Angiostatin and endostatin inhibit endothelial cell migration in response to FGF and VEGF without interfering with specific intracellular signal transduction pathways

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Abstract The anti-angiogenic agents angiostatin and endostatin have been shown to affect endothelial cell migration in a number of studies. We have examined the effect of these agents on intracellular signalling pathways known to regulate endothelial cell migration and proliferation/survival. Both agents inhibited fibroblast growth factor (FGF)-, and vascular endothelial growth factor (VEGF)-mediated migration of primary human microvascular endothelial cells and affected vascular formation in the embryoid body model. However, using phosphospecific antibodies we could not detect any effect of angiostatin or endostatin on phospholipase C-γ (PLC-γ), Akt/PKB, p44/42 mitogen-activated protein kinase (MAPK), p38 MAPK and p21-activated kinase (PAK) activity. Furthermore, using a glutathione S-transferase (GST)-PAK pull-down assay, we could not detect any effect on Rac activity. We conclude that angiostatin and endostatin inhibit chemotaxis, without affecting intracellular signalling pathways known to regulate endothelial migration and proliferation/survival.

Key words: Angiostatin; Endostatin; Cell migration; Angiogenesis

1. Introduction

Angiogenesis denotes the formation of new blood vessels from preexisting vessels. This physiological process is involved in embryonic development and the menstrual cycle. It is also involved in pathological conditions such as tumour progression, rheumatoid arthritis and diabetic retinopathy. Angiogenesis is stimulated by a number of angiogenic factors, including fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) [1,2]. These angiogenic factors bind to their cognate receptor expressed on the surface of endothelial cells and evoke an intracellular signalling response, culminating in the proliferation, migration and eventual differentiation of endothelial cells to form lumen-containing vessels.

The potential pharmacological regulation of angiogenesis is now an area of intense interest. In 1994, O'Reilly et al. described a 38 kDa fragment of plasminogen called angiostatin (AS), which was purified from mice bearing a Lewis lung carcinoma [3]. AS inhibits tumour angiogenesis and induces tumour dormancy by inhibiting endothelial cell proliferation [4] and by inducing apoptosis [5,6]. AS has been shown to bind and block α/β-ATP synthase on the surface of endothelial cells, inhibiting proliferation [7], whilst another study has described an AS binding protein, termed angiomotin, which is required for AS to inhibit endothelial cell chemotaxis [8]. In 1997, O'Reilly et al. isolated endostatin (ES), a 20 kDa fragment of the C-terminal part of the collagen XVIII α1-chain [9]. ES has been reported to bind to α_v- and α_5-integrins [10] and to a low affinity glypican type receptor [11]. Whilst a number of studies of AS and ES action have shown an affect on endothelial cell chemotaxis [12,13], no studies so far have addressed the effect of these compounds on pathways known to regulate cellular migration.

The migration of endothelial cells is a critical component of the angiogenic response, where cells must digest the basement membrane, migrate and proliferate to form a new lumen-containing vessel. Cellular migration is regulated in a spatial and temporal manner by Rho family GTPases [14]. In this paper we have examined the effect of AS and ES on a number of intracellular pathways implicated in endothelial cell physiology. We report that whilst AS and ES can affect cellular migration of primary endothelial cells and vascular development in embryoid bodies, there is no apparent effect on intracellular pathways known to regulate cellular migration and proliferation. We conclude that both AS and ES have a novel effect in perturbing cellular migration.

2. Materials and methods

2.1. Antibodies and reagents

Phosphospecific rabbit polyclonal antibodies against p44/42 MAPK (Thr-202/Tyr-204), p38 MAPK (Thr-180/Tyr-182) Akt/PKB (Ser-473), PLC-γ (Tyr-783) and PAK (PAK1 Thr-423, PAK2 Thr-402) were purchased from Cell Signalling. The monoclonal anti-Rac clone 23A8 was purchased from Upstate Biotechnology. Recombinant human FGF-2 was purchased from Boehringer Mannheim (Mannheim, Germany) and recombinant human VEGF-A_{165} and recombinant human platelet-derived growth factor (PDGF-BB) were purchased from Peprotech (England). Bovine type I collagen was purchased from Vitrogen (Cohesion Technologies, USA). Recombinant human AS and ES, purified from *Pichia pastoris*, were provided by Entremed (Rockville, MD, USA). Endotoxin level for AS was 0.002 EU/mg whilst that for ES was undetectable.

