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TGFβ1 antagonistic peptides inhibit TGFβ1-dependent angiogenesis

Simona Serratì [a], *Francesca Margheri* [a], *Marco Pucci* [a], *Anna Rita Cantelmo* [b], *Rosaria Cammarota* [b], *Javier Dotor* [c], *Francisco Borràs-Cuesta* [c], *Gabriella Fibbi* [a], *Adriana Albini* [b], *Mario Del Rosso* [a,*]

[a] *Department of Experimental Pathology and Oncology and DENOTHE, Center for the Study at Molecular and Clinical Level of Chronic, Degenerative and Neoplastic Diseases to DEvelop NOvel THErapies, University of Florence, Viale G.B. Morgagni, 50, 50134, Florence, Italy*

[b] *Scientific Pole Multimedica IRCCS, Milan, Italy*

[c] *Division of Hepatology and Gene Therapy, Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain*

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ABSTRACT

The role of transforming growth factor beta (TGFβ) in tumor promotion and in angiogenesis is context-dependent. While TGFβ prevents tumor growth and angiogenesis in early phases of tumor development, evidence is accumulating about its pro-angiogenic and tumor promotion activities in late-stages of tumor progression. Here we have studied, in an experimental context previously reported to disclose the pro-angiogenic effects of TGFβ, the blocking activity of TGFβ antagonist peptides. In agreement with previous results, we have observed that TGFβ exerts a powerful pro-angiogenic activity on human normal dermal microvascular endothelial cells (MVEC), by promoting invasion and capillary morphogenesis in Matrigel. No apoptotic activity of TGFβ was observed. By RT-PCR we have shown that TGFβ up-regulates expression not only of plasminogen activator inhibitor type-1 (PAI-1), but also of the urokinase-type plasminogen activator receptor (uPAR), whose inhibition by specific antibodies blunted the TGFβ angiogenic response *in vitro*. The SMAD2/3 and FAK signaling pathways were activated by TGFβ in MVEC, as an early and late response, respectively. The use of two different TGFβ1 antagonist peptides, derived from TGFβ type III receptor sequence and 15-mer phage display technology, inhibited the signaling and pro-angiogenic response *in vitro*, as well as uPAR and PAI-1 up-regulation of MVEC following TGFβ challenge. The anti-angiogenic properties of both inhibitors were evident also in the *in vivo* TGFβ Matrigel Sponge Assay. These results may be relevant to develop a potentially fruitful strategy for the therapy of late-stage-associated tumor angiogenesis.

1. Introduction

Transforming growth factor beta-1 (TGFβ1, referred to as TGFβ throughout the paper), is one of the most important growth factors acting in the tumor microenvironment, that has recently emerged as a putative therapeutic target against cancer and cancer-related angiogenesis [1]. TGFβ is a pleiotropic cytokine with opposing modes of action: its property to inhibit cell proliferation is cell type-dependent, and its role in adult tissue homeostasis and during embryonic

* *Corresponding author*. Tel.: +39 055 4598205.
E-mail address: delrosso@unifi.it (M. Del Rosso).

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development has frequently been reported to be the opposite in different cell types or in subsequent developmental stages [2]. In neoplastic disease, TGFβ suppresses the progression of early lesions, but later this effect is lost and cancer cells themselves produce TGFβ that promotes the metastatic process [3]. TGFβ has been recently shown to modulate a set of pro-metastatic genes that govern the pattern of osteoclast activation in the sites of bone colonization of lung cancer cells and its blockade reduces bone tumor burden and osteolytic metastases [4]. Also angiogenesis regulation reflects the opposite activities of TGFβ. TGFβ induces expression of selected members of the Vascular Endothelial Growth Factor (VEGF) family in normal endothelial [5] and carcinoma cells [6]. Knockouts for TGFβ and its receptors show clear defects in angiogenesis, which often eventuate with death *in utero* [2]. On the other hand, TGFβ inhibits endothelial cell proliferation [7], migration and proteolytic activity [8]. VEGF, an endothelial cell pro-survival factor, has been shown to act in concert with TGFβ to induce endothelial cell apoptosis [9]. In conclusion, TGFβ acts either as inhibitor or as enhancer of neo-vascularization. The opposing effects of TGFβ on angiogenesis have been tentatively explained by the differential involvement of two distinct TGFβ signaling cascades within endothelial cells, the ubiquitous activin receptor-like kinase (ALK)5-SMAD2/3 pathway and the endothelial cell-restricted ALK1-SMAD1/5 pathway [2,10]. Additionally, endothelial cells have TGFβ type III receptors, whose occupancy by TGFβ is essential to favor ALK activity and a successful angiogenic process [11].

Among other activities, phosphorylated SMAD proteins stimulate the production of plasminogen activator inhibitor type-1 (PAI-1), which maintains ECM integrity and favors its accumulation by inhibiting fibrinolysis and preventing plasmin-activation of pro-matrix metallo-proteases (MMPs) [12]. It is now firmly established that a proper activity of the cell-associated plasminogen activation (PA) system, composed by the urokinase-type plasminogen activator (uPA), its receptor (uPAR), and its type-1 inhibitor (PAI-1), is required in the invasion and capillary tube formation steps of angiogenesis [13]. Many data, starting with the pioneer observations of Rifkin and coworkers in early 1990s, indicate a regulative role of TGFβ on uPA and PAI-1 expression in endothelial cells and other cell lines [8,14–18]. However, despite the well established role of uPAR in angiogenesis, information about angiogenic growth factor-dependent regulation of uPAR expression in endothelial cells is limited to Vascular Endothelial Growth Factor [19] and Fibroblast Growth Factor-2 (FGF2) [20].

Here we studied the pro-angiogenic activity of TGFβ on human microvascular endothelial cells (MVEC), as related to TGFβ-dependent modulation of uPAR and of other members of the PA system, and inhibited TGFβ-dependent angiogenesis *in vitro* and *in vivo* by using two peptides, derived from human TGFβ type III receptor and phage display technology, with a high affinity for soluble TGFβ and potent inhibitory effects on TGFβ binding to its receptors [21,23]. The same peptides have been previously used successfully in controlling bleomycin-induced skin fibrosis in C3H mice [23] and in reducing bone colonization of lung cancer cells in a mouse model [4].

2. Materials and methods

2.1. Endothelial cell preparation and characterization

For patients selection, ethics approval and patient consent, as well as for endothelial cell isolation from skin biopsies, refer to D'Alessio et al. [24]. Microvascular endothelial cells were prepared from skin biopsies of 15 healthy patients undergoing surgery for traumatic events at the hands. Where present, cell colonies were detached with EDTA, and CD31-positive cells were subjected to immuno-magnetic isolation with Dynabeads CD31 (Dynal ASA, Oslo, Norway) [24]. Isolated cells were further identified as MVEC by labeling with anti-factor VIII-related antigen, anti-CD105 (endoglin), and re-probing with anti-CD31 antibodies. MVEC resulted negative for immune-staining of CD45 (Leukocyte Common Antigen, LCA). Cells were maintained in complete MCDB medium, supplemented with 30% FCS, 20-μg/ml endothelial cells growth supplement (ECGS), 10 μg/ml hydrocortisone, 15 UI/ml heparin, and antibiotics (100 UI/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml amphotericin), as described [24]. MVEC were used between the 3rd and 7th passage in culture.

