CANCER GENOMICS & PROTEOMICS 2: 265-270 (2005)

Expression of Integrins and Adhesive Properties of Human Endothelial Cell Line EA.hy 926

PATRYCJA BARANSKA, HANNA JERCZYNSKA, ZOFIA PAWLOWSKA, WIKTOR KOZIOLKIEWICZ and CZESLAW S. CIERNIEWSKI

Department of Molecular and Medical Biophysics, Medical University of Lodz, Lodz, Poland

Abstract. *Immortalized endothelial cell lines are very often used as a model of endothelium for studies of various processes connected with its functions. Among the hybrid cells, the EA.hy 926 cell line, derived by the fusion of HUVECs with the continuous human lung carcinoma cell line A549, is presently the best characterized macro-vascular endothelial cell line. Although EA.hy 926 cells retain several endothelial characteristics, our data show some differences between this cell line and primary human umbilical vein endothelial cells (HUVEC). Analysis of their proteomic pattern reveals that there are many proteins expressed only in the immortalized cell line, but several proteins of EA.hy 926 are missed when compared to HUVECs. We observed a distinct profile of integrin expression on the surface of both types of endothelial cells, that may be responsible for diminished EA.hy 926 adhesion and migration to selected adhesive proteins. Studies on proliferation and migration in the presence of VEGF showed lower growth factor responsiveness of EA.hy 926 in comparison with HUVECs, but hybrid endothelial cells can also be converted into a pro-angiogenic phenotype. These studies showed significant similarity of endothelial cell lines with primary HUVECs, but also pointed out marked phenotype differences.*

Vascular endothelial cells mediate interactions between blood cells and vessel walls and play significant roles in many physiologically and pathologically important processes. Thus, the use of human endothelial cells in *in vitro* culture systems has been broadly applied to describe mechanisms explaining early phases of inflammation, angiogenesis, wound healing, hemostasis, atherosclerosis, tumor growth and metastasis. Immortalized endothelial cell lines are very convenient to test these processes, since they can be grown in large quantities and do not lose viability in the course of propagation. Several studies have focused on the comparison of hybrid cells with primary human umbilical vein endothelial cells (HUVECs) in terms of (a) the endothelial phenotype (1), (b) the expression and activity of membrane proteins involved in immune reactions (2) and (c) their response to cytokines (3-5). All these studies showed significant similarity between EA.hy 926 cells and primary HUVECs, but also pointed out marked phenotype differences.

Among the hybrid cells, the EA.hy 926 cell line is currently the best characterized (for review see 6). EA.hy 926 is derived by the fusion of HUVECs with the continuous human lung carcinoma cell line A549 (7). EA.hy 926 cells produce large amounts of tissue-type plasminogen activator (t-PA), plasminogen activator inhibitor type 1 and small amounts of urokinase. Their fibrinolytic characteristics are stable for at least 30 passages (7). EA.hy 926 cells are currently used as an *in vitro* model system for various physiological and pathological processes, especially in angiogenesis research (8, 9). Therefore, in this report we attempted to characterize their adhesive properties in comparison to primary endothelial cells. Our data show that the immortalized EA.hy 926 endothelial cell line retains several endothelial characteristics and can be converted into a pro-angiogenic phenotype. However, they show a significantly different proteomic pattern than that of HUVECs, as well as a distinct profile of integrin expression. The latter is responsible for the diminished adhesion and migration of EA.hy 926 cells when compared to HUVECs, particularly when collagen is used as a protein ligand.

Materials and Methods

Materials. Recombinant human $VEGF_{165}$ was purchased from Sigma. EGF was from Upstate, Lake Placid, NY, USA. A cocktail of protease inhibitors (Complete) was from Roche. All cell culture media were from Sigma. Reagents for 2-D gel electrophoresis were from Amersham Biosciences.