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Abbreviations: AS, angiostatin; EBs, embryoid bodies; ES, endostatin; FCS, foetal calf serum

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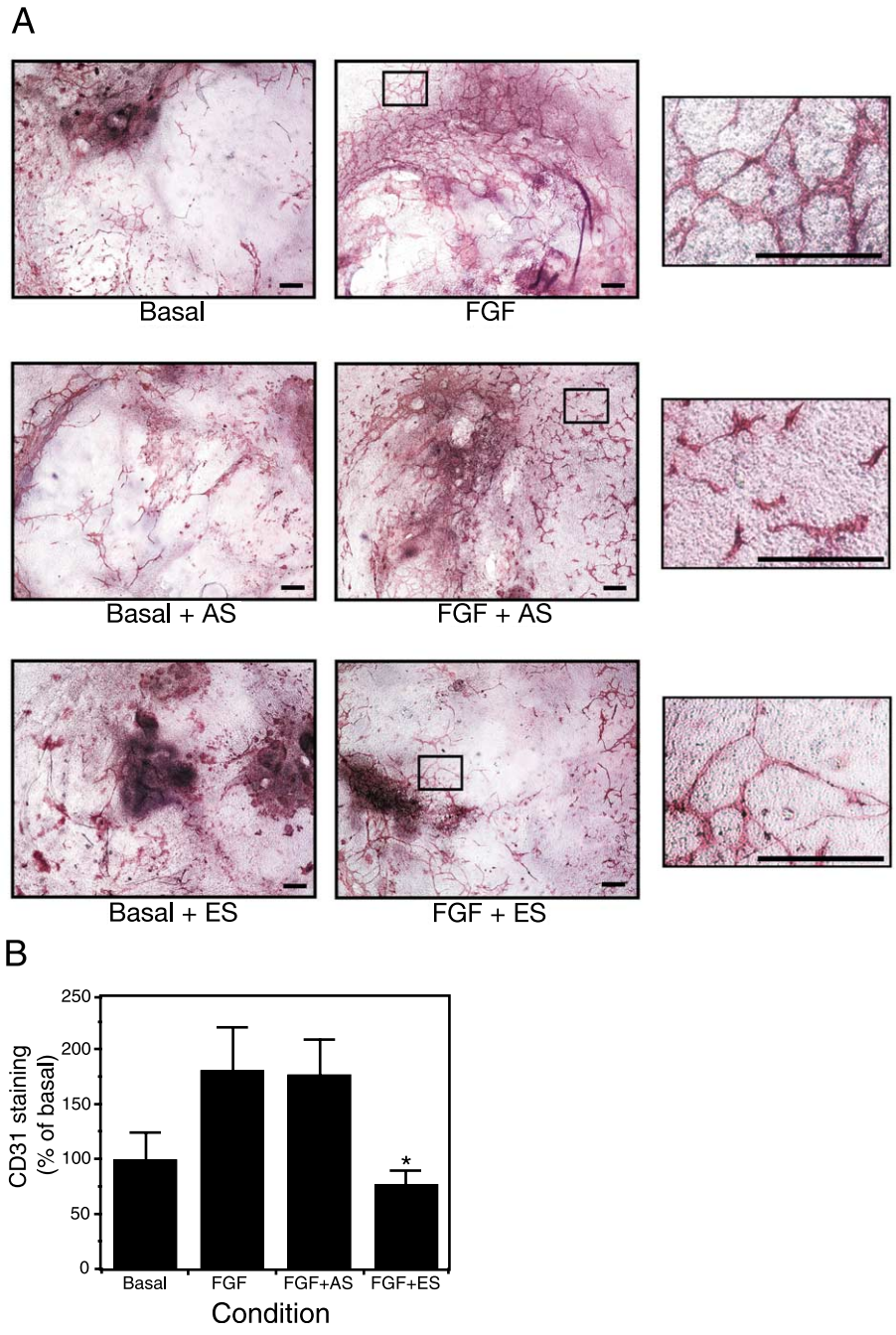

Fig. 1. Effect of AS and ES on vascular development in EBs. A: Murine embryonal stem cells were allowed to differentiate into EBs in the absence or presence of FGF-2. Bodies were incubated with either AS (3 μg/ml) or ES (3 μg/ml) and were allowed to develop for 12 days. Endothelial cells were visualised by staining for CD31. Pictures shown are ×10 magnification. Higher magnifications (×50) of specific areas from the FGF-stimulated conditions are shown. Bars represent 500 μm. B: The amount of CD31 staining was quantified. Results are expressed as the percentage of basal staining (mean ± S.D., $n = 3$) from a single experiment representative of three. *$P < 0.05$ compared with FGF alone, as determined using Student's t-test for unpaired data.