2.2. Migration assays

The Boyden chamber (blind wells) was used to evaluate spontaneous and stimulated invasion (chemoinvasion) through Matrigel matrix (BD Biosciences, Two Oak Park, Bedford, MA)-coated porous filters, as described [24]. In some experiments Matrigel matrix Growth Factors Reduced (GFR) (obtained from the same source) has been used. For spontaneous invasion, 200 μl of cell suspension (6.25×10^3 cells) were placed in the upper compartment of the chamber and migration was allowed to occur for 6 h. For chemoinvasion, test solutions, containing increasing or fixed concentrations of the chemoattractant (recombinant human TGFβ1, cat. 100-21, PeproTech, Rocky Hill, NJ), were dissolved in serum-free medium and placed in the lower wells. In the experiments with TGFβ antagonists, each molecule was added to the lower well, together with the chemotactic substance. In the experiments with anti-uPAR and anti-PAI-1 antibodies, each antibody was added to the upper well (1.5 μg/ml), with the cell suspension. At the end of the migration time the filter was removed and fixed in methanol. Non-migrating cells on the upper surface of the filter were removed with a cotton swab, while migrated cells, adherent on the lower filter surface, were stained with Diff-Quick (Mertz-Dade AG, Dade International, Milan, Italy) and counted by a light microscope (40×) on the whole migration surface per each well. Mobilization was measured by the number of cells moving across the filter. Experiments were performed in triplicate. Migration was expressed as mean ± SD of the number of total cells counted/filter or as the percentage of basal response.

2.3. Proliferation studies

Cell growth was quantified in subconfluent cell monolayers, as described [24]. MVEC were seeded onto 24 multi-well plates (Sarstedt, Verona, Italy) (15×10^3 cells/well) in complete MCDB medium and were left to adhere overnight. Cells were then

extensively washed in phosphate buffered saline and maintained for 24 h in 1% FCS–MCDB medium. Medium was removed and cells were incubated with 1% FCS–MCDB medium containing increasing concentrations of TGFβ for 48 h. The proliferative effect of 30% FCS was defined as an optimal growth. Cells were fixed by adding 1 ml of ice-cold methanol, stained with Diff-Quick and counted. Experiments were performed in triplicate. Values were expressed as percent increase/decrease over basal response.

2.4. *In vitro capillary morphogenesis assay*

Matrigel (0.5 ml; 10–12 mg/ml) was pipetted into 13-mm (diameter) tissue culture wells and polymerized for 30 min to 1 h at 37 °C, as described [24]. MVEC were plated (60×10^3/ml), in complete MCDB medium, supplemented with 30% FCS, and 20 μg/ml ECGS. Capillary morphogenesis was also performed in the presence of 1 ng/ml TGFβ and/or 100–150 μg/ml antagonist peptide, and/or 1.5 μg/ml of anti-uPAR (cat. 3936, American Diagnostica Inc., Stamford, CT), and anti-PAI-1 (cat. sc-59633, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Positive controls were obtained upon stimulation with 10 ng/ml recombinant human VEGF (cat. 100-20 A, PeproTech). Some experiments of capillary morphogenesis were performed also with Matrigel matrix GFR. The effects on the growth and morphogenesis of endothelial cells were recorded after 6 and 24 h with an inverted microscope (Leitz DM-IRB) equipped with CCD optics and a digital analysis system. Results were quantified at 6 h by measuring the percent field occupancy of capillary projections. Six to nine photographic fields from three plates were scanned for each point.

2.5. *RT-PCR analysis of uPAR, uPA and PAI-1*

mRNA levels of uPAR, uPA and PAI-1 were determined by an internal-based semi-quantitative RT-PCR, using procedures previously described [25]. The primers' sequences, the size of products and cycling profile are reported in Table 1. The reaction products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide, followed by photography under ultraviolet illumination using Polaroid positive/negative instant films, and quantified as reported [25].

2.6. *Western blotting*

For signal transduction experiments N-MVEC were grown to 70% confluence and were serum-starved overnight in MCDB supplemented with 2% FCS. Specific treatments (TGFβ alone, antagonist alone, and TGFβ with each antagonist) were performed either for 60 min or 24 h upon MVEC starvation. Confluent monolayers of MVEC, before and after specific treatments, were lysed in a 10 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl, 1% Triton X-100, 15% glycerol, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of aprotinin per 100 ml. 40–100 μg of the cell extract protein were subjected to electrophoresis in SDS-(10% or 12%) polyacrylamide gel under reducing conditions and then blotted to a polyvinylidene difluoride membrane (Hybond-C Extra; Amersham Biosciences, Piscataway, NJ) for 3 h at 35 V. The membrane was incubated with 5% skim milk in 20 mM Tris buffer, pH 7.4, for 1 h at room temperature to block non-specific binding and then probed with primary antibody to phospho-ERK (p42/p44) (200 μg/ml, 1:500) (cat. 9101S, Cell Signaling, Beverly, MA), ERK-2 (200 μg/ml, 1:500) (cat. sc-154), JNK (200 μg/ml, 1:500) (cat. sc-571) and phospho-JNK (200 μg/ml, 1:500) (cat. sc-6254) (Santa Cruz Biotechnology), $p38^{MAPK}$ (250 μg/ml, 1:200) (cat. AB3188, Chemicon International, Temecula, CA), phospho-$p38^{MAPK}$ (250 μg/ml, 1:500) (cat. 44-684G, Biosource International, Camarillo, CA), SMAD5 (100 μg/ml, 1:500) (cat. 9517), SMAD2/3 (100 μg/ml, 1:500) (cat. 3102), phospho-SMAD1/5 (Ser 463/465) (100 μg/ml, 1:500) (cat. 9516), and phospho-SMAD2 (Ser 465/467) (100 μg/ml, 1:500) (cat. 3108) (Cell Signaling Technology, Inc., Danvers, MA), phospho-SMAD3 (1:500) (cat. AB3226, R&D System, Minneapolis, MN), phospho-FAK (tyr576/577) (100 μg/ml, 1:500) (cat. 3281, Cell Signaling), FAK (200 μg/ml, 1:500) (cat. sc-56901), overnight at 4 °C. After incubation with horseradish

Table 1 – PCR primers and cycling conditions.

Primers	Sequence	Size (bp)	Cycling profile
uPA	5′-AAAATGCTGTGTGCTGCTGACC-3′ (sense) 5′-CCCTGCCCTGAAGTCGTTAGTG-3′ (antisense)	704	94 °C, 1 min 56 °C, 1 min 72 °C, 1 min 35 cycles total
uPAR	5′-GGTCACCCGCCGCTG-3′ (sense) 5′-CCACTGCGGTACTGGACA-3′ (antisense)	910	94 °C, 1 min 56 °C, 1 min 72 °C, 1 min 35 cycles total
PAI-1	5′-GAACAAGGATGAGATCAGCACC-3′ (sense) 5′-ACTATGACAGCTGTGGATGAGG-3′ (antisense)	780	94 °C, 1 min 56 °C, 1 min 72 °C, 1 min 35 cycles total
GAPDH	5′-CCACCCATGGCAAATTCCATGGCA-3′ 5′-TCTAGACGGCAGGTCAGGTCCACC-3′	598	94 °C, 1 min 56 °C, 1 min 72 °C, 1 min 35 cycles total

peroxidase-conjugated donkey anti-mouse or anti-rabbit IgG (1:5000) for 1 h (Amersham Bioscience), immune complexes were detected with the enhanced chemiluminescence ECL™ detection system (Amersham Bioscience). The membranes were exposed to autoradiographic films (Hyperfilm MP; Amersham Bioscience) for 1–30 min.