Correspondence to: Zofia Pawlowska, Department of Molecular and Medical Biophysics, Medical University of Lodz, 6/8 Mazowiecka Street, 92-215 Lodz, Poland. e-mail: pawlow@zdn.am.lodz.pl

Key Words: Endothelial cells, adhesive properties, VEGF.

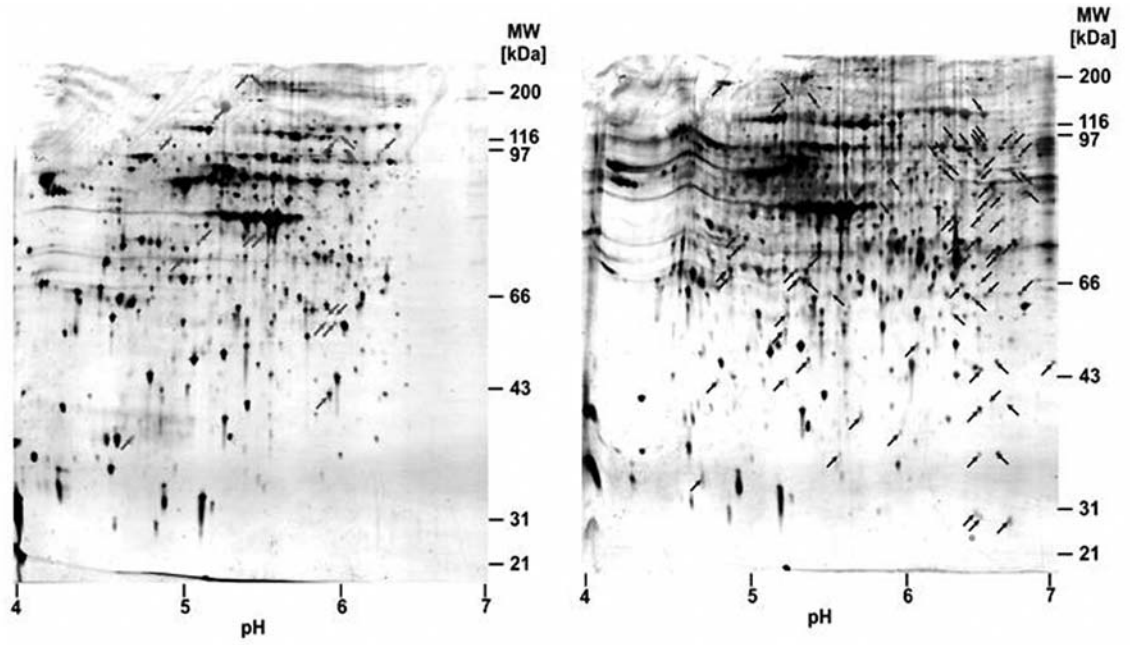

Figure 1. *The typical 2-D gel electrophoresis pattern of endothelial cell line EA.hy 926 and primary human endothelial cells, HUVEC. Proteins were separated using pH 4-7 gradient gels in the first direction and 12.5% PAGE-SDS in the second one. Proteins were visualized by silver staining and digital images of 2-D gels of HUVECs and EA.hy 926 cells were analyzed for changes in protein patterns, measured as percentage of volume after subtracting the background intensity.*

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords by collagenase treatment, according to the previously established protocol (10). The cells were cultured in M199 (Sigma) medium supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), heparin (90 μg/ml), L-glutamine (1 mM), sodium bicarbonate (2 mg/ml), 20% fetal bovine serum (FBS) and epidermal growth factor (EGF, 15 ng/ml). Primary cultures were harvested at confluence with trypsin/EDTA and transferred into gelatin-coated dishes. Primary cultures between passage three and four were used in the experiments.

The human endothelial cell line EA.hy 926 was derived by fusion of human umbilical vein endothelial cells with the continuous human lung carcinoma cell line A549. The cells were cultured in growth medium DMEM (Sigma) containing, HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) and 10% fetal bovine serum. The cells were harvested at confluence with trypsin/EDTA and transferred into cell culture-treated dishes.