2.2. Cell culture

Human dermal microvascular endothelial cells (HDMECs) were isolated from neonatal foreskin as previously described [15]. Cells were routinely cultured on gelatin-coated plastic dishes in endothelial basal media (EBM; Clonetics, USA) containing 10% foetal calf serum (FCS) and 2 ng/ml FGF-2.

2.3. Chemotaxis assay

Chemotaxis was analysed essentially as described [13] using a 48-well modified Boyden chemotaxis chamber (Neuro Probe, Cabin John, USA). Polycarbonate membranes (pore size 8 μM, polyvinylpyrrolidone-free) were coated with bovine collagen type I (150 μg/ml). HDMECs were starved in EBM containing 1% FCS overnight. The cells were trypsinised, resuspended in EBM containing 0.1% bovine serum albumin (BSA) and either AS or ES and placed in the upper chamber. Either FGF or VEGF, with or without AS or ES, was placed in the lower chamber and cells were allowed to migrate towards this for 4 h at 37°C. The number of cells that had migrated to the lower surface of the membrane were counted in three fields using ×400 magnification. Samples were analysed in triplicate on three separate occasions.

2.4. Embryoid bodies

The mouse embryonal stem cell line R1, derived from the strain SvJ129, was a kind gift of Dr. A. Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada). Embryonal stem cells were cultured in medium composed of Dulbecco's modified Eagle's medium/glutamax (Invitrogen, Rockville, MD, USA) supplemented with 15% heat-inactivated FCS, 25 mM HEPES, 1.2 mM Na-pyruvate (Invitrogen), 19 μM monothioglycerol (Sigma, St. Louis, MO, USA) and 1000 U/ml recombinant leukaemia inhibitory factor (LIF; Chemicon International, Harrow, UK). Cells were cultured at 37°C with 5% CO_2. Medium was changed every day and splitting was done every second or third day. To induce differentiation, LIF was withdrawn from the medium. Aggregation was induced by placing 20 μl drops of 1200 cells on the lid of a non-adherent tissue culture dish in the presence or absence of FGF-2 (20 ng/ml). The day when LIF was removed from the media and droplets were formed was denoted day 0. The drops were left hanging on the lid for 4 days, in 37°C and 5% CO_2, whereafter they were plated one by one in eight-well glass culture slides (Falcon, Frankling Lakes, NJ, USA) in the presence or absence of FGF-2. The inhibitors AS (3 μg/ml) or ES (3 μg/ml) were added as indicated. Media including inhibitors were changed every fourth day. On day 12, the embryoid bodies (EBs) were fixed for 10 min in 4% paraformaldehyde, blocked for endogenous peroxidase and non-specific binding before incubation of primary antibody rat anti-mouse CD31 (Becton Dickinson (BD) Biosciences, Erembodegem, Belgium) overnight at 4°C. Secondary biotinylated antibody goat anti-rat IgG (Vector Laboratories, Burlingame, CA, USA) was added followed by streptavidin–horseradish peroxidase (HRP) (Vector) and the chromogen substrate, 3-amino-9-ethylcarbazole (AEC; Vector Laboratories). Pictures were taken with an inverted Nikon Eclipse Microscope (Nikon, Kanagawa, Japan). Quantification of CD31 staining was performed with Easy Image analysis software (Tekno Optik AB, Stockholm, Sweden).

2.5. Western blotting

Cells were seeded at 2×10^5 cells per well of gelatin-coated six-well plates in growth medium and cultured for 48 h at 37°C. The medium was changed to EBM containing 1% FCS and cells incubated for another 24 h at 37°C. Cells were stimulated with agonist, rinsed in ice-cold phosphate-buffered saline (PBS) and lysed in 250 μl Laemmli sample buffer containing 1 mM phenylmethylsulphonyl fluoride (PMSF). Lysates were sonicated for 5 s, heated at 95°C for 5 min and centrifuged at $14\,000\times g$ for 15 min. Supernatant was transferred to a fresh Eppendorf tube. Approximately 50 μg of total cellular protein was resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 10% gel. Proteins were detected by Western blotting as previously described [16].