Also TGFβ-dependent apoptosis of MVEC was evaluated by Western blot, using the following monoclonal antibodies: anti-caspase-3 (100 μg/ml, 1:500) (cat. sc-7272, Santa Cruz Biotechnology) and anti-PARP-1 (100 μg/ml, 1:500) (cat. ALX-804-210, Alexis Biochemicals, Farmingdale, NY). MVEC were grown to 70% confluence and were serum-starved overnight in MCDB medium supplemented with 2% FCS. Specific treatments (increasing concentration of TGFβ: 10, 100 pg/ml, 1, 10 ng/ml) were performed for additional 6, 24 and 72 h after MVEC starvation. Treatment with H_2O_2 (200 mM final concentration, apoptosis positive control) was performed for 1 h after starvation.

2.7. Immunocytochemistry

TGFβ-dependent apoptosis of MVEC was also evaluated by immunocytochemistry. N-MVEC were grown on glass slides to 70% confluence and were serum-starved overnight in MCDB medium supplemented with 2% FCS. Cells were incubated in the presence or absence of increasing concentrations of TGFβ (10, 100 pg/ml, 1, 10 ng/ml). α-bisabolol (Fluka and Riedel-De Haen, Sigma Chemical Company, Milan, Italy) (initial concentration 250 μM), was diluted 1:80 in order to induce apoptosis in MVEC over a 6–24 h treatment (positive control). Treatment with H_2O_2 for 1 h was used as a further positive control for apoptosis. MVEC were then stained with the fluorescent DNA-binding dye Hoechst 33342 (10 μg/ml) (Invitrogen, San Giuliano Milanese, Italy) for 15 min at room temperature. Condensed and fragmented nuclei, characteristic of apoptotic cells, were counted with a fluorescence microscope (Leyca DC-200, Leyca Microsystem Imaging Solutions Ltd, Cambridge, UK).

2.8. Antagonist TGFβ peptides

We have used two TGFβ antagonist peptides developed by Digna Biotech (Pamplona, Spain), one derived from its type III receptor, as previously reported [21]: peptide p144 (TSLDASII-WAMMQN, 1580.86 kDa); the other one derived from phage display library technology, as previously reported [22]: peptide p17 (KRIWFIPRSSWYERA, 1995.6 kDa). While p17 is water soluble, p144 is hydrophobic. The p144 stock solution was usually sonicated to reduce the particle size and to increase solubility before *in vitro* assays. After sonication, around 20% of p144 is dissolved in aqueous solutions (J. Dotor, personal communication). The final concentration of 100 μg/ml antagonist peptides was chosen on the basis of previous results [21,22] and of preliminary data indicating that at 250 μg/ml each peptide became toxic to MVEC.

2.9. Matrigel Sponge Assay (in vivo angiogenesis)

TGFβ (1 ng/ml), alone or in addition to heparin (50 U/ml), in the presence or absence of the TGFβ inhibitor peptides p144 and p17, was added to unpolymerized Matrigel at 4 °C at a final volume of 0.6 ml. The Matrigel suspension was carefully injected subcutaneously into the flanks of C57/BL6 male mice (Charles River, Calco [Lecco], Italy) using a cold syringe. As the Matrigel warms to body temperature, it polymerizes to a solid gel, which then becomes vascularized within 4 days in response to the angiogenic substance. The extent of vascularization is quantified by measuring the hemoglobin content of the recovered sponges. Groups of 4–8 pellets were injected for each treatment. The reagents for all treatments were added to the Matrigel solution prior to injection. Four days after injection, the pellets were removed, minced and diluted in water to measure the hemoglobin content with a Drabkin reagent kit (Sigma). Samples were also fixed in formalin and embedded in paraffin, for histological analysis and stained with hematoxylin-eosin.

2.10. Statistical analysis

Results are expressed as means ± SD for (n) experiments. Multiple comparisons were performed by the Student–Newman–Keuls test, after demonstration of significant differences among medians by non-parametric variance analysis according to Kruskal–Wallis.

3. Results

3.1. TGFβ promotes Matrigel invasion and capillary morphogenesis of human MVEC

The property of MVEC to move within ECM is the basic requirement to form ordered cords of endothelial cells appointed to give origin to blood vessels. The left panel of Fig. 1A shows the results of the Matrigel chemoinvasion assay under the effect of increasing TGFβ concentrations in the lower well of the migration chamber. Since the maximal effect was obtained at 1 ng/ml TGFβ, such a concentration was used throughout the whole study. The insets of the left panel of Fig. 1A show the appearance of the lower side of the Matrigel-coated porous filter after scraping of the upper face and staining of migrated MVEC. Invasion assays performed with Matrigel matrix GFR gave similar results. On the contrary, TGFβ did not stimulate MVEC proliferation (right panel of Fig. 1A). The final angiogenic event requires MVEC to organize in interconnecting tubular structures to form the new capillaries. Other studies have previously shown that TGFβ stimulates a capillary tube patterning by endothelial cells embedded in collagen type I [26] and fibrin matrices [27]. To assay whether also in our experimental conditions TGFβ stimulated capillary morphogenesis in MVEC, we have studied the capillary tube patterning in Matrigel, a method currently used in our laboratory [24]. In this assay MVEC produce elongated processes which eventuate in the formation of anastomosing cords of cells resembling a capillary plexus [24]. Fig. 1B shows that, after 6 h from plating on Matrigel, MVEC produced abundant networks of branching cords of cells (upper left panel). By 24 h (lower left panel), MVEC formed an interconnected network of anastomosing cells that, by low power light microscopy, had a ''honeycomb'' appearance. The