The culture flasks were maintained at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Sample preparation and 2-D polyacrylamide gel electrophoresis. HUVECs and EA.hy 926 were rinsed three times with phosphate-buffered saline (PBS) and centrifuged at 200 xg for 5 min. Then, cell lysates were prepared using Lysis Buffer (7 M urea, 2 M tiourea, 4% CHAPS, 1% DTT, 2% Pharmalyte, and Complete Inhibitors). A buffer volume approximately equal to the packed cell volume was used. To improve resolution and recovery of proteins in the 2-D gel electrophoresis, cell extracts were treated with a PlusOne 2D Clean up Kit (Amersham Biosciences). The concentration of proteins in the supernatant was determined using PlusOne 2D Quant Kit and bovine serum albumin as a standard. The cell lysates were stored at –70˚C until analyzed.

The proteins were separated by 2-D gel electrophoresis using ready-made gels with immobilized pH gradients (Amersham Biosciences), as described previously (11). For the first dimension, samples containing 75 – 350 μg of soluble proteins in the Lysis Buffer were mixed with the IPG Reswelling Solution (6 M urea, 2 M tiourea, 1% CHAPS, 0.4% DTT, 0.5% Pharmalyte) to obtain the final volume of 450 μl. Then, they were loaded onto 24-cm immobilized pH linear gradient strip gels (pH 4-7). The IEF strips were allowed to rehydrate for 5 h, and isoelectric focusing was performed according to the manufacturer's protocol by a gradual increase of voltage (30 V for 5 h, 500 V for 1 h, 1000 V for 1 h, followed by 70 kVh at 8000 V) using an IPGphor system (Amersham Biosciences). SDS electrophoresis was performed on 12.5% polyacrylamide gels using the EttanDalt vertical system. Protein spots were visualized by staining with silver according to the method compatible with the analysis of proteins by mass spectrometry (12). Silver-stained gels were digitized using an Image Scanner. Computerized 2-D gel analysis was performed using the Image Master 2D software package Version 3.0. Digital images of 2-D gels with proteins of HUVECs and EA.hy 926 were analyzed for changes in protein patterns measured as the percentage of volume after subtracting the background intensity.

Cell adhesion. Adhesion of HUVECs and EA.hy 926 was tested using 96-well plates coated with fibronectin (10 μg/ml), collagen type I (20 μg/ml), fibrinogen (100 μg/ml) or gelatine (10 mg/ml) and blocked with 1% BSA in PBS. Cells were harvested with

