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Effects of abciximab and tirofiban on vitronectin receptors in human endothelial and smooth muscle cells

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Abstract

Glycoprotein IIb/IIIa blockade by abciximab and tirofiban, non-peptidergic inhibitors, leads to sustained clinical benefits in the treatment of acute coronary syndromes. The purpose of this study was to clarify the functional effects of abciximab and tirofiban on vascular vitronectin receptors, αvβ3- and αvβ5-integrins. Integrin expression and 7E3 binding in human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells were observed in the following intensity: αvβ3 — human umbilical venous endothelial cells > human umbilical venous smooth muscle cells > human iliac arterial smooth muscle cells/αvβ5 — human iliac arterial smooth muscle cells > human umbilical venous smooth muscle cells > human umbilical venous endothelial cells. 7E3 binding correlated with αvβ3-expression in all cell types. Integrin-mediated cell functions were analysed with adhesion and spreading assays on vitronectin. In human umbilical venous endothelial cells, these functions were mediated by αvβ3 and in human iliac arterial smooth muscle cells by αvβ5. In human umbilical venous smooth muscle cells, both vitronectin receptors were involved. Abciximab potently inhibited αvβ3-mediated cell adhesion and spreading. With tirofiban, no significant inhibition of vascular cell functions was observed. The present data demonstrate that vitronectin–cell interactions in vascular cells are mediated via two distinct integrin-receptors, αvβ3 and αvβ5. Abciximab, which solely inhibits αvβ3-mediated cell functions, may be particularly effective in human endothelium and in β3-integrin expressing vascular smooth muscle cells.

Keywords: (Human); Smooth muscle cell, Vascular; Endothelial cell; Integrin; Vitronectin; Glycoprotein IIb/IIIa-inhibitor

1. Introduction

Abciximab, a mouse–human chimeric monoclonal Fab fragment (c7E3), is an effective anti-thrombotic agent directed against platelet glycoprotein IIb/IIIa (αIIb/β3). In three large, clinical, coronary intervention trials (EPIC, EPILOG, and EPISTENT), abciximab significantly reduced post-procedural mortality, myocardial infarction, and the need for revascularization after 30 days and 6 months in patients with coronary artery disease (EPIC-Investigators, 1994; Topol et al., 1994, 1999; EPILOG-Investigators, 1997; EPISTENT-Investigators, 1998; Lincoff et al., 1999). Chimeric 7E3 is a non-specific β3-integrin antagonist with equivalent affinity and functional blockade of platelet glycoprotein IIb/IIIa and αvβ3-integrin receptors (Tam et al., 1998). Tirofiban is a synthetic, short-acting, high selective non-peptide inhibitor of the platelet glycoprotein IIb/IIIa receptor (Peerlinck et al., 1993). In the RESTORE trial, tirofiban administration reduced the incidence of the composite endpoints of death, myocardial infarction, and revascularization after 2 and 7 days in patients undergoing coronary interventions with acute coronary syndromes (RESTORE-Investigators, 1997). At 30 days, a strong beneficial trend of tirofiban could be documented after readjusting the endpoint including death, myocardial infarction, and urgent interventions to allow comparison with the other trials (Lincoff and Korngold, 1999). The long-term clinical effects of Tirofiban treatment after 6 months are more controversial. A benefit was shown in the PRISM PLUS study among patients treated

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with tirofiban and heparin (PRISM-PLUS-Investigators, 1998). In contrast, the RESTORE and PRISM trials failed to demonstrate sustained beneficial effects of tirofiban (RESTORE-Investigators, 1997; PRISM-Investigators, 1998).

Integrins are cell surface heterodimeric glycoproteins expressed on platelets, human endothelial cells, human vascular smooth muscle cells, fibroblasts, and monocyte-derived macrophages (Hynes, 1987; Coller et al., 1991; Conforti et al., 1992; Hall et al., 1994; Stern et al., 1996; Bilato et al. 1997). The expression, distribution, and function of integrins vary significantly in different cell types (Wayner et al., 1991; Clyman et al., 1992; Ruegg et al., 1998). In the pathogenesis of atherosclerosis and restenosis after coronary intervention, integrins mediate important cell–matrix interactions such as adhesion, spreading, migration and proliferation of both vascular endothelial and smooth muscle cells (Hoshiga et al., 1995; Liaw et al., 1995a; Slepian et al., 1998). Vitronectin has been identified as an important extracellular matrix protein for integrin-mediated functions in vascular cells and an accumulation of vitronectin was demonstrated in human atherosclerotic tissues (Lupu et al., 1993; Brown et al., 1994; Van Zanten et al., 1994). $\alpha v\beta 3$- and $\alpha v\beta 5$-integrins are two major vitronectin receptors (Wayner et al., 1991). $\alpha v\beta 3$-integrins have been shown to be involved in migration and neointima formation by vascular smooth muscle cells after balloon injury of the rat aorta and carotid artery (Graf et al., 1996; Slepian et al., 1998). Hoshiga et al. (1995) demonstrated an increased expression of $\alpha v\beta 3$-integrin in the endothelium and in neointimal vascular smooth muscle cells of atherosclerotic coronary arteries. The $\alpha v\beta 5$-vitronectin receptor also has been implicated in cell–matrix interactions of vascular smooth muscle cells and macrophages, which are crucial in atherosclerotic events (Wayner et al. 1991; Bilato et al. 1997).

With regard to the crossreactivity of c7E3, it has been speculated that vascular $\alpha v\beta 3$-inhibition might contribute to the long-term clinical benefit of this agent, whereas a mechanism for the long-term effects of tirofiban through potential vascular interactions of this agent has not been investigated (Nakada et al., 1997). The objective of this study was to define a putative mechanism for the long-term therapeutic effects of c7E3 and tirofiban through their antagonising blockade of interactions with vascular vitronectin receptors $\alpha v\beta 3$ and $\alpha v\beta 5$, which could diminish atherosclerotic and restenotic processes. $\alpha v\beta 3$- and $\alpha v\beta 5$-integrin expression was first examined in different vascular cell types: Human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells. The functional importance of these integrins in each cell type was determined by performing adhesion and spreading assays on vitronectin using integrin-specific blocking monoclonal antibodies. Cell surface binding of 7E3 was also studied. The effects of abciximab and tirofiban on adhesion and spreading of human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells on vitronectin were also evaluated.

2. Materials and methods

2.1. Cell culture

Human vascular smooth muscle cells were prepared from iliac arteries according to the explant technique of Ross and Kariya (1988). Tissues were derived from donors of the liver transplant program, which has been approved by the local institutional review board. Human umbilical vascular smooth muscle cells were prepared from umbilical veins after enzymatic digestion to remove endothelial cells, also using the explant method. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum, 15 mM HEPES-buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM L-glutamine. Cells were identified as vascular smooth muscle cells through their characteristic hill-and-valley growth pattern. Studies were performed with cells at passages 3 to 7. Human umbilical venous endothelial cells were isolated by enzymatic digestion as described earlier (Graefe et al., 1993). The cells were cultured in Medium 199 × containing 20% foetal calf serum, 150 mM HEPES-buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 mM L-glutamine, and 10 ng/ml acidic fibroblast growth factor. Studies were performed with cells at passage 2 or 3.