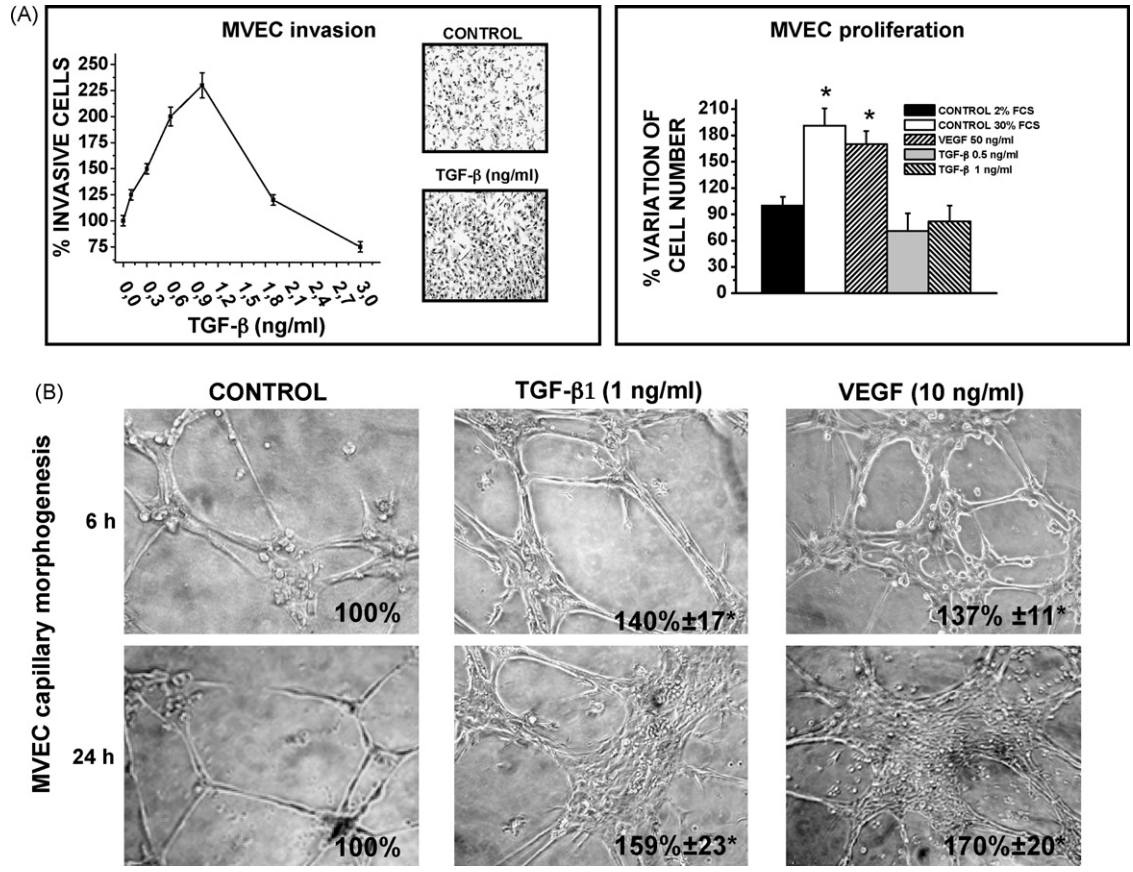

Fig. 1 – *In vitro* angiogenic activity of TGFβ on MVEC. (A) The left part of the panel shows the concentration-dependence of TGFβ-chemoinvasion of MVEC through Matrigel-coated porous filters in blind-well Boyden chamber assay. Data are expressed as percent increase of cell migration by TGFβ, with respect to the migration of control unstimulated cells considered as 100%. Insets show the typical aspect of the lower face of the filters in control unstimulated MVEC and under stimulation with 1 ng/ml TGFβ. The right part of the panel shows MVEC growth under the effect of TGFβ at the shown concentrations. Stimulation with 30% FCS and with 10 ng/ml VEGF was used as positive controls. Data are expressed as percent increase of cell number with respect to the number of control unstimulated cells considered as 100%. Data are the mean (±SD) of three different experiments performed in triplicate. $^{*}p < 0.05$, significantly different from control. (B) Capillary morphogenesis assay of MVEC seeded on Matrigel. 60×10^3 cells were plated on Matrigel-coated (0.5 ml; 10–12 mg/ml) 13 mm tissue culture wells and photographed after 6 and 24 h from plating. Positive control was obtained upon stimulation with 50 ng/ml VEGF (panels C and F). Six to nine photographic fields from three plates were scanned for each point, considering as 100% the area occupied by untreated MVEC. The data are expressed as percent modification (±SD) with respect to control. $^{*}p < 0.05$, significantly different from control. The results shown are representative of three different experiments. Two experimental replicas for invasion capillary morphogenesis assays performed with Matrigel matrix GFR gave similar results.

ability to form a complete network can be strengthened by the addition of angiogenic growth factors to culture medium. In the presence of 1 ng/ml TGFβ the capillary morphogenesis increased by about 40% and 59% at both 6 and 24 h, respectively (central panels), an effect that was comparable to that elicited by exogenous addition of 10 ng/ml VEGF, used as a positive control (right panels). The use of Matrigel matrix GFR gave similar results.

3.2. *TGFβ does not induce apoptosis of MVEC*

TGFβ has been shown to promote endothelial cell apoptosis *in vitro* [9], which has been suggested to be required for pruning the formation of vascular networks. We have checked for TGFβ-dependent apoptosis in MVEC. Fig. 2A shows the negative results obtained with Hoechst 33342 dye staining, using α-bisabolol and H_2O_2 treatment of MVEC as positive controls. It is noteworthy that under our experimental conditions neither TGFβ nor the peptide inhibitors alone, as well as the association of TGFβ and peptide inhibitors, stimulated apoptosis of MVEC. Results obtained with caspase-3 activation, shown in the Western blot reported in Fig. 2B, were in agreement with those obtained with immunocytochemistry. The use of anti-PARP-1 antibodies in Western blotting gave results similar to those obtained with anti-caspase-3 (not shown).

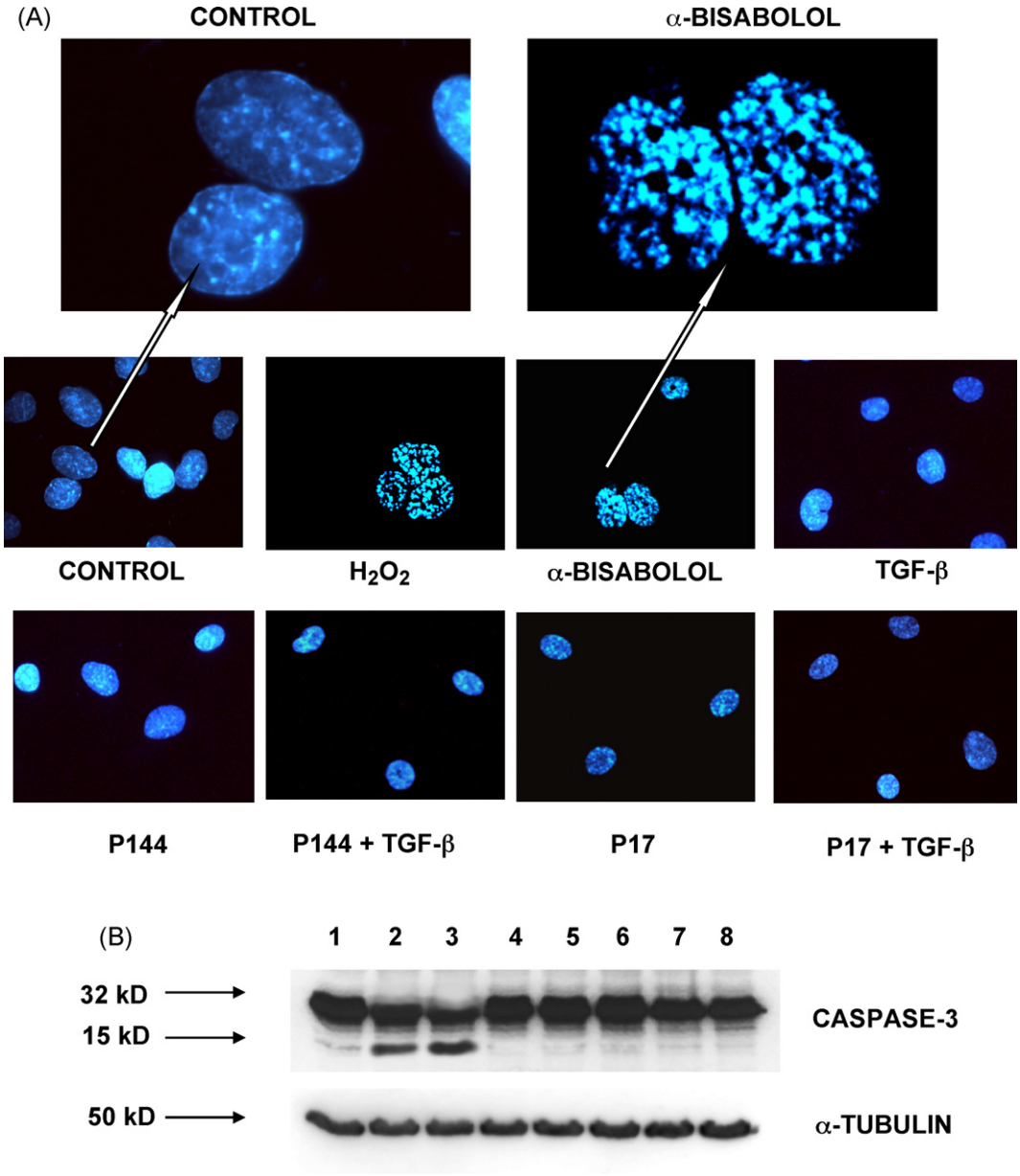

Fig. 2 – TGFβ-dependent apoptosis in MVEC. (A) Immunocytochemistry of MVEC stained with the fluorescent DNA-binding dye Hoechst 33342 in the conditions reported in the figure and described under Section 2. Condensed and fragmented nuclei, characteristic of apoptotic cells, were evaluated with a fluorescence microscope. (B) Western blotting for caspase-3 activation. Lane 1: control unstimulated MVEC; lane 2: pro-apoptotic effect of H_2O_2 (1 h); lane 3: pro-apoptotic effect of α-bisabolol (6 h); lane 4, stimulation with TGFβ (1 ng/ml for 6 h); lane 5: p144 alone (150 μg/ml for 6 h); lane 6: p144 (150 μg/ml) and TGFβ (1 ng/ml) for 6 h; lane 7: p17 alone (100 μg/ml for 6 h); lane 8: p17 (100 μg/ml) and TGFβ (1 ng/ml) for 6 h. The results obtained by incubating MVEC for longer times (6, 24 and 72 h) with the relevant substances gave similar results, as well as Western blotting with anti-PARP-1 antibodies (not shown). Molecular weights are shown on the left. α-Tubulin was used as a reference loading control. The results shown are representative of three different experiments performed in triplicate.