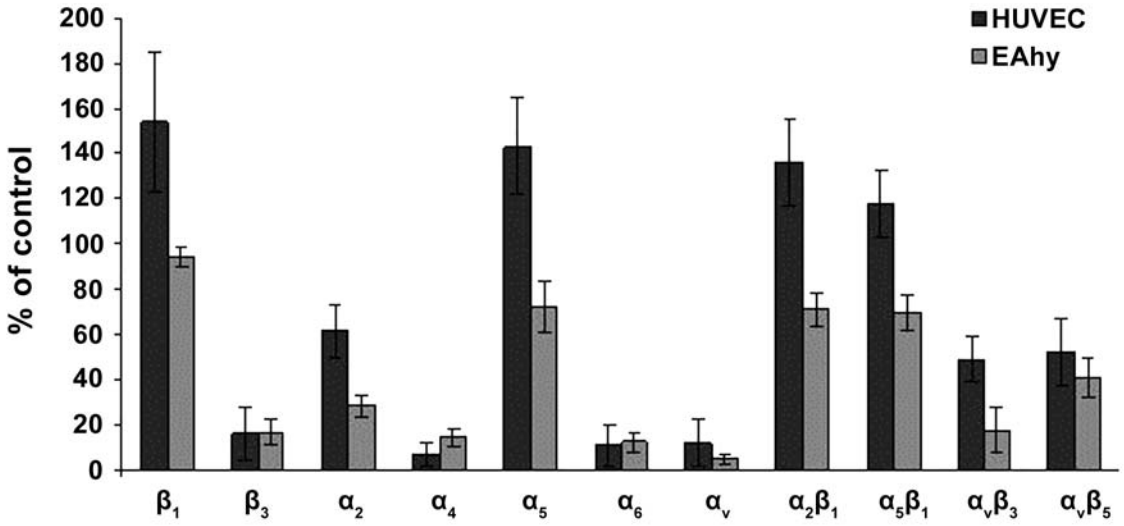

Figure 2. *Integrin expression on the surface of HUVECs and EA.hy 926 cells. Endothelial cells were labeled with monoclonal antibody against integrin subunits and whole receptors conjugated with fluorescent dye and fluorescence intensity was measured using a flow cytometer.*

trypsin/EDTA, washed with PBS and resuspended in the adhesion buffer (FBS-free growth medium DMEM or M199 containing 0.5% BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.2 mM $MnCl_2$). The cells were plated ($2.5x10^4$/well) in the wells containing 100 µl adhesion buffer and allowed to attach for 30 min. They were then washed gently three times with the adhesion buffer to remove nonadherent cells, as described previously (13). The number of adherent cells was determined with CyQuant Proliferation Assay Kit (Invitrogen).

Flow cytometry. The cells were harvested with trypsin/EDTA, resuspended in culture medium and washed twice in order to remove trypsin. To analyze integrin expression, HUVECs and EA.hy 926 (10^6/ml) were incubated in the dark at room temperature for 30 min with mouse monoclonal antibodies (10 µg/ml) conjugated with fluorescein (FITC) or phycoerythrin (PE): anti-α_2 /FITC, anti-α_4 /PE, anti-β_3 /FITC (Pharmingen), anti-$\alpha_v\beta_5$ /FITC, anti-$\alpha_v\beta_3$ /FITC, anti-α_v /FITC (Chemicon), anti-β_1 /FITC (DAKO) anti-α_6 /PE (Serotec) or primary antibodies anti-α_5, anti-$\alpha_5\beta_1$, anti-$\alpha_2\beta_1$ followed by secondary antibodies anti-mouse IgG conjugated with FITC (Chemicon). Fluorescence of the labeled cells was measured using a flow cytometer (FACSCalibur, Becton Dickinson).

Cell proliferation. Five hundred µl aliquots of the cell suspension ($5x10^4$ cells/ml) in growth medium were seeded in triplicate into 48-well plates. After attachment, the medium was replaced either with M199 with 2% FBS, or DMEM with 2% FBS. Proliferation of the cells was evaluated in the presence or absence of $VEGF_{165}$ (20 ng/ml) after 96 h using the CyQuant Proliferation Assay Kit (Molecular Probes).

"*Wound healing-like*" *assay (cell migration).* Cells were grown to confluence in medium using 24-well plates. Then, the cells were starved for 4 h in medium without serum and wounded by scraping away a swath of cells with a 200-µl pipette tip. After scraping, the cells were rinsed twice with PBS to remove wound-derived loose and dislodged cells. The cells were cultured in replaced serum-free medium with or without VEGF. Images were recorded immediately (time zero), 4 h, 8 h, 24 h, 48 h and 72 h later and stored for the analysis. Cells without treatment served as the control. The movement of cells into the denuded area was evaluated with an inverted Nikon phase-contrast microscope at a magnification of 400x, and photographed with a digital camera. The migration of wounded cells was quantified using an image analysis of a minimum of 7 fields of view of the denuded area examined at random. The lesion area in each field of view was measured and, using the data from time 0 (T_0 wound area), the wound area was then converted to give mean % recovery from 3 identically treated plates (%R) using the equation (14):

$$\%R = \left[1 - \frac{\text{wound area at } T_t}{\text{wound area at } T_0} \right] \times 100$$

Where: T_0 - the wounded area at 0 h; T_t - the wounded area (4 – 72) h post-injury.

