cAMP inhibits TGFβ1-induced in vitro angiogenesis

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Abstract Transforming growth factor-β (TGFβ1) is a proangiogenic factor both, in vitro and in vivo, that is mainly involved in the later phases of angiogenesis. In an attempt to identify genes that participate in this effect, we found that TGFβ1 downregulates expression of adenylate cyclase VI. In addition, cAMP analogs (8-Bromo-cAMP) and forskolin (an adenylate cyclase activator) also reduced TGFβ1-induced in vitro angiogenesis in mouse endothelial cell lines and in primary cultures of human umbilical vein endothelial cells on collagen gels. Induction of Ets-1 and plasminogen activator inhibitor-1 (PAI-1) by TGFβ1 was blocked by these cAMP agonists and activators, in the absence of effects on endothelial cell viability. Moreover, the signal transduction pathways stimulated by TGFβ1 were unaffected. Thus, Smad2 was normally phosphorylated and translocated to the nucleus in the presence of forskolin. In contrast, transfection studies using the PAI-1-promoter indicated that these cAMP analogues inhibit transcriptional stimulation by TGFβ1. Electrophoretic mobility shift assay showed that Smad2/3 were bound normally to a TGFβ1-response region in the presence of the cAMP analogs. In all, these data suggest that the cAMP pathway inhibits the transcriptional activity of Smads, that could be responsible for the block of the TGFβ1-induced in vitro angiogenesis caused by this second messenger.

Keywords: Angiogenesis; Transforming growth factor-β; cAMP; Adenylate cyclase VI; Endothelial

1. Introduction

Angiogenesis or the formation of new blood vessels from pre-existing vasculature occurs in normal situations such as embryonic development, wound healing and during the female reproductive cycle. However, activated blood vessel growth is also found in many diseases, such as tumor progression, diabetic retinopathy or arthritis [1,2]. In the last few years, several studies have led to the discovery of inducers and inhibitors of angiogenesis [3–5]. Among the inducers there are factors such as vascular endothelial growth factor (VEGF), FGF-1 and 2 that induce angiogenesis in vivo and in vitro. In contrast, other factors such as transforming growth factor-β (TGFβ) induce angiogenesis in vivo and in vitro but inhibit endothelial cell proliferation in vitro [3–5].

TGFβ1 is a 25-kDa peptide belonging to a family of multifunctional cytokines that control the development and homeostasis of most tissues by regulating diverse cellular functions, such as proliferation and differentiation [6–8]. The receptors for this family are basically two transmembrane serine/threonine kinases, termed receptor type I and type II. The binding of the ligand causes the heterodimerization of receptors I and II followed by the activation by phosphorylation of receptor I. This receptor then phosphorylates and activates the Smad family of proteins, which transduce the signal to the nucleus [6,7,9,10]. The role of TGFβ in angiogenesis was first shown by new capillary formation after injection of the factor in mice [11,12] and when applied to chicken chorioallantoic membrane (CAM) [13]. Moreover, TGFβ1 and TGFβ2 are expressed during the development of angiogenically active tissues [14,15]. This proangiogenic activity of TGFβ has been confirmed by experiments using knock-out mice. The knock-out of TGFβ1, the type II receptor and the type I receptor activin receptor-like kinase 1 (ALK1) are lethal at 10.5 days of gestation due to defective vasculogenesis (the initial formation of the primitive vasculature in the embryo), along with defective endothelial cell differentiation and inadequate capillary tube formation [16]. Morevoer, Smad5 knock-out mice also die due to defects in vasculogenesis and angiogenesis [17,18]. Finally, mutations in the human ALK1 gene and in the endoglin gene, a TGFβ1-binding protein which presents TGFβ1 to the type I and II receptors, all cause hereditary hemorrhagic telangiectasia, a disease characterized by vascular malformations [19,20]. Knock-out mice for endoglin also show a defective angiogenesis and die at embryonic day 11.5 [21]. In vitro, TGFβ inhibits endothelial cell proliferation in two-dimensional cultures [22–25], but induces tube formation when endothelial cells are cultured inside three-dimensional collagen gels [26–28]. Finally, TGFβ1 promotes the in vitro differentiation of embryonic stem cells to the endothelium as well as the formation of cord-like structures [29]. The signaling mechanisms implicated in the pro-angiogenic effect of TGFβ are now beginning to be resolved. Thus, it seems clear that at the start of angiogenesis, TGFβ works through the Alk1 receptor type I and Smad1 phosphorylation, which induces endothelial cells to proliferate and degrade the extracellular matrix, while in the last phase of the angiogenesis TGFβ signals through the Alk5 receptor and Smad2/3, blocking proliferation and enhancing extracellular matrix deposition [30]. It has also been shown that