2.6. Analysis of Rac activation

Escherichia coli harbouring the plasmid pGEX2TK containing the PAK-Crib domain (56–272) fused to glutathione S-transferase (GST) was kindly provided by Dr. Collard (Netherlands Cancer Institute). Fusion protein was purified as previously described [16]. For the analysis of Rac activation, cells were washed in ice-cold PBS and lysed in 50 mM Tris–HCl pH 7.4 containing 10 mM $MgCl_2$, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Lysates were clarified by centrifugation and approximately 500 μg of total cellular protein incubated with 50 μg of GST-Pak-Crib domain fusion protein for 1 h at 4°C with end-over-end mixing. The beads were collected by centrifugation and washed four times in lysis buffer. The beads were resuspended in Laemmli sample buffer and boiled for 5 min. Proteins were resolved by SDS–PAGE using a 15% gel, transferred electrophoretically and visualised using electrochemoluminescence (ECL).

2.7. Thymidine assay

Mitogenicity was determined by the incorporation of [^{3}H]thymidine as previously described [16].

3. Results and discussion

3.1. AS and ES affect angiogenesis in the embryoid body model

Embryoid bodies (EBs) are murine embryonic stem cells which develop vascular structures in response to treatment with angiogenic growth factors. The EBs have been shown to represent a useful in vitro model of angiogenesis [17]. In the presence of FGF-2, a characteristic twisted network of long and slender vessels is formed. We were interested in the ability of AS and ES to affect the endothelial vasculature in the EBs. Fig. 1A shows the characteristic capillary plexus of endothelial cells in the presence of FGF-2. When AS was added to the culture, in the presence of FGF-2, the vessel network appeared truncated and disjointed, although the number of endothelial cells were not affected, compared to FGF-2 alone (Fig. 1B). In the presence of ES, the plexus was considerably reduced and the vessels were thinner and more stretched. The number of endothelial cells were reduced to basal levels in the presence of ES (Fig. 1B).

3.2. AS and ES inhibit the migration of human endothelial cells stimulated with FGF and VEGF

AS and ES have been previously shown to affect a number of endothelial responses including cellular migration, which may explain the anti-angiogenic effects of these agents in vivo. We utilised human dermal microvascular endothelial cells (HDMECs) to study the effect of both agents on FGF-

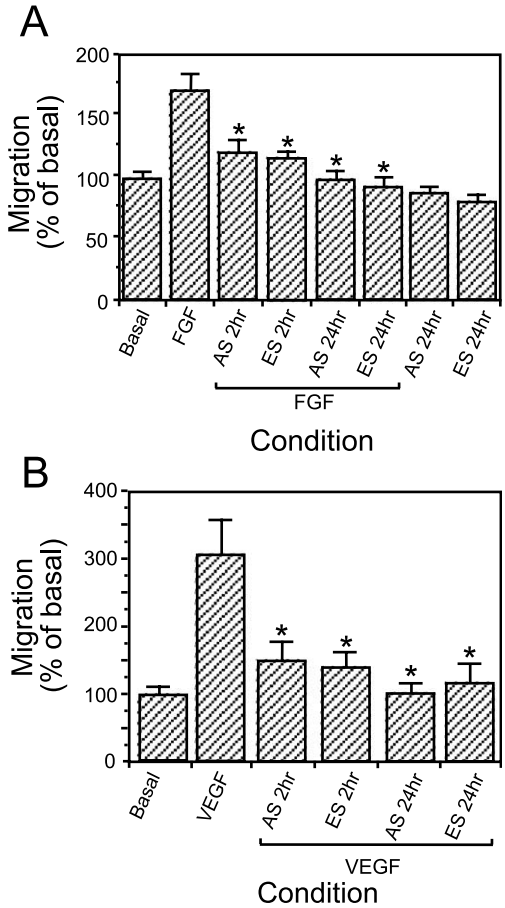

Fig. 2. Effect of AS and ES on endothelial cell chemotaxis. HDMECs were preincubated with either AS (3 μg/ml) or ES (3 μg/ml) for the times shown. Cells were allowed to migrate towards either, A: FGF (10 ng/ml), or B: VEGF (30 ng/ml), using a modified Boyden chamber for 4 h at 37°C. Results are expressed as the percentage of basal response (mean ± S.D., $n=3$) from a single experiment representative of three. *$P<0.01$ compared with either FGF or VEGF alone, as determined using Student's t-test for unpaired data.