Results

To compare the protein composition of HUVECs and EA.hy 926 cells, total protein was extracted from both types of cultured cells and then separated by 2-D gel electrophoresis. The first dimension was run using several pH ranges and 12.5 % gels were used for the second dimension. Following silver staining, the 2-D protein patterns were matched by computer analysis. Whereas the overall 2-D patterns of HUVECs and EA.hy 926 cells were largely similar, numerous protein changes were reproducibly detected. Figure 1 shows representative 2-D gels obtained after separation of proteins using, in the first direction, a pH gradient ranging from

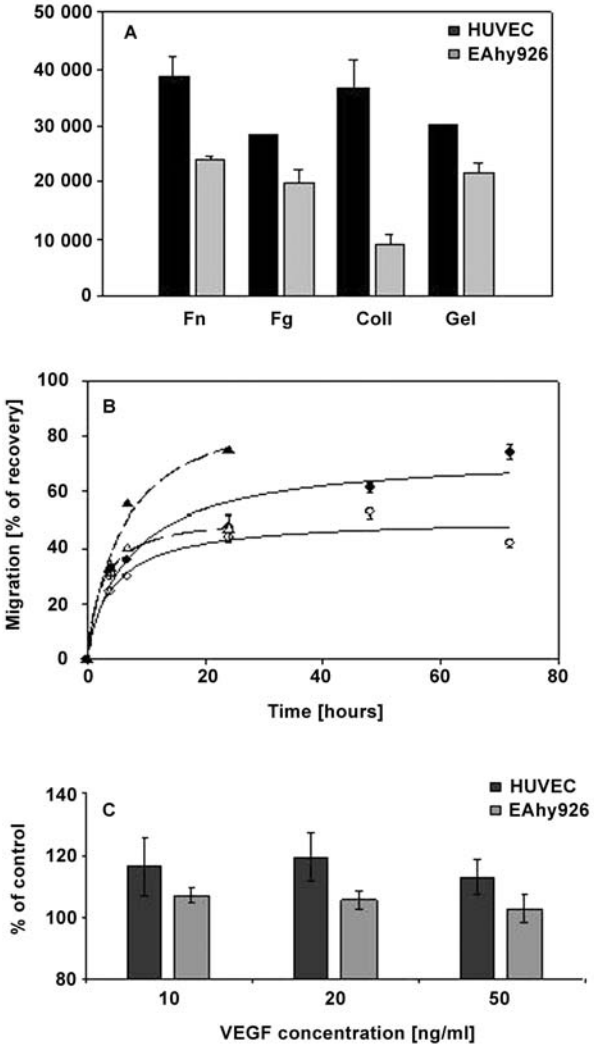

Figure 3. *Comparison of HUVECs and EA.hy 926 cell adhesion, migration and proliferation. Cells were plated in fibronectin (Fn)-, collagen type I (Coll)-, fibrinogen (Fg)- or gelatine (Gel)-coated wells and were allowed to attach for 30 min. Then, nonadherent cells were removed and the number of adherent cells was determined with Proliferation Assay Kit (A). Confluent cells were wounded and cultured in serum-free medium with (◆ - EA.hy 926, ▲ - HUVEC) or without VEGF. Images were recorded immediately and 4-72 h later and stored for the analysis. Cells without treatment served as control (◇ - EA.hy 926, △ - HUVEC). Migration of cells into the wounded area was estimated by quantification of the % of recovery (%R) (B). Proliferation of endothelial cells was determined after 96-h incubation in experimental media with 2% FBS and various concentrations of VEGF (10, 20, 50 ng/ml).*

4.0 to 7.0. We selected 18 and 66 distinct protein spots that showed consistent differences in expression levels and can be found specifically only in either HUVECs or EA.hy 926 cells, respectively. It indicates that there is a significant difference in protein profile between these two types of cells, explaining their distinct behavior in functional tests.