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during the last phase TGFβ can indirectly stimulate the PI3K/Akt and the p42/p44 MAPK pathways, which is essential for cell survival and formation of capillary-like structures [31]. However, although some of the signaling mechanisms have been identified, the molecules that participate in the proangiogenic action of TGFβ are largely unknown. Using a mouse vascular endothelial cell model (1G11 cell line), which rapidly forms capillary-like structures in collagen and responds to TGFβ1, we examined the action of this angiogenic cytokine. We found a negative effect of cAMP on the proangiogenic action of TGFβ1, and that one of the genes down-regulated by TGFβ1 is adenylate cyclase VI.

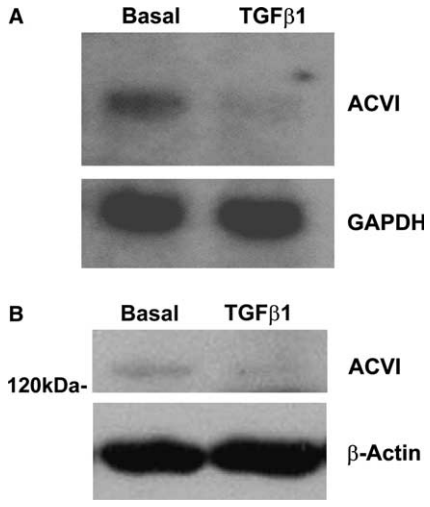

Fig. 1. TGFβ1 blocks adenylate cyclase VI mRNA and protein. (A) 1G11 cells immersed in collagen gels were cultured for 4 h in the presence of DMEM alone (Basal) or 25 ng of TGFβ1/ml (TGFβ1). Cells were lysed and total RNA was isolated. Gels were loaded with 25 μg of RNA and after blotting, adenylate cyclase VI (ACVI) and GAPDH were detected by hybridization using specific probes. A representative autoradiogram is shown. (B) 1G11 endothelial cells immersed in collagen gels were cultured for 48 h in the presence of DMEM alone (Basal) or 25 ng of TGFβ1/ml. Cells were lysed and adenylate cyclase VI was detected by immunoblotting with a specific antibody. β-Actin is shown as a loading control. A representative Western blot of three different experiments is shown.

2. Materials and methods

2.1. Materials

Human recombinant TGFβ1 was obtained from R&D Systems, and forskolin and 8-Bromo-cAMP were from Sigma, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was from Calbiochem. Cell culture media, FBS, glutamine and antibiotics were obtained from Gibco-BRL. The other reagents were of analytical or molecular biology grade and purchased from Sigma or Roche.

2.2. Cell culture and transfections

Murine lung capillary endothelial cells (cell line 1G11) were obtained from Alberto Mantovani and Annunciata Vecchi (Instituto Ricerche Farmacologiche Mario Negri, Milan, Italy) [32]. They were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20% FBS; 50 U of penicillin, 50 μg of streptomycin sulfate, 150 μg of endothelial cell growth supplement (Becton–Dickinson) and 100 μg of heparin (Sigma)/ml; 1% non-essential amino acids; and 2 mM sodium pyruvate. When indicated, 8-Br-cAMP or forskolin were added 10 min before the addition of TGFβ1.

1G11 cells were transiently transfected with a plasmid containing the TGFβ-responsive region of the plasminogen activator inhibitor-1 (PAI-1) promoter (3TP-lux) [33] and a β-galactosidase vector using Lipofectamine 2000 (Invitrogen). Luciferase assays were carried out using the Luciferase Assay System (Promega) 48 h after transfection and 16 h after treatment with 5 ng of TGFβ1/ml. β-Galactosidase activity was measured using a Luminescent β-galactosidase Detection Kit II (Clontech).

Human umbilical vein endothelial cells (HUVEC) were obtained as previously described [34] and were cultivated in M199 (Gibco) supplemented with 20% FCS, 100 μg/ml heparin and 150 μg/ml of endothelial cell growth supplement (Becton–Dickinson).

2.3. Tubulogenesis assays and cell survival

To induce capillary tube formation, 1G11 cells grown to confluence were trypsinized and resuspended in 2× DMEM. Cells were added to a type I collagen solution (Becton–Dickinson; 4 mg/ml) to achieve a cell concentration of 3×10^6 cells/ml and a final collagen concentration of 2 mg/ml. Sixty microliters of this preparation was placed in 24-well plates and incubated for 45 min at 37 °C in a humidified incubator to allow polymerization and DMEM alone or DMEM containing 25 ng of TGFβ1/ml was added where indicated. When indicated, 1 mM 8-Br-cAMP was added 10 min before TGFβ1. Protein extracts were obtained from the gels as described [31].