2.2. Matrix components, peptides, and antibodies

Human vitronectin (Biosource) was resuspended in sterile water to a concentration of 0.5 mg/ml and stored frozen until use. Synthetic peptides with the sequence GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) and GRGESP (Gly-Arg-Gly-Glu-Ser-Pro) were obtained from Bachem, resuspended in sterile water at 10 mM and stored frozen until use. LM609, a neutralising $\alpha v\beta 3$ antibody, was purchased from Chemicon; P1F6 against $\alpha v\beta 5$ was from Gibco. As a control immunoglobulin G (IgG) an unspecific monoclonal mouse-IgG from Sigma was used. Tirofiban (Aggrastat) was purchased from MSD Sharp & Dohme, 85530 Haar, Germany; Chimeric 7E3 Fab and the monoclonal antibody 7E3 were kindly provided by Marian T. Nakada, PhD, Centocor, Malvern, PA.

2.3. Flow cytometry

The expression of integrins was evaluated by indirect immunofluorescence using flow cytometric techniques, as shown previously (Slepian et al., 1998). Cells were harvested by short trypsinisation of subconfluent monolayers.

After washing with cold phosphate-buffered saline (PBS), cells were incubated in PBS (PBS without Ca^{2+} and Mg^{2+}) containing 5% bovine serum albumin to suppress non-specific binding for 20 min. Then, cells were incubated with primary antibody for 20 min, washed with PBS, and resuspended in the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (goat-anti-mouse, Sigma) for 20 min. Cells were fixed with 4% paraformaldehyde, washed, resuspended in PBS and analysed for fluorescence with a Becton-Dickinson FACScalibur flow cytometer. The *x* and *y* axes represent log fluorescent intensity and cell number, respectively. The results are representatives of three separate experiments.

2.4. Reverse-transcription and polymerase chain reaction (RT-PCR) assay for transcripts of αv, β3, and β5 integrins in human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, human iliac arterial smooth muscle cells, and iliac arterial tissue

Pre-confluent (60–70% confluent) human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells (4×10^3 cells/ qcm) were lysed in 40 ul/qcm Trizol from Gibco. Vascular tissue from human iliac arteries, explanted from donors of the liver transplant program, was manual sequestered in fluid nitrogen and lysed in 10 ml Trizol. Each sample was mixed with 200 μl chloroform per 1000 μl Trizol by permanent moving for 15 s and centrifuged at $12{,}000 \times g$ for 15 min at 4°C to extract total RNA. The colourless upper aqueous phase was mixed with 500 μl isopropanol per 1000 μl Trizol and centrifuged at $12{,}000 \times g$ for 10 min at 4°C to precipitate the RNA. The RNA pellet was washed twice with 1000 μl of 75% ethanol per 1000 μl Trizol and centrifuged at $12{,}000 \times g$ for 8 min at 4°C, dried, and dissolved in diethylpyrocarbonate (DEPC) water.

RT-PCR was performed as described previously (Regitz-Zagrosek et al., 1996). RNA (1 μg) was reverse-transcribed by using 200 U Superscript™ (Gibco) in 50 mM Tris–HCl; pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 10 mM dithiotreitol (Gibco); 0.5 mM deoxynucleotide triphosphate (dNTPs — Gibco) and 100 ng/20 μl random hexamer. The PCR mix contained 2 μl of the cDNA product; 10 mM Tris–Cl; pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 8 pmol of each primer and 1.5 U Taq DNA polymerase (Gibco) in a total volume of 25 μl. The primer was separated from the rest by wax. PCR was performed in a Biozym PTC-100 thermal cycler, as a hot start PCR. After initial denaturation at 95°C for 5 min, PCR amplification was performed by using denaturation steps for 30 s at 95°C, annealing for 40 s, primer extension for 30 s at 72°C, and a final extension for 10 min at 72°C. αv was amplified for 34 cycles with an annealing temperature of 54°C, β3, β5, and pyruvate dehydrogenase (PDH) for 30 cycles with an annealing temperature of 58°C. The PCR products were electrophoresed on 2% agarose gel. The results are representatives of three separate experiments.

2.5. Immunohistochemistry

The labelled avidin–biotin method (LAB) was used for detection as described previously (Ashizawa et al., 1996). Antibody-solutions against integrins were used in frozen arterial iliac tissues at a titer 1:300 (clone P1F6), 1:400 (clone LM609), 1:2000 (7E3) and monoclonal antibody against α-smooth muscle actin at a titer 1:500 (Dako). For control experiments, tissues were stained with non-specific monoclonal antibody, or without first specific antibody. Biotinylated secondary antibodies were applied (Zymed) followed by incubation with streptavidin-peroxidase. Peroxidase activity was detected using aminoethyl carbazole as a chromogen (liquid AEC Kit, Zymed). Slides were then counterstained with hematoxylin.

2.6. Adhesion assay

The efficiency of cell attachment was determined by measuring the number of cells that adhered to a substrate, as described previously (Liaw et al., 1994). The test adhesive substrate (vitronectin) was diluted in sterile water. To determine the optimal vitronectin coating density and cell number, experiments with increasing concentrations of vitronectin and cell number were performed. The concentration and cell number around the maximal calculated increase in adhesion was chosen for blocking experiments. A total of 100 μl of vitronectin (10 μg/ml) per well was added to 96-well plates (Falcon #35 3075) and was placed overnight at 4°C. Wells were blocked with 10 mg/ml bovine serum albumin at 37°C for 1 h. Cells were detached by trypsinisation (0.25% trypsin in PBS), washed once in DMEM containing 10% foetal calf serum and centrifuged for 4 min with 1200 U. The cell pellet was washed once in 0.4% foetal calf serum-medium and repelleted by centrifugation. Cells were resuspended in medium containing 0.4% foetal calf serum. A total of 30,000 cells were placed in each well and allowed to adhere at 37°C for 60 min. Cells were pre-incubated with antibodies or pharmacological substances for 30 min at room temperature and gently mixed by permanent moving. Peptides were added in the wells immediately before cells. Non-adherent cells were rinsed off with PBS and the remaining cells were fixed with 4% paraformaldehyde for 5 min, then stained with 0.5% toluidine blue in 4% paraformaldehyde for 5 min and rinsed with water. Cells were solubilized by the addition of 100 μl of 1% sodium dodecylsulphate (SDS) and quantified in a microtiter plate reader at 590 nm. Experiments described were performed in quadruplicate and repeated a minimum of two times.