3.3. *TGFβ-induced MVEC invasion and capillary morphogenesis depend on up-regulation of uPAR*

Left panel of Fig. 3A shows uPAR and PAI-1 mRNA increase 24 h after TGFβ stimulation of MVEC, as revealed by RT-PCR. No modification was observed for uPA mRNA. The use of TGFβ peptide antagonists prevented TGFβ-dependent uPAR and PAI-1 up-regulation (right panel of Fig. 3A). TGFβ-stimulated MVEC increase of Matrigel invasion (Fig. 3B) and capillary morphogenesis (Fig. 3C) were inhibited by incubating MVEC in the upper well of the migration chamber and upon seeding on the Matrigel bed, respectively, with the anti-uPAR antibody (1.5 μg/ml, American Diagnostica, cat. 3936), which inhibits uPA/uPAR interaction. Also the treatment with anti-PAI-1

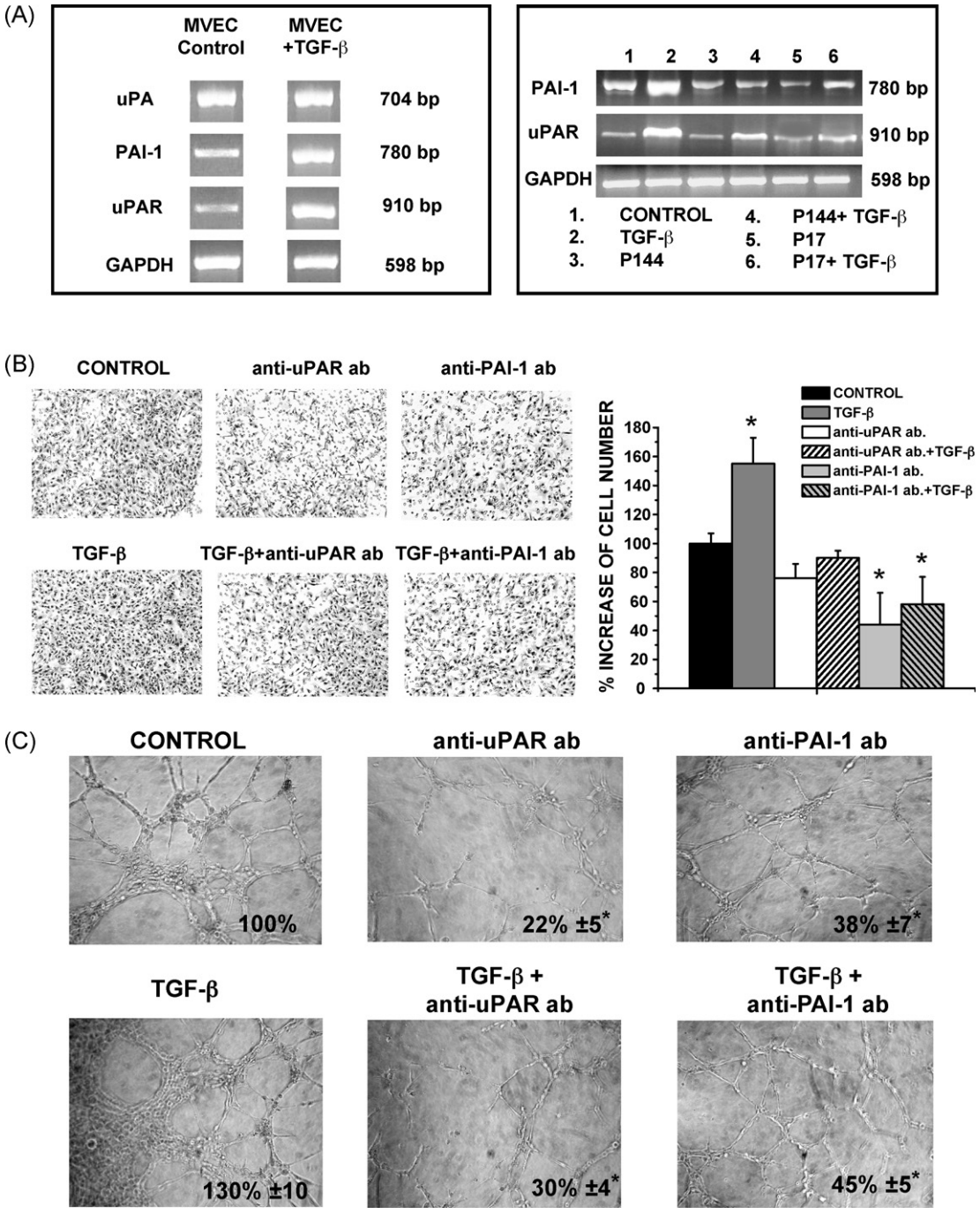

Fig. 3 – TGFβ-dependent up-regulation of uPAR and PAI-1 and their involvement in TGFβ-stimulated *in vitro* angiogenesis. (A) Left part: RT-PCR of uPA, uPAR and PAI-1 mRNA in MVEC, following 24 h of stimulation with TGFβ (1 ng/ml). 3 μg of total RNA were reversely transcribed, and then amplified using the primers reported in Table 1. GAPDH served as a control. Numbers on the right represent the size of each RT-PCR reaction product in terms of base pairs. Right part: RT-PCR showing modulation of TGFβ-dependent up-regulation of uPAR and PAI-1 by TGFβ antagonist peptides. Each experimental condition is reported in the figure and in the text. Refer to the figure of the left side for symbols. The results shown in the panel are representative of three different experiments. (B) uPAR and PAI-1 function in both spontaneous and TGFβ-dependent stimulation of MVEC Matrigel invasion. The left side of the panel shows the aspect of the lower side of the migration filters of a typical experiment performed in the reported conditions. Migration is expressed as % modification (±SD) of the number of invasive cells with respect to control unstimulated MVEC considered as 100%. The right side of the panel shows the quantification of three different experiments performed in triplicate. $^{*}p < 0.05$, significantly different from control. (C) uPAR and PAI-1 function in TGFβ-dependent stimulation of MVEC capillary morphogenesis. The percent decrease/increase of capillary morphogenesis is referred to control unstimulated cells taken as 100%. $^{*}p < 0.05$, significantly different from control. The results shown are representative of three different experiments.

antibody produced results similar to those obtained with anti-uPAR antibody, either in MVEC invasion (Fig. 3B) or in MVEC capillary morphogenesis assay (Fig. 3C). Collectively, these data indicate that TGFβ stimulates the invasive and differentiation steps of *in vitro* angiogenesis of MVEC mainly by uPAR and PAI-1 up-regulation. The results obtained with TGFβ stimulation and with peptide inhibitors of TGFβ–TGFβ receptor interaction suggest that PAI-1 is mainly a product