For HUVEC, 2×10^5 cells were seed in triplicate into a 24-well plate coated with 300 μl of rat tail collagen type I (1.2 mg/ml) in M199 and 10% FCS and allowed to attach for at least 2 h. Following this, cells were overlayed with an additional 300 μl of collagen. Once the collagen had polymerized, cells were fed with M199 medium, 10% FCS, 100 μg/ml heparin, 150 μg/ml of endothelial cell growth supplement, 25 ng/ml of TGFβ1 in the absence or presence of 1 mM 8-Br-cAMP. Following

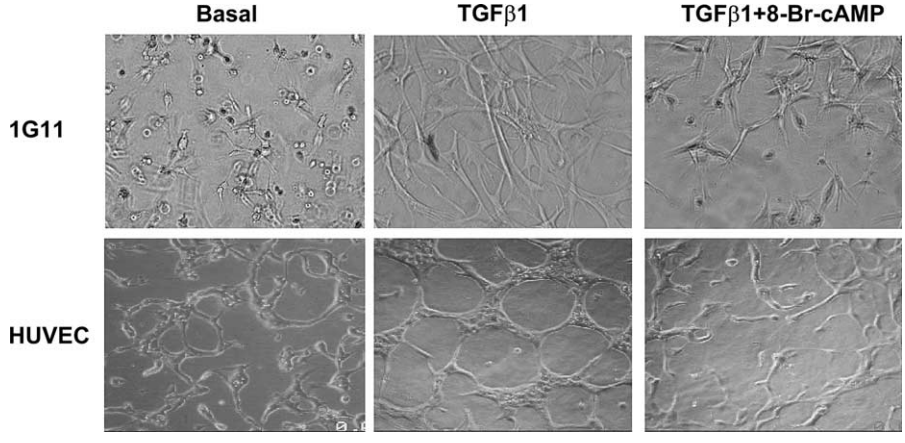

Fig. 2. cAMP blocks TGFβ1-induced in vitro angiogenesis in 1G11 and HUVEC cultures in type I collagen gels. 1G11 or HUVEC were cultured on collagen gels and TGFβ1 (25 ng/ml) alone or in the presence of 1 mM 8-Br-cAMP were added for 24 h. Gels were examined by phase-contrast microscopy.

completion of the network-formation (24 h) three images per well were captured on a Leica inverted phase-contrast microscope DMIRBE equipped with digital capture software.

To measure the viability of 1G11 cells after 24 h in collagen gels, propidium iodide (1 μg/ml; Sigma) was added and incubated for 10 min at 37 °C. Phase-contrast or immunofluorescence images were obtained using an immunofluorescence microscope.

2.4. Suppression substractive hybridization PCR-based technique

To identify genes expressed differentially by the presence of TGFβ1, 1G11 cells immersed in type I collagen gels were incubated for 4 h in the absence or presence of 25 ng of TGFβ1/ml. Cells were lysed and poly(A)$^+$ was isolated. The subtracted library was obtained using a kit of Clontech based on the suppression subtractive hybridization PCR-based technique [35]. The cDNAs obtained were subcloned in a TOPO cloning vector (Invitrogen) and sequenced.

2.5. Western blot analysis

Cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in Triton X-100 lysis buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 100 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 1 μg/ml of leupeptin, 4 μg/ml of aprotinin and 1% Triton X-100) for 15 min at 4 °C. Western blots were performed as described [36]. The blots were incubated with polyclonal anti-adenylate cyclase V/VI antibody (Santa Cruz), polyclonal anti-Ets-1 antibody (Santa Cruz), polyclonal anti-PARP antibody (Cell Signaling), polyclonal anti-phospho-Smad2 antibody (Upstate Biotechnology), polyclonal anti-phospho-CREB (Cell Signaling), monoclonal anti-β-actin antibody (Sigma) or polyclonal anti-ERK2 [37] in blocking solution overnight at 4 °C.

2.6. Northern blot

Total RNA from cells was extracted using the phenol/chloroform method [38], and Northern blot with 20 μg of RNA was performed as described [36]. Blots were hybridized to the mouse PAI-1 cDNA (a generous gift from Dr. Pura Muñoz-Canoves) or rat GAPDH cDNA labelled with [α-^{32}P]dCTP (Amersham Pharmacia).