2.7. Cell spreading

The ability of cells to spread was determined by measuring the ratio of cells that spread after a defined time, as described previously (Yamamoto and Yamamoto, 1994). 96-well plates (Falcon #35 3075) were coated with vitronectin (10 μg/ml) overnight at 4°C. Optimal concentrations of vitronectin were evaluated as described above. To suppress non-specific bindings, plates were blocked with 1% bovine serum albumin in PBS for 1 h at 37°C. Cells were placed in 0.4% foetal calf serum-medium at a concentration of 2000 cells per well in 100 μl. They were allowed to spread for 40 min at 37°C. At this point in time, usually more than 40% of cells were spread. In experiments using antibodies or pharmacological substances, these were added after a minimal time of attachment after 10 min. After the spreading period, cells were fixed with 2% paraformaldehyde for 20 min at room temperature and stained with 0.5% toluidine blue in 4% paraformaldehyde for 5 min. Spread cells were designated as cells having a nucleus recognisable by microscopy or a noncircular shape. Four high power fields were counted per each well (magnification × 200). Experiments were performed in duplicate and at least three times.

2.8. Statistical analysis

Results of spreading and adhesion experiments are reported in the mean of percentage of untreated control (100%) + standard error of the mean (S.E.M.). Analysis of variance with paired and unpaired *t*-tests was performed for statistical analysis, as appropriate. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. αVβ3- and αvβ5-integrin expression and binding of 7E3 on human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells

We employed flow cytometry in order to identify the presence of αvβ3- and αvβ5-receptors and the binding of 7E3 on the cell surfaces in human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells. The mean channel fluorescence for human umbilical venous endothelial cells was: αvβ3-integrin (LM609) 76.86 and αvβ5-integrin (P1F6) 9.25, non-specific IgG 6.32, which indicates a remarkable expression of αvβ3-integrin on the surface of human umbilical venous endothelial cells (Fig. 1). 7E3 (mean channel fluorescence: 67.13) binding was comparable with αvβ3-integrin expression determined by LM609. Co-treatment with increasing concentrations of tirofiban (5–100 μg/ml) had no effects on 7E3 surface binding (data not shown). Flow cytometry analysis in human umbilical venous smooth muscle cells revealed detectable levels of surface expression for both αvβ3- and αvβ5-integrins (mean channel fluorescence: LM609-14.64 and P1F6-6.60), relative to unspecific IgG-controls (2.74) (Fig. 1). A modest difference was observed between the presence of αvβ3 and αvβ5 on the human umbilical venous smooth muscle cells surface, and the 7E3 interaction was consistent with LM609 binding (mean channel fluorescence 7E3: 14.06) (Fig. 1). Flow cytometry in human iliac arterial smooth muscle cells demonstrated increased levels of αvβ5-expression (P1F6: 39.84) compared to αvβ3 (LM609: 9.18), with 6.38 mean channel fluorescence of unspecific IgG (Fig. 1). The high affinity of 7E3 to αvβ3-integrin was also observed in human iliac arterial smooth muscle cells (mean channel fluorescence 7E3: 7.88).

3.2. Expression of αv, β3, and β5 transcripts in human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, human iliac arterial smooth muscle cells, and iliac arterial tissue

RT-PCR assays were performed to investigate the expression of αv-, β3-, and β5-integrins mRNAs in the different cell lines and in vascular tissue from human iliac arteries. Expression of β3 mRNA was markedly decreased in human iliac arterial smooth muscle cells (Fig. 2). Smaller level of β5 transcripts was detectable in human umbilical venous endothelial cells. No remarkable differences could be observed in the expression of αv mRNA in all cell lines (Fig. 2). Vascular tissue from human iliac arteries represented a homogenous expression of PCR products for all three integrins (Fig. 2).

3.3. Immunohistochemistry of integrin αvβ3, αvβ5 and of 7E3 IgG in human iliac arteries

Immunohistochemistry was performed with two different monoclonal antibodies directed against the human αvβ3- and αvβ5-integrins (LM609 and P1F6) and with 7E3 IgG on frozen sections of 10 different atherosclerotic human iliac arteries. Expression of αvβ5, detected using P1F6 was diffuse, and low along the endothelium and less intensive in the media of iliac arteries than staining with 7E3 IgG and LM609 (Fig. 3A). Strong staining with 7E3 IgG was observed in the endothelium along the lumen in atherosclerotic iliac arteries (Fig. 3B). The neointima of the atherosclerotic iliac arteries showed low staining of 7E3 IgG, whereas the media was stained intense and also