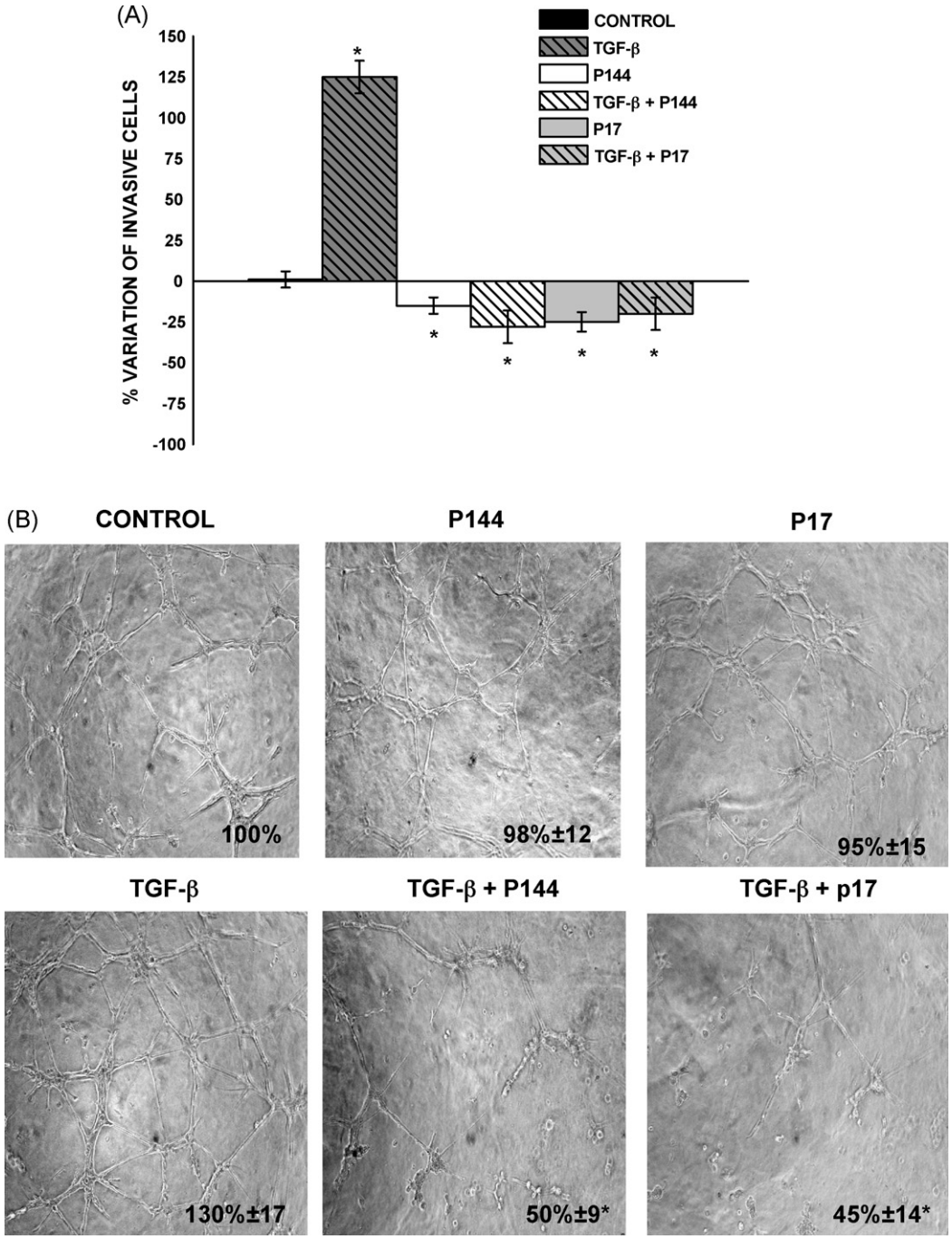

Fig. 4 – Effect of TGFβ antagonist peptides on TGFβ-dependent angiogenesis *in vitro*. (A) Activity of TGFβ antagonist peptides on MVEC invasion. Data shown represent the mean % modification (±SD) of the number of invasive cells with respect to control unstimulated MVEC, obtained in three different experiments performed in triplicate. $^{*}p < 0.05$, significantly different from control. (B) Activity of TGFβ antagonist peptides on MVEC capillary morphogenesis. The panels of the figure show capillary-like organization of MVEC after 24 h under each experimental condition. The data show a typical experiment out of three experiments performed in triplicate. Six to nine photographic fields from three plates were scanned for each point, considering as 100% the area occupied by untreated MVEC. The data are expressed as percent modification (±SD) with respect to control. $^{*}p < 0.05$, significantly different from control.

of MVEC themselves, rather than being picked-up from Matrigel, which has been reported to contain small amounts of entrapped PAI-1 and uPA [28].

3.4. TGFβ antagonist peptides inhibit TGFβ-dependent Matrigel invasion and capillary morphogenesis of MVEC

Fig. 4A shows the results of Matrigel chemoinvasion experiments performed by stimulating MVEC invasion with TGFβ added to the lower well of the chamber, in the presence or in the absence of various TGFβ antagonist peptides added to the cell suspension in the upper well of the migration chamber throughout the whole experiment. It is evident that both TGFβ antagonist peptides efficiently inhibited TGFβ-stimulated MVEC invasion. It is interesting that each peptide showed a basal MVEC invasion inhibiting activity even in the absence of exogenous TGFβ addition. It is likely that such an effect has to be related to antagonization of the TGFβ constitutively present within the Matrigel [28], which forms a very thin layer over the migration filter. Also capillary morphogenesis was inhibited by TGFβ antagonist peptides, as shown in Fig. 4B. In this case, in the absence of TGFβ stimulation antagonist peptides did not show any inhibiting activity, which was fully expressed following exogenous TGFβ addition. It is likely that the