2.7. Immunofluorescence studies

Cells were cultured on glass coverslips for 24 h, depleted of growth factors and treated with the different factors. Cells were rinsed three times with PBS and fixed in 3% paraformaldehyde for 30 min. After four washes with PBS, they were permeabilized with PBS–0.2% Triton X-100 for 5 min, rinsed four times with PBS and blocked for 30 min at room temperature in PBS containing 2% bovine serum albumin. Coverslips were incubated with mouse monoclonal anti-Smad2/3 antibody (BD Transduction Laboratories) in blocking solution for 1 h at room temperature, followed by Texas red anti-mouse (Molecular Probes) for 1 h at room temperature. Coverslips were mounted using Mowiol (Calbiochem) and immunofluorescence was visualized with a Nikon Eclipse E800 microscope.

2.8. Electrophoretic mobility shift assay

Preparation of the nuclear extract was performed as described by Ausubel et al. [39]. The DNA probe that responded to TGFβ1 was that described by Dennler et al. [40], with the following sequence: 5′-TCGAGAGCCAGACAAG GAGCCAGACAAGGAGCCAGA-CAC-3′. The oligonucleotides were ^{32}P-end-labeled using T4-polynucleotide kinase (MBI-Fermentas). The gel mobility shift assays were performed in a 15-μl reaction volume, containing 1 μg of double-stranded poly(dI–dC), 25 000 cmp of labeled DNA probe, 5 μg of protein nuclear extract, 10 mM HEPES, pH 7.9, 10% glycerol, 50 mM KCl, 0.1 mM EDTA and 0.25 mM dithiothreitol. The mixture (without the labeled DNA) was incubated for 15 min at room temperature. After the addition of the labeled DNA, the reaction mixture was incubated for another 15 min at room temperature, immediately loaded on a 5% polyacrylamide gel and run in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8) for 3–4 h. The gels were dried and autoradiographed.

Supershifts experiments were performed by incubating nuclear extracts, poly(dI–dC) and end-labeled probe as detailed above followed by incubation for 15 min at 37 °C in the presence of 0.25 μg of Smad2/3 antibody (BD Transduction Laboratories) or Smad4 (a generous gift from Dr. Joan Massagué). The samples were loaded on a 5% polyacrylamide gel, dried and autoradiographed.

3. Results

To identify genes involved in the pro-angiogenic effect of TGFβ1, we generated a subtracted cDNA library from 1G11 cells, immersed on collagen type I gels and treated for 4 h with TGFβ1, using the suppression subtractive hybridization PCR-based technique [35]. Among the genes repressed by TGFβ1, we have found the adenylate cyclase VI cDNA [41,42].