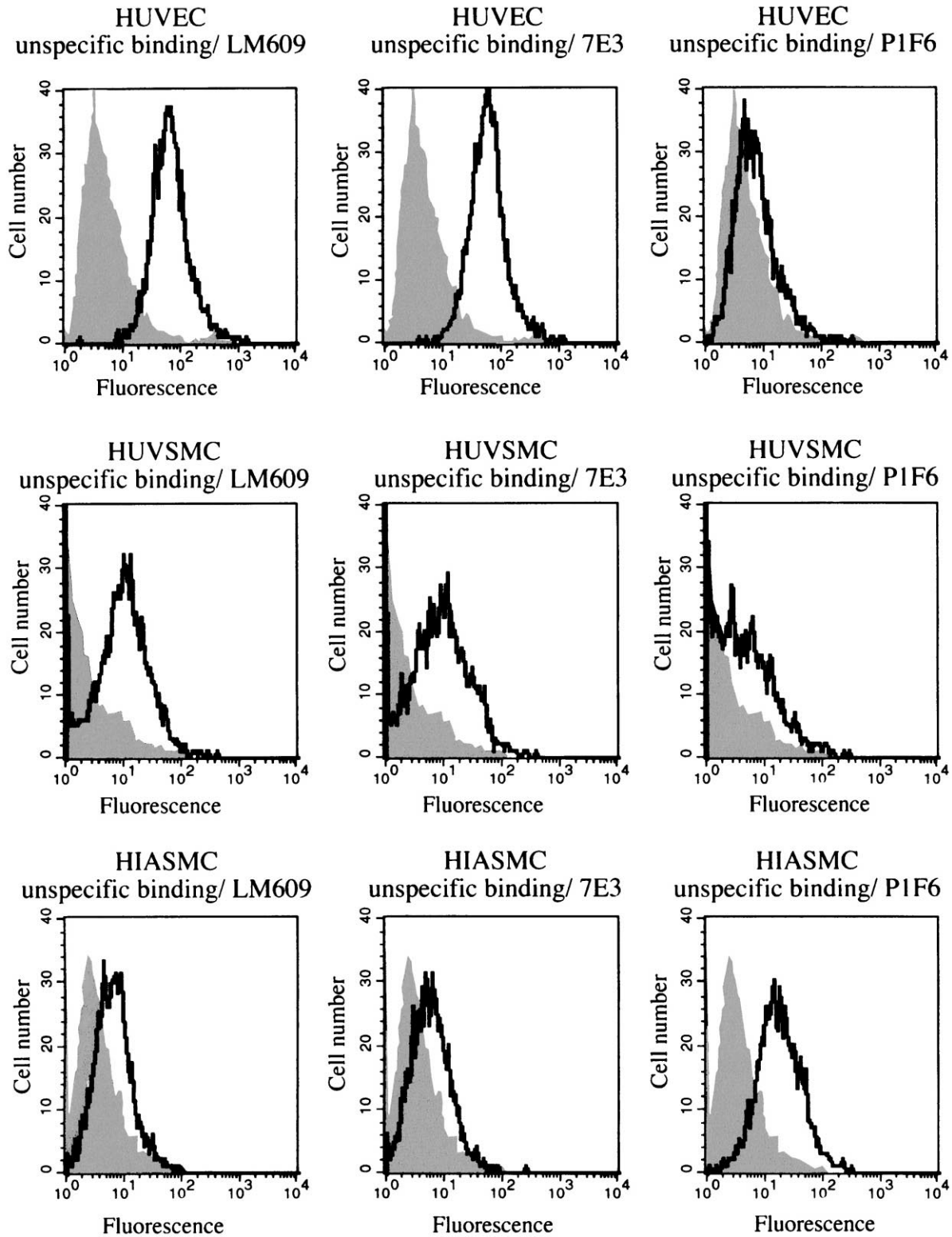

Fig. 1. Expression of integrin receptors on cultured human umbilical venous endothelial cells (HUVEC), human umbilical venous smooth muscle cells (HUVSMC), and human iliac arterial smooth muscle cells (HIASMC) by flow cytometry. Cells were incubated with an unspecific mouse-IgG (grey area), or with LM609 (anti-αvβ3), P1F6 (anti-αvβ5) and 7E3-IgG (5 μg/ml). After incubation with secondary anti-mouse FITC-conjugated antibody, cells were prepared for flow cytometry as described in Section 2. The *x*-axis corresponds to fluorescence intensity on a logarithmic scale and the *y*-axis corresponds to the cell number. Graphs are representatives of three separate experiments.

the adventitia revealed diffuse signals (Fig. 3B). The distribution of αvβ3-expression using LM609 correlated with staining of 7E3 IgG, although expression in the media was less intense (Fig. 3C). In the neointima of atherosclerotic

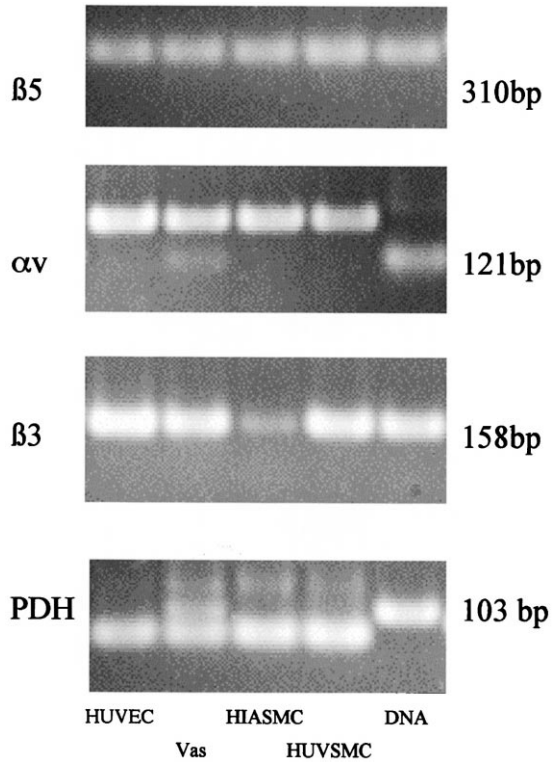

Fig. 2. Representative RT-PCR assay demonstrates presence of transcripts for αv-, β3- and β5-integrins in human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, human iliac arterial smooth muscle cells, and human vascular tissue from human iliacal arteries. PDH, a ubiquitously non-regulated gene, was used as an internal standard for RNA loading. Results are representatives of three separate experiments.

arteries, staining with smooth muscle cell marker (α-actin) was low, whereas the media was stained intensely (Fig. 3D).

3.4. Effect of c7E3 and tirofiban on αvβ3- and αvβ5-integrin mediated adhesion to vitronectin

To determine the importance of different integrin expression for the integrin-mediated cell functions, cell adhesion experiments were performed with human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells. The effects of inhibition of integrin–matrix interactions on cell adhesion were investigated with monoclonal antibodies directed against αvβ3- and αvβ5-integrins (LM609 5 μg/ml and P1F6 5 μg/ml), with linear RGD/ RGE peptides (GRGDSP 0.1 mM and GRGESP 0.1 mM), with tirofiban (60 μg/ml), with c7E3 Fab (ReoPro 5 and 60 μg/ml) and with an unspecific IgG (60 μg/ml) on vitronectin-coated plates (10 μg/ml). Fig. 4 summarises the results of the adhesion experiments in human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells.