Since EA.hy 926 cells are frequently used as model cells in *in vitro* experiments to analyze the pro-angiogenic activity of different compounds, we next focused on their adhesive properties and their migrative function upon treatment with VEGF. First, the expression of integrin receptors in EA.hy 926 cells was measured and compared with that characteristic for HUVECs by flow cytometry (Figure 2). There were significantly lower expressions of the major integrin subunits in EA.hy 926 cells when compared to HUVECs, particularly α_2, α_5 and β_1, which were reduced by 40-55%. Similarly, expressions of the major integrin receptors in EA.hy 926 cells, $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$, were reduced by the same extent.

EA.hy 926 cells showed significantly reduced binding to fibrinogen, fibronectin, collagen and gelatine when compared to HUVECs (Figure 3A). Their ability to migrate was only slightly reduced (Figure 3B). VEGF stimulation significantly increased HUVECs proliferation and migration. Although these processes were also stimulated by the growth factor in EA.hy 926 cells, the differences were not statistically significant (Figure 3B,C).

Discussion

Since human endothelial cell lines are very convenient *in vitro* models to study different processes in the human microvasculature, they have been widely used to address different issues of endothelial cell biology. A number of immortalized cell lines were developed from different tissues and characterized in terms of their phenotypes. Among them, EA.hy 926, HMEC-1, HPMEC-ST1.6R and ISO-HAS-1 appeared to be the most similar in behavior to HUVECs under different conditions (1). EA.hy 926 is a particularly extensively used and characterized cell line, mostly as an *in vitro* model in studies of different issues of angiogenesis. The properties of EA.hy 926 cells were compared with HUVECs in several studies showing that: (a) EA.hy 926 cells express endothelial cell markers to the same extent as HUVECs, particularly vWF, PECAM-1, ICAM-1, VCAM-1, E-selectin, as well as Flt-1 and KDR (1, 2); (b) they form capillary-like tubes in matrigel (8); (c) they release fibronectin and PAI-1 upon activation with TNFα (3, 15); (d) and upon activation with cytokines, they interact with a number of cells (T cells, IY-cells, J6-cells, neutrophils) (16).

The present study indicates that, despite these phenotypic similarities, EA.hy 926 cells show significantly changed proteomic profiling when compared to HUVECs. They lack a number of proteins expressed by HUVECs, but they also exhibit many additional cellular proteins, which normally are not detectable in HUVECs. Interestingly, EA.hy 926 cells show markedly reduced expression of all the major integrin receptors that are reported to be involved in

angiogenesis, namely the β_1 integrin subfamily and $\alpha_v\beta_3$. This is in contrast to such adhesion molecules as ICAM-1, VCAM-1 and E-selectin, which basically show the same pattern in both EA.hy 926 cells and HUVECs (2). However, the cell surface expression of E-selectin upon TNFα stimulation is much lower in EA.hy 926 cells than in HUVECs (4). EA.hy 926 cells were significantly less responsive to VEGF as compared to primary endothelial cells when analyzed either by "wound healing" or proliferation assays. The effect of VEGF treatment can be masked by the phenotype of hybrid cells, which reveals a greater extent of proliferation and migration, as compared to HUVECs, without any additional stimulators. As we determined, in our preliminary study, even a very low serum concentration (1%) in the experimental medium was sufficient to maintain the proliferation and migration processes of EA.hy 926 cells (data not shown).

Acknowledgements

This study was performed with the contribution of the Centre of Excellence in Molecular Medicine MolMed supported by the European Commission.

References

1 Unger RE, Krump-Konvalinkova V, Peters K and Kirkpatrick CJ: *In vitro* expression of the endothelial phenotype: comparative study of primary isolated cells and cell lines, including the novel cell line HPMEC-ST1.6R. Microvasc Res *64*: 384-397, 2002.