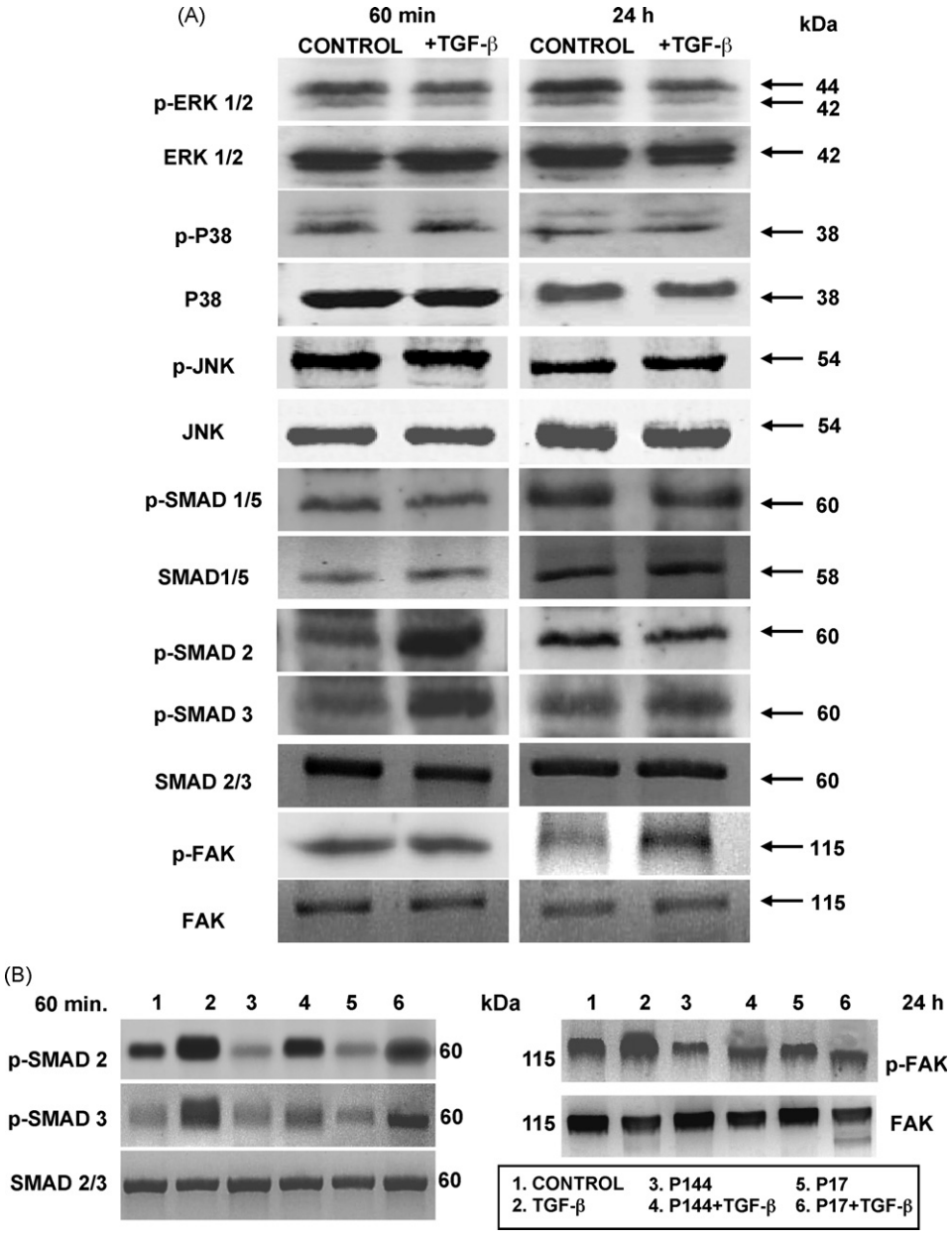

Fig. 5 – TGFβ signaling pathways in MVEC and activity of TGFβ antagonist peptides. (A) TGFβ-stimulated signaling pathways in MVEC. Involvement of signaling pathways was studied following short-term (60 min) and long-term (24 h) stimulation MVEC with TGFβ. Molecules of each pathway are shown as total and phosphorylated form (p-). (B) Activity of TGFβ antagonist peptides on SMAD2/3 and FAK signaling pathways. In both panels molecular weights are reported in kDa. Results are indicative of five different experiments.

thickness of the gel prevents antagonist peptides to reach Matrigel-entrapped TGFβ, which is instead easily inhibited once added together with inhibiting peptides.

3.5. *TGFβ-dependent signaling in MVEC is inhibited by antagonist TGFβ peptides*

The signaling pathways to be studied under TGFβ stimulation were selected on the basis of data recently reviewed by Leask [29]. Data shown in Fig. 5A indicate that after 1 h of TGFβ stimulation only the classic SMAD2/3 pathway was triggered in MVEC. However, upon a 24 h TGFβ stimulation only the FAK pathway resulted activated, while all the others, including SMAD2/3, did not show any change. Therefore, inhibiting experiments with TGFβ antagonist peptides were focused only on phospho-SMAD2/3 inhibition after short-term treatment and on phospho-FAK inhibition after long-term treatment. All the peptides inhibited SMAD2/3 and FAK phosphorylation at the relevant times (Fig. 5B). These data, coupled with the property of TGFβ antagonist of inhibiting TGFβ-induced uPAR and PAI-1 up-regulation (Fig. 3A), suggest that TGFβ promotes MVEC invasion and capillary morphogenesis by uPAR and PAI-1 up-regulation which follows phospho-SMAD2/3 and FAK phosphorylation, and that TGFβ antagonists inhibit TGFβ pro-angiogenic effects by preventing SMAD2/3 and FAK phosphorylation and the subsequent uPAR and PAI-1 up-regulation.

3.6. *TGFβ inhibitor peptides inhibit angiogenesis in Matrigel Sponge Assay in vivo*

In order to measure TGFβ *in vivo* angiogenesis, we performed a Matrigel Sponge Assay. Vascularization was seen in samples recovered from injected TGFβ-containing Matrigel sponges. In contrast, vascularization was inhibited by the addition of p144 and p17 as shown by decreases in hemoglobin content. Angiogenesis was induced by the addition of TGFβ alone (Fig. 6A), however, the efficiency was improved by the co-addition of heparin (Fig. 6B). The angiogenic effect of TGFβ was blocked by addition of the inhibitory peptides p144 and p17 (Fig. 6A–B). Both peptides alone have no effect when used in absence of pro-angiogenic stimuli (Fig. 6C).