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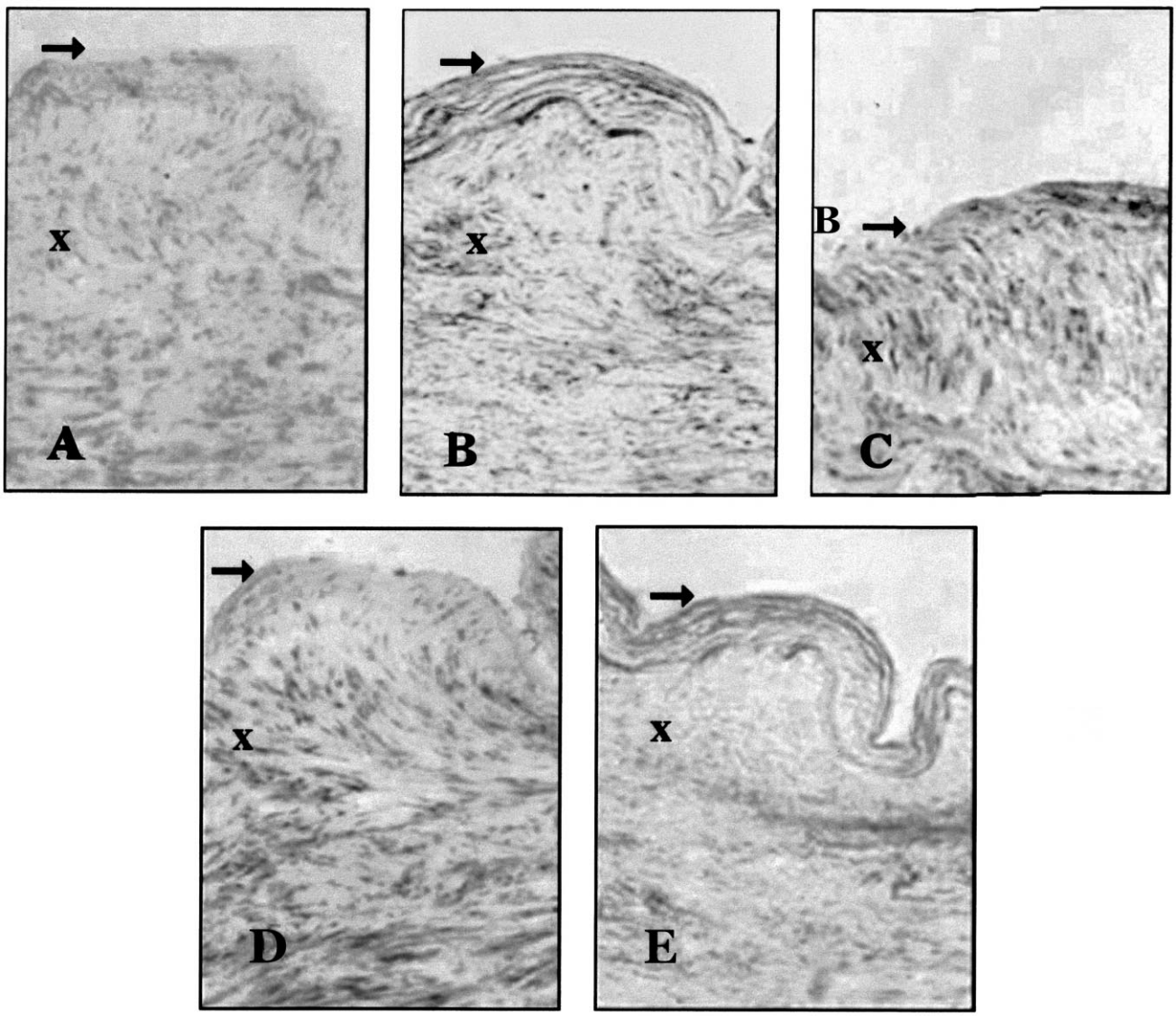

Fig. 3. Immunhistochemistry of human adult iliacal atherosclerotic arteries: (A) with P1F6 (antibody against αvβ5), (B) with 7E3, (C) with LM609 (antibody against αvβ3), (D) with antibody against smooth muscle α-actin, (E) with non-immune serum, counterstained with hematoxylin. (►) endothelium; (→) vascular smooth muscle cells; Magnification × 400.

bilical venous smooth muscle cells, this could not be observed in human iliac arterial smooth muscle cells (65% of control with $P < 0.01$ vs. control). Linear RGD also blocked cell adhesion in human iliac arterial smooth muscle cells (42% of control with $P < 0.01$ vs. control).

3.5. Effect of c7E3 and tirofiban on αvβ3- and αvβ5-integrin mediated spreading on vitronectin

Cell spreading of human umbilical venous endothelial cells and vascular smooth muscle cells involves αvβ3- and αvβ5-integrin receptors on vitronectin matrices. We characterised the inhibition of αvβ3- and αvβ5-integrin–matrix interactions by testing the effects of LM609 (5 μg/ml), P1F6 (5 μg/ml), c7E3 (5 and 60 μg/ml), tirofiban (60 μg/ml), RGD (0.1 mM) and RGE (0.1 mM) on human umbilical venous endothelial cell, human umbilical venous smooth muscle cell, and human iliac arterial smooth muscle cell spreading on vitronectin (10 μg/ml). Increased inhibition was not observed using higher antibody concentrations (LM609 and P1F6 25 μg/ml) in any cell type. Fig. 5 summarises the results of spreading experiments in the different cell lines. Untreated control values, expressed as the ratio of total cell number/spreaded cells in % were: human umbilical venous endothelial cells, 40.2 ± 2.7%; human umbilical venous smooth muscle cells, 40.4 ± 5.1%; human iliac arterial smooth muscle cells, 50.3 ± 3.2%.

In human umbilical venous endothelial cells, LM609 significantly inhibited cell spreading on vitronectin (46.5% of control with $P < 0.01$ vs. control). Consistently, pretreatment with c7E3 blocked cell spreading on the extra-

