media, was significantly higher in the co-cultures of EC and SMC compare to SMC cultured alone (data not shown).

DISCUSSION

We have reported a direct co-culture system of EC on top of the cultured SMC in order to study the effects of the physical contact on the expression of different angiogenic factors.

We found that in co-cultured EC the gene expression and protein synthesis of VEGF was significantly higher than the control groups. The protein synthesis of PDGF-BB and TGF-β was significantly lower than the control groups.

In co-cultured SMC the gene expression of VEGF, PDGF-AA and -BB, and TGF-β, was significantly higher, and the gene expression of bFGF and its receptor was significantly lower than the control groups. In co-cultured SMC, the protein level of PDGF-BB and TGF-β was significantly higher and the protein level of VEGF was significantly lower than the control groups.

The congruity between gene and protein expression in EC for VEGF and in SMC for

PDGF-BB and TGF-β strengthen that these changes may be of biological significance.

EC play an important role in the development of the vascular system, in directing the migration, proliferation and differentiation of vascular SMC, and, in keeping the SMC in their normal quiescent and differentiated form [Vernon et al., 1997]. The quiescence state of EC and SMC is probably due to a balance between stimulatory and inhibitory growth factors (such as FGF, VEGF, PDGF, TGF-β, and others) [Folkman and D'Amore, 1996; Pepper, 1997; Vernon et al., 1997; Carmeliet and Collen, 1998; Darland and D'Amore, 1999; Hungerford and Little, 1999; Tomanek and Schatteman, 2000; Conway et al., 2001].

It has been shown that the microenvironment created by soluble factors, extracellular matrix and the presence/absence of associated cells affect the morphology, proliferation and gene expression of vascular cells in vitro [Saunders and D'Amore, 1992]. It has been observed that transmembrane co-culture of EC and SMC cause the inhibition of the proliferation of EC. Further more, the conditioned media which are affected by the co-cultures inhibit the growing EC [Orlidge and D'Amore, 1987; Saunders and D'Amore, 1992]. Also Saunders [1992] have postulated that the inhibition of EC proliferation depends on the direct contact of EC and SMC. In a transmembrane co-culture system of EC and SMC, however, the physical contact between the two cell types is prevented by a filter membrane or by the space between the chamber and the bottom of the culture dish. Thus the cells are not in direct contact with each other all along the cell layer, which may influence the observations.

Fillinger et al. have demonstrated that SMC, co-cultured with EC and in a conditioned media system, were characterized by long, filamentous projections and a more spindle-shaped appearance [Fillinger et al., 1993, 1997]. In the present study, a similar phenotype for SMC in contact with EC was observed. Fillinger et al. [1997] have reported that, with time, EC decrease the SMC proliferation, both in a co-culture and in a conditioned media model. A limitation in the studies of the cell–cell interactions by the use of cell-conditioned media is that this media only contains soluble molecules, which could become ineffective during the transferral. It also prevents feedback signaling between the cells.

Other studies have demonstrated the interactions between the vascular cells by the use of a microcarrier system and have suggested the existence of important humoral interactions. This technique provides a rapid separation of the two cell populations without the use of proteolytic enzymes to conserve the surface proteins. The technique is limited to direct cell-cell contact only at the touching point of the microcarrier beads on the layer of the SMC. The EC on the solid plastic microcarrier beads cannot establish a confluent monolayer on the top of the SMC [Davies et al., 1985a, 1988; Davies, 1986].

In the present study, both the gene expression and the protein synthesis were studied after 72 h and the results indicate that the gene and protein expression for some of the factors, such as VEGF and bFGF in co-cultured SMC, and PDGF-BB, TGF-β in co-cultured EC, does not follow the same pattern at this time point.

Up-regulation/down-regulation of some of these factors may be present only during the first hours of co-culture and rapidly thereafter becomes down/up-regulated. This process can occur because of the effect of the other stimulators/inhibitors in the co-culture system released by EC and (or) SMC. For example, in the case of TGF-β, Sato et al. [1990] have demonstrated that the activation of TGF-β occurred rapidly after co-culture and after 12 h the amount of TGF-β decreased. The explanation by Sato et al. [1990] is, when the EC and SMC contact each other, the EC express plasminogen (PA)-plasmin, which in turn activates TGF-β in SMC. At high concentration of TGF-β, plasminogen activator inhibitor-1 (PAI-1) synthesis will rise and plasmin formation decrease, resulting a decreased TGF-β level. A similar regulatory process may be considered for other factors.

The selection of 72 h may imply that some rapid changes passed unnoticed and this is a limitation of this study, since no time course was made. However, the protein levels represent a sum effect of the production during the experiment.

The sensitivity of the proteins to protease digestion and also storage of both proteins and mRNA may be considered too.

The differences between culture media and plasma, the presence of the neighboring cells and environmental conditions can give rise to functional and structural differences between the cells both in culture and in vivo, such as level

of gene expression, growth rate and morphology. Although no co-culture method can perfectly mimic or replicate the in vivo conditions of EC and SMC, the culture system presented here pays regard to many of the most important factors, including physical cell–cell contact, luminal/abnuminal orientation of EC/SMC, and the presence of a confluent EC monolayer. The cell separation technique described in this article facilitated the study of gene and protein expression of different factors in the separated EC and SMC from the co-culture, without affecting the cell signaling pathways.

The co-culture model presented in this article is not only a single "sandwich" of two different cell types, but also a very complicated system, in which still many different factors involved need to be explored. This co-culture model allows direct cell interaction. The separation technique we report here allows separation of the cell types for further analysis or assay. In our experiment, gene expression of angiogenic factors changed in the co-culture of EC and SMC compared to the single cultures. These effects must be considered when attempting to model in vivo phenomena in tissue culture.

The co-culture model described in this paper is useful for investigating the effects of physical contact between EC and SMC. This co-culture system may be superior to the other co-culture models such as conditioned media model and also offers unique and potentially important contributions to vascular wall biology.

Studies of the cellular and molecular interactions in the vessel wall will improve the understanding of the developmental regulation of the normal vascular system and pathophysiological processes such as atherosclerosis and intimal hyperplasia. Through the understanding of the normal control mechanisms in the vascular system and the interactions between the cells in this system, new strategies may be developed for the treatment or prevention of different vascular diseases.

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