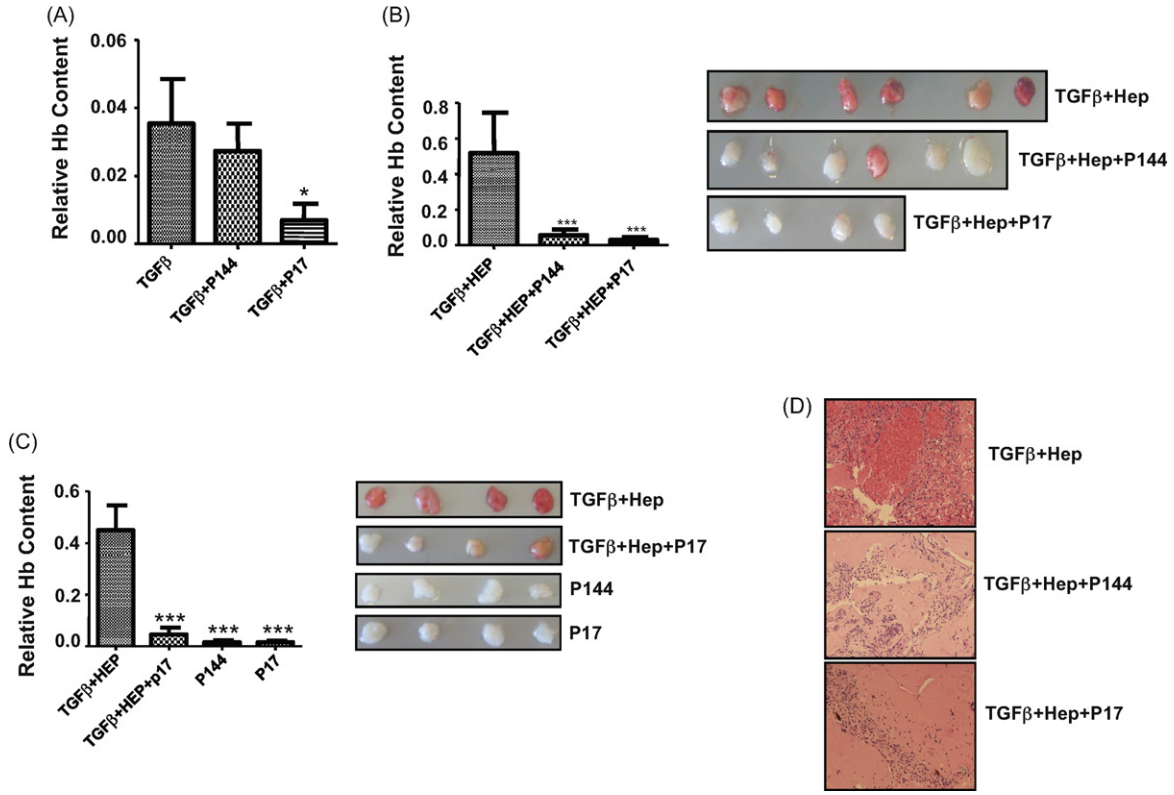

Fig. 6 – Matrigel Sponge Assay (*in vivo* angiogenesis). (A) Angiogenesis in a Matrigel Sponge Assay by the addition of matrigel containing TGFβ (1 ng/ml) alone or in the presence of the inhibitory peptides p144 and p17 (100 and 150 μg/ml, respectively). (B) Effect on TGFβ-induced angiogenesis of the inhibitory peptides p144 and p17 in the presence of heparin. Both peptides significantly blocked the pro-angiogenic effects of TGFβ (graphs are shown as mean ± SE; $^{***}p < 0.001$, Student t-test). On the right of the panel: representative photographs of individual Matrigel sponges recovered at autopsy in the experimental conditions shown on the left side of the panel. (C) The effect on TGFβ-induced angiogenesis of the inhibitory peptide p17 in the presence of heparin. Both peptides have no effects in absence of pro-angiogenic factors. The right side of the panel shows representative photographs of individual Matrigel sponges recovered at autopsy shown in the left part of the panel. (D) Histological analysis of Hematoxylin-eosin stained Matrigel sponges recovered from experiments shown in panel B. TGFβ-induced invasion of inflammatory cells and angiogenesis in the Matrigel sponge. In the presence of p144 or p17 the pro-angiogenic effects of TGFβ are reduced.

To analyze vascularization and cellular content, we used a hemotoxylin-eosin staining of sections from the recovered samples seen in Fig. 6B. In Matrigel sponges containing TGFβ, we observed invasive inflammatory cells and vascularization (Fig. 6D, upper panel), however, both vascularization and inflammation were reduced by the presence of p144 as well as p17 (Fig. 6D, medium and lower panels).

4. Discussion

We have shown that two peptides, derived from human TGFβ type III receptor and phage display technology respectively, with a high affinity for soluble TGFβ and potent inhibitory effects on TGFβ binding to its receptors [21,22] inhibited TGFβ-dependent MVEC invasion and capillary morphogenesis, by inhibiting TGFβ-dependent SMAD2/3 and FAK phosphorylation and up-regulation of uPAR and PAI-1. The same peptides exhibited a noticeable anti-angiogenic activity in the Matrigel Sponge Angiogenesis model *in vivo*. The *in vivo* anti-angiogenic properties of p17 and p144 were evident both in TGFβ-dependent angiogenesis and in heparin-enhanced TGFβ-dependent angiogenesis. Additionally, we observed that TGFβ promotes angiogenesis *in vitro* without exerting any pro-apoptotic effect. Antibodies against of uPAR and PAI-1 resulted in inhibition of TGFβ-dependent *in vitro* angiogenesis. Collectively, these evidences suggest that TGFβ antagonist peptides exert a strong inhibition on TGFβ-induced angiogenesis, which depends on inhibition of TGFβ binding to its receptor, reduction of SMAD2/3 and FAK phosphorylation and of the subsequent up-regulation of uPAR and PAI-1 expression in MVEC.

To study the angiogenic activity of TGFβ we have used a Matrigel-based model, both *in vitro* (invasion, capillary patterning) and *in vivo* (Matrigel Sponge Assay). Our data are in agreement with previous results obtained with TGFβ in collagen type I capillary morphogenesis of rat epididimal fat pad endothelial cells [26], and with those reported in fibrin gels for bovine aortic endothelial cells [27]. The similarity of results we have obtained with Matrigel matrix and Matrigel matrix GFR simplifies the interpretation of the data.

The best-described classical TGFβ signaling pathway involves TGFβ binding to its receptor, a type I/II heteromeric serine/threonine kinase, and induction of SMAD2/3 phosphorylation, which we have observed upon TGFβ stimulation in our human MVEC. Smads have intrinsic transcription-inducing activity [30]. In the basal state, SMAD2/3 are found in the cytoplasm but after activation, they form a complex with SMAD4 and are shuttled to the cytoplasm where they bind DNA at a number of transcription sites such as AP-1 or Sp-1 [31]. TGFβ has also been reported to regulate gene expression via activation of mitogen-activated protein kinases (MAPK), particularly p38 [32]. Although the stress-activated protein kinase p38 has been shown to be activated by TGFβ in endothelial cells from other species and tissues [33–37], human MVEC did not show any p38 activation under our experimental conditions. Similarly, we did not observe JNK activation, which has been previously described in TGFβ stimulation of bovine aortic endothelial cells [27]. FAK activation occurring 24 h after TGFβ challenging in MVEC is consistent with previous data [38]. Levels of FAK protein expression and/or activation have been correlated to phenotypic changes that affect cell differentiation and function, notably adhesion and migration, in a number of tissues [39–42]. Targeted FAK deletion in vascular endothelial cells leads to apoptosis and aberrant cell movement whereas FAK over-expression results in increased angiogenesis [43,44].

It is now generally accepted that TGFβ promotes late-stage tumor progression [45]. The ability of TGFβ to promote tumorigenesis was initially attributed to its activity on tumor-associated fibroblasts [46], able to stimulate the growth of epithelial cells and angiogenesis by direct cell-to-cell contact and by release of soluble factors, such as TGFβ. The loss of TGFβ responsiveness in mouse fibroblasts results into the loss of invasion of invasive prostate and squamous cell carcinoma of the forestomach [47]. Angiogenesis stimulation is considered one of the major tumor-promoting activity of TGFβ. The pro-angiogenic activity of TGFβ presumably combines direct and indirect effects. TGFβ induces fibroblasts to over-express VEGF, which acts on endothelial cells to stimulate proliferation and migration [48]. In endothelial cells ALK5 mediates a TGFβ-dependent recruitment of ALK1 into a TGFβ receptor complex and is required for optimal ALK1 activation and subsequent angiogenesis stimulation [10]. The type III TGFβ accessory receptor is essential for vascularity and is required for efficient TGFβ/ALK1-SMAD1/5 signaling, which indirectly inhibits TGFβ/ALK5-SMAD2/3 signaling [11]. Here we have shown that there are differences in the level of inhibition of p-SMAD2 and p-SMAD3 by the two antagonist peptides used. As shown in Fig. 5B, coincubation with TGFβ and p144 or p17 inhibited p-SMAD3, but not as much p-SMAD2. This is in line with a previous report showing pro-angiogenesis activity of the former [49]. The intense search for identification of the switch in TGFβ signaling pathway from one of tumor suppression to one of tumor promotion has led to development of TGFβ antagonists for inhibition of tumor progression and of TGFβ-dependent angiogenesis (for a review, [45]). However, systemic inhibition of TGFβ raises important safety concerns, since this factor displays pleiotropic effects including a well established anti-tumor activity, whose inhibition could result into tumor progression [50]. Therefore, results on TGFβ inhibition in the control of cancer-associated angiogenesis must be considered in their contextual dependence. From the TGFβ standpoint the most important issue is how to restore lost tumor suppressor function while eliminating or preventing acquired pro-oncogenic/angiogenic effects. Therefore, late-stage invasive, metastatic disease, which is typically characterized by locally or systemically elevated TGFβ levels, coupled with diminished responsiveness of tumor cells to its suppressor functions [51,52], has the chance to be the appropriate target for a therapeutic use of TGFβ antagonist peptides. Peptide p17 may have a better clinical implication due to its aqueous solubility. Experiments are in progress to evaluate the efficacy of each peptide and of their combination in the control of tumor development in mice. Immediately after homogeneous preparations of TGFβ were available, its role as a multi-functional regulatory molecule became appreciable and the contextual dependence of the role of TGFβ during carcinogenesis and angiogenesis represents a paradigm [3,53].

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