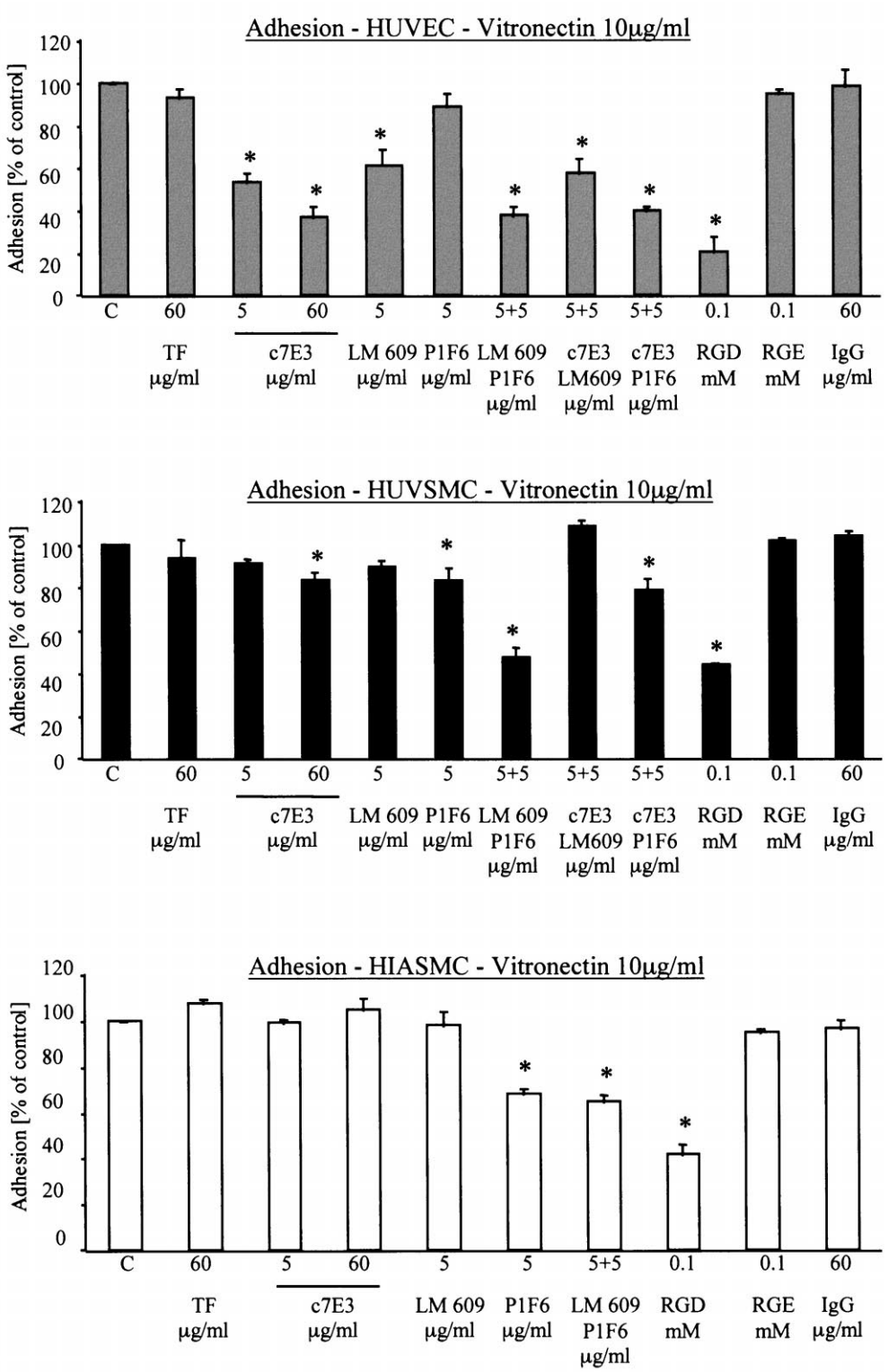

Fig. 4. Adhesion of human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells (30,000/well) to vitronectin-coated plates (10 µg/ml). Cells were pre-incubated with tirofiban, LM609 (anti-αvβ3), P1F6 (anti-αvβ5), c7E3, unspecific mouse-IgG, linear RGD-, or linear RGE-peptides for 30 min and then allowed to adhere for 1 h at 37°C. Experiments were performed in quadruplicate, with three different sets of cells. $^{*}P < 0.05$ compared to cells (control) cultured without anti-integrin antibodies and synthetic peptides by the *t*-test.

cellular matrix in low and high concentrations (5 µg/ml: 45.5% of control/60 µg/ml: 42.3% of control with $P <$ 0.01/0.01 vs. control). Blockade of αvβ5 by P1F6 failed to inhibit spreading of human umbilical venous endothelial

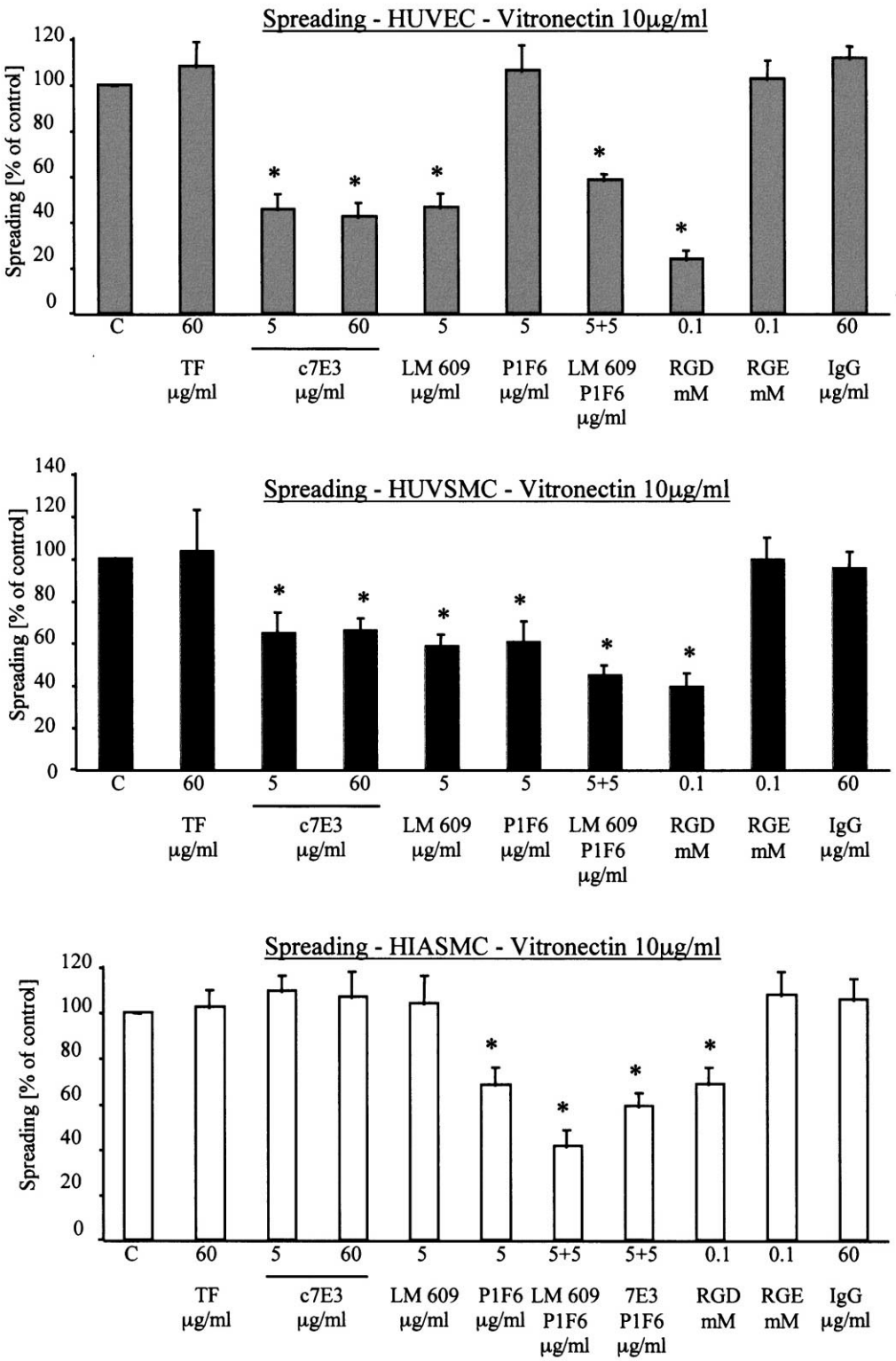

Fig. 5. Effects of tirofiban, LM609 and P1F6 (anti-integrins), c7E3 and linear RGD-, RGE-peptides on the cell spreading of human umbilical venous endothelial cells, human umbilical venous smooth muscle cells, and human iliac arterial smooth muscle cells on vitronectin-coated plates (10 µg/ml). Cells were allowed to spread for 40 min. For antibodies and synthetic peptides, these were added after a minimal time of attachment after 10 min. Cells were fixed with 2% paraformaldehyde at room temperature and stained with 0.5% toluidine blue in 4% paraformaldehyde for 5 min. Rounding cells and spread cells were counted in four randomly chosen high-power fields, with three different sets of cells. $^{*}P < 0.05$, compared with the control cells by the *t*-test.