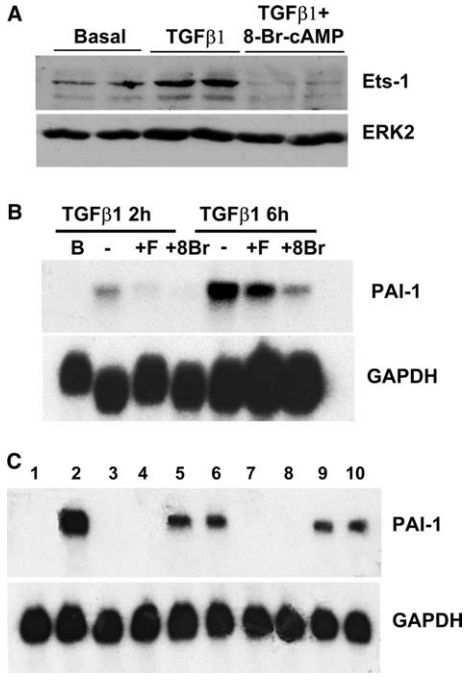

Fig. 3. cAMP prevents TGFβ1 induction of Ets-1 and PAI-1. (A) 1G11 endothelial cells immersed in collagen gels were cultured for 48 h in the presence of DMEM alone (Basal) or 25 ng of TGFβ1/ml either alone or with 1 mM 8-Br-cAMP. Cells were lysed and Ets-1 was detected by immunoblotting with a specific antibody. ERK2 is shown as a loading control. Lines 1 and 2, 3 and 4, and 5 and 6 correspond to duplicates. A representative Western blot of three different experiments is shown. (B) Depleted 1G11 endothelial cells cultured on plastic plates were stimulated with 25 ng of TGFβ1/ml alone or in the presence of 25 μM forskolin (F) or 1 mM 8-Br-cAMP (8Br) for 2 or 6 h or not stimulated (B). Cells were lysed and total RNA was isolated and analyzed by Northern blot. After blotting, PAI-1 or GAPDH (as a loading control) were detected by hybridization using a specific probe. A representative autoradiography is shown. (C) Depleted 1G11 endothelial cells cultured on plastic plates were incubated with different treatments. 1, DMEM alone for 6 h; 2, 25 ng of TGFβ1/ml for 6 h; 3, 25 ng of TGFβ1/ml for 6 h in the presence of 5 μg/ml of actinomycin D; 4, 25 ng of TGFβ1/ml for 6 h in the presence of 5 μg/ml of actinomycin D and 1 mM 8-Br-cAMP; 5, 25 ng of TGFβ1/ml for 3 h, and addition of 5 μg/ml of actinomycin D for additional 3 h; 6, 25 ng of TGFβ1/ml for 3 h, and addition of 5 μg/ml of actinomycin D and 1 mM 8-Br-cAMP for additional 3 h; 7, 25 ng of TGFβ1/ml for 6 h in the presence of 100 μM DRB; 8, 25 ng of TGFβ1/ml for 6 h in the presence of 100 μM DRB and 1 mM 8-Br-cAMP; 9, 25 ng of TGFβ1/ml for 3 h, and addition of 100 μM DRB for additional 3 h and 10, 25 ng of TGFβ1/ml for 3 h, and addition of 100 μM DRB and 1 mM 8-Br-cAMP for additional 3 h. After these treatments, RNA was obtained, and PAI-1 or GAPDH were detected by hybridization using a specific probe. A representative autoradiography of two independent experiments is shown.

Northern and Western blot experiments confirmed that adenylate cyclase VI mRNA and protein were repressed by TGFβ1 (Fig. 1). Adenylate cyclases are responsible for the production of cAMP in response to extracellular signals. Given the observed negative effect of TGFβ1 on adenylate cyclase VI, we evaluated the effect of cAMP on an in vitro model of angiogenesis stimulated by TGFβ1. 1G11 cells cultured on collagen type I gels formed tubular structures after 24 h incubation with TGFβ1 (Fig. 2). In contrast, pre-incubation in the presence of 8-Bromo-cAMP, a cell permeable analog of cAMP more resistant to phosphodiesterases, blocked the pro-angiogenic effect. We obtained the same results using primary cultures of HUVEC (Fig. 2): TGFβ1 caused the reorganization of the cells on collagen type I gels. In contrast, incubation in the presence of 8-Br-cAMP blocked the reorganization.

To confirm this inhibitory effect of cAMP on TGFβ1-induced in vitro angiogenesis, we studied the induction of a typical angiogenic marker, transcription factor Ets-1 [28,31,43]. TGFβ1 stimulated Ets-1 expression, but this induction was completely abolished by 8-Br-cAMP (Fig. 3A). A known TGFβ immediate responsive gene at the transcriptional level is PAI-1 [44]. To evaluate whether the blocking effect of 8-Br-cAMP on TGFβ1 actions was exerted at the transcriptional level, we performed Northern blot experiments using the cDNA for PAI-1 as a probe. Increasing cAMP levels by incubating cells either with forskolin or with 8-Br-cAMP inhibited the stimulation of PAI-1 mRNA by TGFβ1 in 1G11 cells (Fig. 3B). In order to discart an effect of cAMP on mRNA stability, we incubated 1G11 cells in the presence of TGFβ1 for 6 h. At the same time or 3 h later, 5 μg/ml of actinomycin D or 100 μM DRB were added to the samples to stop RNA synthesis (without change of the culture medium) for additional 3 h in the absence or presence of 1 mM 8-Br-cAMP. RNA was obtained and PAI-1 or GAPDH were detected by Northern blot. As is observed in Fig. 3C, presence of 8-Br-cAMP did not modify mRNA PAI-1 decay rate. Thus, cAMP not only affected the morphological reorganization of endothelial cells to form tubular structures, but TGFβ1 early induced genes were also blocked.

To identify the mechanisms involved in the effect of cAMP on TGFβ1-stimulated in vitro angiogenesis and gene expression, we first evaluated a possible effect of cAMP on endothelial cell viability. 1G11 cells cultured on collagen gels were incubated in the presence of 1 mM 8-Br-cAMP and 25 ng of TGFβ1/ml for 24 h. Cell viability was evaluated by propidium iodide staining (labeling the nuclei of dead cells with condensed chromatin). As shown in Fig. 4A, no differences were observed between cultures in the absence or presence of 8-Br-cAMP. We also analyzed PARP cleavage as a measure of apoptosis. No significant differences were observed between