2 Lidington EA, Moyes DL, McCormack AM and Rose ML: A comparison of primary endothelial cells and endothelial cell lines for studies of immune interactions. Transplant Immunol *7*: 239-246, 1999.

3 Pellegatta F, Radaelli A, Ferrero E, Toninelli E, Vidal MJ, Chierchia SL and Zocchi MR: Inducible nitric oxide synthase modulates fibronectin production in the EA.hy926 cell line and cultured human umbilical vein endothelial cells. J Cardiovasc Pharmacol *24*: 1014-1019, 1994.

4 Galley HF, Blaylock MG, Dubbels AM and Webster NR: Variability in E-selectin expression, mRNA levels and sE-selectin release between endothelial cell lines and primary endothelial cells. Cell Biol Intern *24*: 91-99, 2000.

5 Al-Nedawi KN, Czyz M, Bednarek R, Szemraj J, Swiatkowska M, Cierniewska-Cieslak A, Wyczolkowska J and Cierniewski CS: Thymosin β_4 induces the synthesis of plasminogen activator inhibitor-1 in cultured endothelial cells and increases its extracellular expression. Blood *103*: 1319-1324, 2004.

6 Bouis D, Hospers GA, Meijer C, Molema G and Mulder NH: Endothelium *in vitro*: a review of human vascular endothelial cell lines for blood vessel-related research. Angiogenesis *4*: 91-102, 2001.

7 Emeis JJ and Edgell CJ: Fibrinolytic properties of a human endothelial hybrid cell line (Ea.hy 926). Blood *71*: 1669-1675, 1988.

8 Bauer J, Margolis M, Schreiner C, Edgell CJ, Azizkhan J, Lazarowski E and Juliano RL: *In vitro* model of angiogenesis using a human endothelium-derived permanent cell line: contributions of induced gene expression, G-proteins, and integrins. J Cell Physiol *153*: 437-449, 1992.

9 Cieslak M, Niewiarowska J, Nawrot M, Koziolkiewicz M, Stec WJ and Cierniewski CS: DNAzymes to beta 1 and beta 3 mRNA down-regulate expression of the targeted integrins and inhibit endothelial cell capillary tube formation in fibrin and matrigel. J Biol Chem *277*: 6779-6787, 2002.

10 Jaffe EA, Nachman RL, Becker CG and Minick CR: Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J Clin Invest *52*: 2745-2756, 1973.

11 Pawlowska Z, Baranska P, Jerczynska H, Koziolkiewicz W and Cierniewski CS: Heat shock proteins and other components of cellular machinery for protein synthesis are up-regulated in vascular endothelial cell growth factor-activated human endothelial cells. Proteomics *5*: 1217-1227, 2005.

12 Shevchenko A, Wilm M, Vorm O and Mann M: Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem *68*: 850-858, 1996.

13 Isogai C, Laug WE, Shimada H, Declerck PJ, Stins MF, Durden DL, Erdreich-Epstein A and DeClerck YA: Plasminogen activator inhibitor-1 promotes angiogenesis by stimulating endothelial cell migration toward fibronectin. Cancer Res *61*: 5587-5594, 2001.

14 Slevin M, Kumar S and Gaffney J: Angiogenic oligosaccharides of hyaluronan induce multiple signaling pathways affecting vascular endothelial cell mitogenic and wound healing responses. J Biol Chem *277*: 41046-41059, 2002.

15 Swiatkowska M, Cierniewska-Cieslak A, Pawlowska Z and Cierniewski CS: Dual regulatory effects of nitric oxide on plasminogen activator inhibitor type 1 expression in endothelial cells. Eur J Biochem *267*: 1001-1007, 2000.

16 Thornhill MH, Li J and Haskard DO: Leucocyte endothelial cell adhesion: a study comparing human umbilical vein endothelial cells and the endothelial cell line EA-hy-926. Scand J Immunol *38*: 279-286, 1993.

Received June 17, 2005
Accepted June 28, 2005