cells. The combination of LM609 and P1F6 did not produce an additive inhibitory effect on human umbilical venous endothelial cell spreading as observed in adhesion experiments, whereas the treatment with P1F6 additionally to LM609 seemed to diminish the inhibitory effect of αvβ3 blockade. Tirofiban again demonstrated no effect on spreading of human umbilical venous endothelial cells. Treatment with RGD peptides led to a pronounced inhibition of human umbilical venous endothelial cell spreading on vitronectin, which indicated a RGD dependent cell–matrix interaction for cell spreading in human endothelial cells (23.6% of control with $P < 0.01$ vs. control). Control experiments with RGE peptides had no detectable effect on human umbilical venous endothelial cell spreading. In human umbilical venous smooth muscle cells, LM609 (5 μg/ml–58.3% of control with $P < 0.01$ vs. control) and c7E3 (5 μg/ml–64.9% of control/ 60 μg/ml–66.2% of control with $P < 0.01$/ 0.01 vs. control), as well as blockade of the αvβ5-receptor with P1F6 (5 μg/ml: 60.3% of control with $P < 0.01$ vs. control) showed significant inhibition of cell spreading, implicating the importance of both integrin-receptors for this function in human umbilical venous smooth muscle cells. No blockade of human umbilical venous smooth muscle cell spreading could be detected with tirofiban. In addition, an additive blocking effect of LM609 and P1F6 could be observed (44.7% of control with $P < 0.01$ vs. control). As demonstrated in human umbilical venous endothelial cells, the cell spreading of human umbilical venous smooth muscle cells was also inhibited by RGD pre-treatment (39.1% of control with $P < 0.01$ vs. control). In human iliac arterial smooth muscle cells, LM609, c7E3, and tirofiban failed to inhibit cell spreading, whereas P1F6 significantly reduced spreading of human iliac arterial smooth muscle cells (68.7% of control with $P < 0.05$ vs. control). The additive inhibitory effect of the antibodies against αvβ3- and αvβ5-integrins (LM609/P1F6: 41.4% of control) was reproducible in human iliac arterial smooth muscle cells, as well as the blocking of spreading through RGD sequences (68.9% of control with $P < 0.05$ vs. control). The less intense RGD inhibition, compared to the combination of LM609 and P1F6, might be due to the binding characteristics of the linear RGD peptide to its receptors. Furthermore, the combination of c7E3 and P1F6 produced also additional inhibitory effects (58.9% of control), compared to P1F6 alone.

4. Discussion

The expression of the vitronectin receptor αvβ3-integrin is well-characterised in human vascular smooth muscle cells and endothelial cells (Conforti et al., 1992; Bilato et al., 1997). Additionally, αvβ5-integrin was identified as a vitronectin receptor on vascular smooth muscle cells, which regulated cell–matrix interactions (Wayner et al., 1991; Bilato et al., 1997). Both vitronectin receptors mediate cell adhesion and cell spreading, which are required for cell migration and proliferation induced by attachment to vitronectin and osteopontin. Therefore, these receptors contribute to crucial morphogenic cell changes seen during atherosclerotic lesion formation (Smith et al., 1990; Brown et al., 1994; Liaw et al., 1994; Giachelli et al., 1995). Variable expression of αvβ3- and αvβ5-integrins has been described for different cell types (Wayner et al., 1991; Clyman et al., 1992; Conforti et al., 1992). We observed high expression of αvβ3-integrin on human endothelial cells, which was lower on human umbilical venous smooth muscle cells. The lowest expression of αvβ3 was found in smooth muscle cells from human iliac arteries. An opposite pattern of expression was seen for αvβ5, which was increased in human iliac arterial smooth muscle cells, intermediate in human umbilical venous smooth muscle cells, and low in human umbilical venous endothelial cells. These results of surface expression were corroborated by RT-PCR studies. We observed a low level of β3-transcripts in human iliac arterial smooth muscle cells and a low level of β5-transcripts in human umbilical venous endothelial cells. These findings reveal that vitronectin receptors are heterogeneously expressed on different vascular cell types. αvβ3-integrin was identified by Conforti et al. (1992) as one of the major integrins expressed by human umbilical venous endothelial cells, which is consistent with our data. Expression and function of αvβ3-integrin in vascular smooth muscle cells is controversial. Low expression of β3-integrins has been reported in cultured human vascular smooth muscle cells (Skinner et al., 1994), while Liaw et al. (1995b) described strong expression and identified a major functional role in dedicated vascular smooth muscle cells. Studied cell lines, culture conditions, and the investigated species may account for divergent findings of expression levels. The dominance of αvβ5 as the major vitronectin receptor in human iliac arterial smooth muscle cells might indicate a distinct pattern of vascular integrin expression in adult vascular smooth muscle cells. With respect to heterogeneity of expression in human endothelial and smooth muscle cells, we characterised the functions of both receptors in adhesion and spreading assays using specific blocking antibodies. Consistent with the observed pattern of integrin expression, we demonstrated a significant inhibition of human umbilical venous endothelial cells adhesion and spreading on vitronectin through LM609, whereas αvβ5-blockade by P1F6 failed to inhibit these cell–matrix interactions. In iliac vascular smooth muscle cells, the opposite effect was observed. αvβ3 inhibition did not show any major effects on adhesion and spreading. Similarly, in accordance with the FACS and RT-PCR data, adhesion and spreading were suppressed by P1F6. Both integrins were expressed in umbilical vascular smooth muscle cells, and cell spreading was mediated by both αvβ3- and αvβ5-integrins, while the αvβ5-receptor seemed to be

predominant in regulating adhesive cell–matrix interactions to vitronectin. When LM609 and P1F6 were added simultaneously, additional inhibition of either adhesion and/or spreading could be observed in all cell types, indicating both receptors were functionally relevant. The level of expression might contribute to the functional dominance, which furthermore seems to change the adhesion/spreading process of one cell type (Bilato et al., 1997). Adhesion and spreading in all the three cell types were significantly suppressed by linear RGD peptides, implicating the importance of this peptide sequence in the integrin vitronectin binding site. In summary, the present data confirm a major functional role of the vitronectin receptor αvβ3 in human umbilical venous endothelial cells (Conforti et al., 1992; Ruegg et al., 1998). Whereas an intermediate distribution of αvβ3- and αvβ5-integrins in umbilical vascular smooth muscle cells was observed, αvβ5 was the dominant vitronectin receptor in iliac vascular smooth muscle cells. Previous published studies have focused on the function of the vitronectin receptor αvβ3 in human vascular smooth muscle cells (Brown et al., 1994; Stouffer et al., 1998). Bilato et al. (1997) have found αvβ5-integrin involved in attachment of human aortic vascular smooth muscle cells on vitronectin, whereas P1F6 inhibited adhesion in combination with LM609 only. Our findings in human iliac vascular smooth muscle cells are the first demonstration, that αvβ5-integrin regulates dominantly cell functions mediated through vitronectin in human vascular smooth muscle cells and substitutes αvβ3 in these cells.