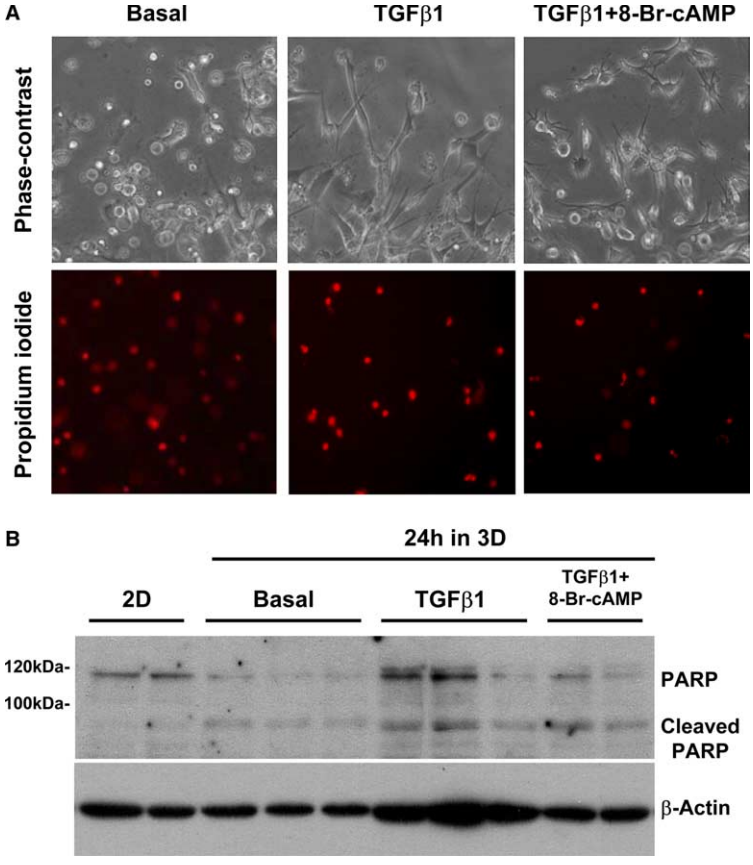

Fig. 4. cAMP does not affect 1G11 cells viability. (A) 1G11 were cultured on collagen gels and DMEM (Basal), TGFβ1 (25 ng/ml) alone or in the presence of 1 mM 8-Br-cAMP were added for 24 h. After propidium iodide addition for 10 min, gels were examined by phase-contrast or immunofluorescence microscopy. (B) Proliferative 1G11 endothelial cells (2D) or immersed in collagen gels (24 h in 3D) were cultured for 24 h in the presence of DMEM alone (Basal) or 25 ng of TGFβ1/ml either alone or with 1 mM 8-Br-cAMP. Cells were lysed, and PARP was detected by immunoblotting with a specific antibody. β-Actin is shown as a loading control. Results obtained from two independent samples of proliferative 1G11 cells, three of Basal, three of TGFβ1 and two of TGFβ1 + 8-Br-cAMP are shown.

cells incubated for 24 h with TGFβ1 in the absence or presence of 1 mM 8-Br-cAMP (Fig. 4B). When cells were maintained for 4 days with the same factors, 8-Br-cAMP increased apoptosis (data not shown).

Next, we analyzed the effect of cAMP on the TGFβ1 signalling pathways. 1G11 cells were pre-incubated in the presence of forskolin followed by TGFβ1 addition for 1, 2, 4 or 8 h. We did not detect any difference in Smad2 phosphorylation by TGFβ1 in the absence or presence of forskolin, in conditions where CREB was phosphorylated by high levels of cAMP (Fig. 5A). Smad2/3 are translocated to the nucleus after their phosphorylation by the TGFβ receptor type I. Thus, one possibility was that cAMP modified the translocation of Smad2/3 in response to TGFβ. To test this hypothesis we performed immunofluorescence studies using antibodies against total Smad2/3 (Fig. 5B). In the basal state, Smad2/3 was mainly located in the cytoplasm. In contrast, incubation with TGFβ1 for 1 h caused the translocation of Smad2/3 to the nucleus, which was not affected by incubation with either forskolin or 8-Br-cAMP.

We also evaluated a possible effect of cAMP on the transcriptional activity of Smads. We transfected 1G11 cells with a plasmid containing the TGFβ-responsive region of the PAI-1 promoter [33]. TGFβ1 caused a threefold increase in the activity of the promoter in these cells, whereas incubation in the presence of 8-Br-cAMP abolished this induction (Fig. 6A). This effect on the promoter activity could be due to a lack of binding of the transcriptional activators Smad2/3–Smad4 to the specific zones of the promoter. To test this possibility, we performed electrophoretic mobility shift assays (EMSA) using nuclear extracts from 1G11 cells non-treated or treated for 1 h with TGFβ1 or with this factor in the presence of forskolin, using the TGFβ-responsive region of the PAI-1 promoter as a probe [40]. Incubation with TGFβ1 caused a shift in the mobility of the probe due to the presence of Smad2/3 present in the nuclear extracts, as confirmed by super-shift assays (Fig. 6B). However, the presence of forskolin in the incubation medium did not alter the pattern of

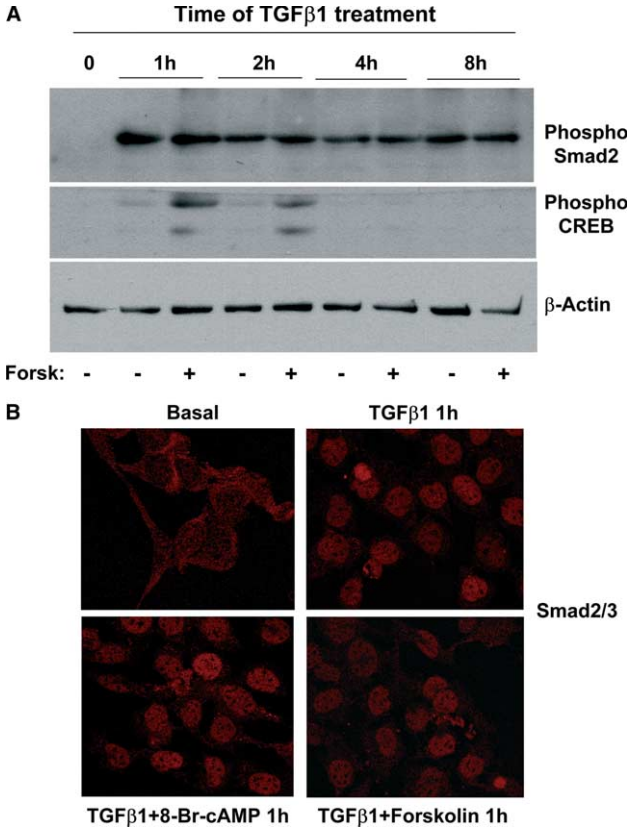

Fig. 5. cAMP does not affect TGFβ1 signaling. (A) Depleted 1G11 cells were pre-incubated in the absence (−F) or presence (+F) of 25 μM forskolin for 10 min. After that, 25 ng of TGFβ1/ml was added for the indicated periods of time. Cells were lysed and phosphoSmad2, phosphoCREB or β-actin were immunodetected by Western blot. A representative autoradiogram of three independent experiments is shown. (B) Depleted 1G11 cells were pre-incubated in the absence or presence of 1 mM 8-Br-cAMP or 25 μM forskolin for 10 min. After this time, cells were incubated for 1 h in the absence (Basal) or the presence of 25 ng of TGFβ1/ml. Cells were fixed and Smad2/3 immunolocalized.