The two glycoprotein IIb/IIIa inhibitors abciximab and tirofiban have been shown to provide sustained clinical benefits (EPIC-Investigators, 1994; Topol et al., 1994; EPILOG-Investigators, 1997; EPISTENT-Investigators, 1998; PRISM-PLUS-Investigators, 1998; Lincoff et al., 1999). Abciximab, a chimeric monoclonal antibody Fab fragment, reduced ischemic long-term complications in high- and low-risk patients undergoing coronary interventions (EPIC-Investigators, 1994; Topol et al., 1994; EPILOG-Investigators, 1997; EPISTENT-Investigators, 1998; Lincoff et al., 1999). Tirofiban, a non-peptidergic small-molecule agent, improved clinical long-term outcome in the PRISM PLUS trial in patients with acute coronary syndromes treated medically or with angioplasty (PRISM-PLUS-Investigators, 1998). In the RESTORE and PRISM studies, tirofiban had no beneficial effects after 6 months (RESTORE-Investigators, 1997; PRISM-Investigators, 1998). Whereas tirofiban seems to block solely the glycoprotein IIb/IIIa platelet receptor, abciximab has been demonstrated to inhibit also αvβ3-integrin mediated cell functions (Tam et al., 1998). It has been shown that abciximab binding to αvβ3 blocked cell adhesion of M21 melanoma cells, which express the αvβ3-receptor but not the glycoprotein IIb/IIIa receptor (Tam et al., 1998). Treatment of human aortic smooth muscle cells with murine 7E3 immunoglobulin G reduced thrombospondin-induced proliferation by approximately 60% (Stouffer et al., 1998). Furthermore, inhibition of αvβ3-integrin function resulted in reduction of neointima formation of the carotid artery in hamster and rabbit injury models (Choi et al., 1994; Matsuno et al., 1994). These studies proposed that direct vascular β3-inhibition contributes to the long-term clinical benefits of c7E3. We demonstrated surface binding of 7E3 in both cell types, which correlated with αvβ3-expression detected by LM609. The major inhibitory effects of c7E3 were observed for human umbilical venous endothelial cells adhesion and spreading and for spreading in human umbilical venous smooth muscle cells. In human iliac arterial smooth muscle cells, c7E3 failed to produce any major blocking effects, since αvβ3 was not mainly involved in these processes. Furthermore, the additional inhibitory effect of c7E3 and P1F6 co-treatment supports the binding of c7E3 to αvβ3. With respect to the cellular integrin expression in our study and in previous reports, c7E3 seems to mediate the major inhibitory effects at the endothelial lumen and in αvβ3-expressing vascular smooth muscle cells (Hoshiga et al., 1995). Localisation of αvβ3-expressing vascular smooth muscle cells in human atherosclerotic lesions and non-atherosclerotic arteries is controversial. Stouffer et al. (1998) reported a detectable expression of β3-integrins in the neointima of balloon-injured brachial arteries of a baboon 1 week after injury, which was undetectable in normal baboon brachial arteries. Srivatsa et al. (1997) demonstrated corresponding upregulation of αvβ3-expression after 7 days of arterial injury in a porcine coronary model and described persisting high levels in SMC of the media up to 21 days. Hoshiga et al. (1995) observed detectable αvβ3-expression in non-atherosclerotic and atherosclerotic human coronary arteries. We found αvβ3-staining along the endothelium of human iliac atherosclerotic arteries. Also the media was stained, which underscores the presence of αvβ3-expressing SMC in atherosclerotic lesions. 7E3 immunoreactivity corresponded with αvβ3-expression, detected by LM609, indicating that αvβ3 is the main vascular target of 7E3 in human atherosclerotic tissue. The αvβ5-integrin was also expressed in atherosclerotic human arteries, but the intensity of the staining was diffuse and lower compared to LM609. αvβ3 has also been identified as a critical osteopontin receptor elevated in a coordinate fashion with osteopontin after vascular injury (Liaw et al., 1995b). Osteopontin appears to be another important matrix protein deposited after endothelial injury or during vascular remodeling in vivo (Liaw et al., 1995b; Wang et al., 1996; Panda et al., 1997; Srivatsa et al., 1997). These findings support that αvβ3 and its matrix partners play a major role in atherosclerotic and restenotic mechanisms, whereas the role of αvβ5 is not well-defined yet. We also conclude that abciximab, which has potent blocking effects on αvβ3-functions in vitro may also have a major inhibitory in vivo impact on human atherosclerotic vasculature.

The potential beneficial long-term effect of tirofiban has not yet been established. We studied potential direct vascular effects in human endothelial and smooth muscle cells. Tirofiban had no direct inhibitory effect on adhesion or spreading on vitronectin in these cells, which might imply an indirect effect on the vascular wall. Tirofiban is a selective and specific glycoprotein IIb/IIIa inhibitor (Peerlinck et al., 1993). There is growing evidence that glycoprotein IIb/IIIa activation and subsequent platelet activation regulates important vascular mechanisms. Activated platelets have been estimated to accelerate thrombin generation and release numerous adhesive proteins and mitogens like platelet derived growth factor (PDGF) and thrombospondin (Holmsen, 1994; Walsh and Schmaier, 1994). These factors are known to stimulate vascular smooth muscle cells migration and proliferation (Stouffer et al., 1998; Goetze et al., 1999). Tirofiban and abciximab have been implicated in decreasing thrombin generation and may further enhance long-term outcome by inhibiting the release of proliferative signals derived from platelets (Reverter et al., 1996; Keularts et al., 1998). This might be an alternative pathway of indirect vascular effects of tirofiban, which requires further investigations.

In summary, abciximab effectively inhibits αvβ3 mediated functions in cultured human endothelial cells and β3-integrin expressing human vascular smooth muscle cells. Tirofiban had no direct inhibitory effects on vascular cells. Abciximab does not interfere with αvβ5, another vitronectin receptor, which can partially substitute αvβ3-mediated functions in human vascular smooth muscle cells, indicating that αvβ3 and αvβ5 share common cellular functions as vitronectin receptors in human vascular cells. We also demonstrated that 7E3 has binding sites on endothelial and vascular smooth muscle cells in atherosclerotic tissue. These findings support the hypothesis that sustained clinical benefits of abciximab might be partially mediated by its direct functions on the vascular wall, whereas tirofiban seems to act via indirect mechanisms.

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