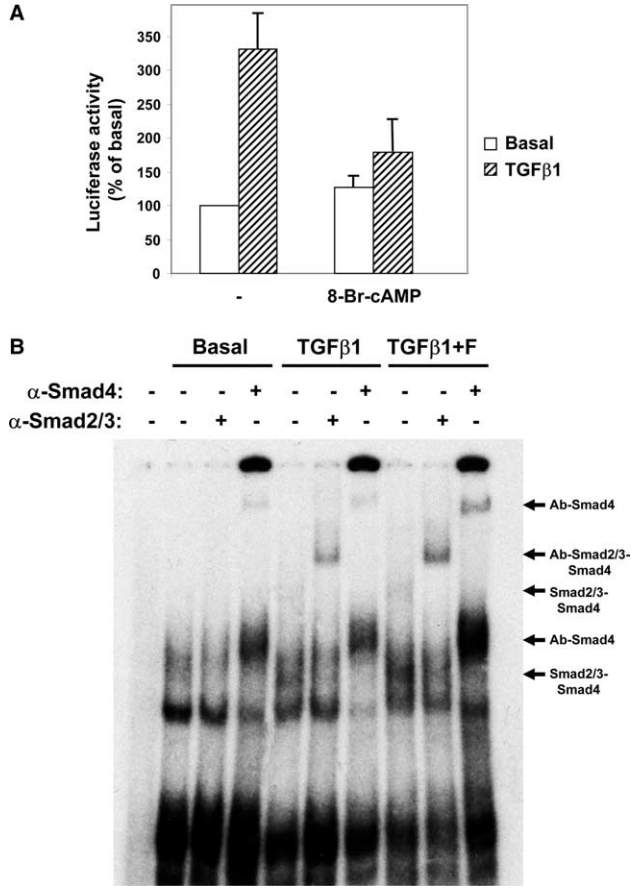

Fig. 6. cAMP blocks TGFβ1-induced promoter transcription but does not affect Smad2/3 binding to DNA. (A) 1G11 cells were transiently transfected with 3TP-lux reporter and β-galactosidase transfection control, incubated for 24 h in complete medium, rinsed with PBS and incubated for additional 16 h in the presence of 1 mM 8-Br-cAMP in the absence of growth factors (Basal) or the presence of 5 ng of TGFβ1/ml. Cells were harvested, and luciferase and β-galactosidase activities were measured. The results are means ± S.E.M. of three different experiments. (B) A TGFβ-responsive region of the PAI-1 promoter was incubated with 5 μg of nuclear extracts from 1G11 cells treated for 1 h in the absence (Basal) or the presence of 25 ng of TGFβ1/ml alone or in the presence of 25 μM forskolin (TGFβ1 + F). An antibody against Smad4 or Smad2/3 was added or not and samples were analyzed on a 5% polyacrylamide gel. Arrows indicating the complexes Smad4, Smad2/3–Smad4 and the supershift caused by the antibodies are included.

bands in the EMSA or the bound levels of Smad2/3-containing complexes.

4. Discussion

Agents that increase cAMP levels, such as prostaglandins, β-adrenergic agonists or phosphodiesterase inhibitors, all decrease endothelial cell barrier permeability by promoting attachment to the basal membrane [45,46]. Moreover, increased cAMP levels block the pro-angiogenic effects of several factors, such as migration, proliferation and in vitro angiogenesis of HUVEC induced by VEGF and bFGF [47,48], in vitro angiogenesis stimulated by collagen I of dermal microvascular endothelial cells [49], and in vivo angiogenesis stimulated by bFGF in the CAM assay [50,51]. Here, we show that this inhibitory effect of cAMP is also observed for a distinct angiogenic factor, TGFβ1. In contrast to VEGF or bFGF, which stimulate the first phases of angiogenesis and induce matrix degradation, proliferation and migration of endothelial cells, TGFβ1 participates in the later phases of angiogenesis, when endothelial cells become more quiescent [3,30,52]. Thus, TGFβ1 blocks endothelial cell proliferation, increases basement membrane deposition and causes morphological organization to form the capillaries. The capacity of cAMP to block angiogenesis stimulated by both types of factors implies that this second messenger affects a common mechanism for angiogenesis, such as the reorganization and migration of the endothelial cells. Alternatively cAMP may induce apoptosis [51]. However in our model, apoptosis only occurs after much longer incubation times, implicating other mechanisms involved in the anti-angiogenic response.

Our results also show that cAMP affects the ability of TGFβ1 to stimulate gene transcription. Thus, PAI-1 mRNA and promoter activity are decreased in conditions where TGFβ-induced Smad2/3 phosphorylation, translocation to the nucleus and binding to DNA were not altered by cAMP activation. cAMP also blocks TGFβ1-induced transcription for other genes in endothelial cells, such as c-sis [53], but the mechanisms that cause this block of TGFβ1-dependent transcription remain unknown. Recently it has been described that the inhibitory effect of cAMP on TGFβ-induced transcription in HaCaT keratinocytes is mediated by diminishing the association of Smad3 with transcriptional coactivators CBP and p300 [54]. cAMP may also induce the recruitment of a repressor of the Smads complex that blocks transcription, similarly to the recruitment of ATF3 for Id1 repression by TGFβ in epithelial cells [55]. Finally, a third possibility is the induction of the phosphorylation of Smads by cAMP, which would reduce its transcriptional activity. This negative regulation of the transcriptional activity of Smads by phosphorylation has already been described for Ca^{2+}/calmodulin-dependent protein kinase II and for PKC [8].

Our results also indicate that one of the effects of TGFβ1 on endothelial cells is to decrease the levels of adenylate cyclase VI. This enzyme is highly expressed in endothelial cells [56] and its negative regulation is responsible for the down-regulation of the levels of cAMP by Ca^{2+} [46]. Other pro-angiogenic stimuli decrease cAMP levels in a similar way. Thus, activation of integrins α1β1 and α2β1 by collagen I decreases cAMP levels and PKA activity in human dermal microvascular endothelial cells [49], and activation of α5β1 by vitronectin suppresses PKA activity in HUVEC [50]. The mechanisms responsible for these effects are unknown, but the TGFβ-induced decrease in the levels of adenylate cyclase VI may contribute to the suppression of cAMP during angiogenesis.

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