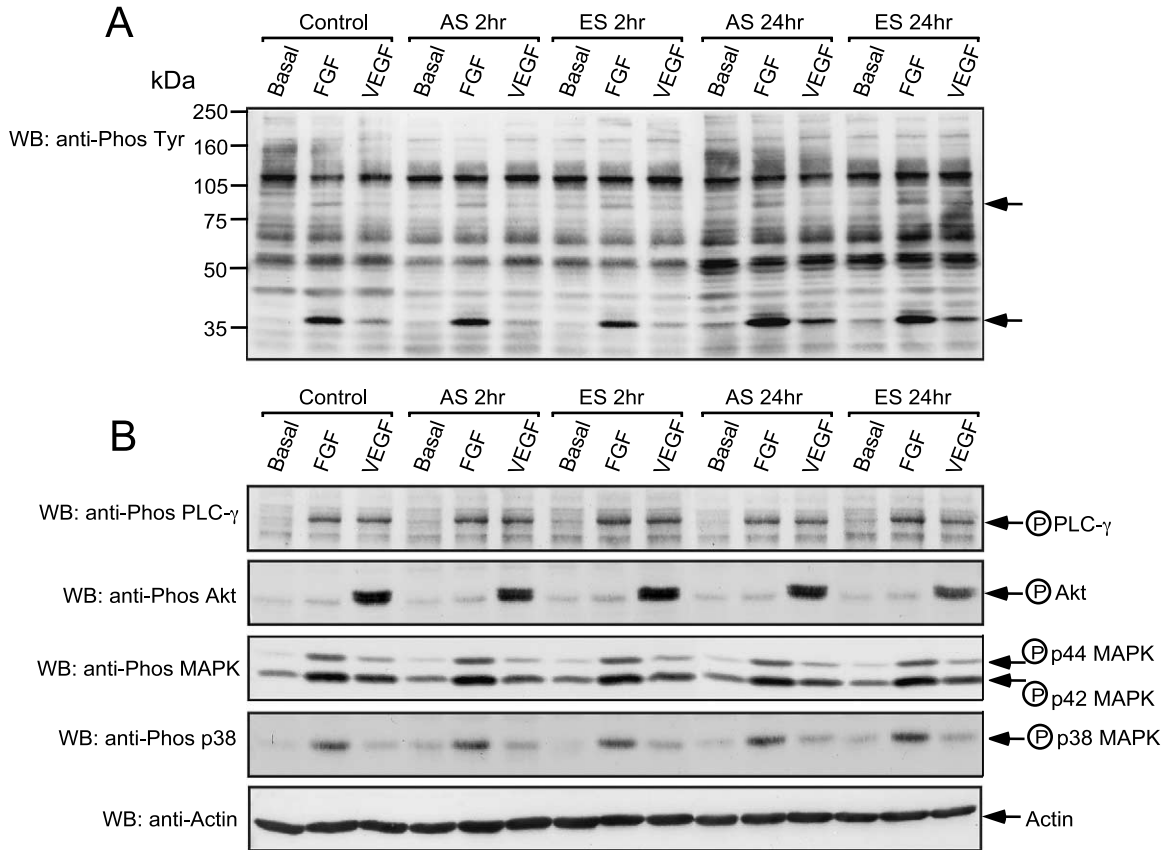

Fig. 3. Effect of AS and ES on intracellular signalling pathways. HDMECs were preincubated with either AS (3 μg/ml) or ES (3 μg/ml) for the times shown. Cells were stimulated with either FGF (10 ng/ml) or VEGF (30 ng/ml), lysed and proteins separated by SDS–PAGE followed by Western blotting. A: Analysis of tyrosine phosphorylation of cellular proteins. Arrows indicate bands of approximately 90 and 42 kDa. B: Analysis of the phosphorylation status of PLC-γ, Akt/PKB, p44/42 MAPK and p38 MAPK. Actin is used as a loading control. Results shown are representative of three separate experiments.

and VEGF-stimulated cell migration. Fig. 2 shows that both AS and ES inhibited HDMEC migration in response to FGF and VEGF. Preincubation with the inhibitors for either 2 or 24 h produced a similar effect in inhibiting migration, confirming that long-term exposure to these agents was not required to affect migration.

3.3. *AS and ES do not affect intracellular signalling pathways stimulated by FGF and VEGF*

FGF and VEGF stimulate a number of intracellular signalling pathways leading to an angiogenic response in endothelial cells [1,2]. We sought to determine if AS and ES affected a number of these signalling pathways which may explain their known effects on endothelial cells. Analysis of the tyrosine phosphorylated proteins in cell extracts from FGF- and VEGF-stimulated HDMECs (Fig. 3A), revealed that FGF induced the phosphorylation of proteins of approximately 90 and 42 kDa, probably FGF-receptor substrate 2 (FRS2) and p42 MAPK respectively. VEGF induced a weaker phosphorylation of only the 42 kDa protein. Preincubation with either AS or ES did not affect the tyrosine phosphorylation status of these proteins (Fig. 3A). Using phosphospecific antibodies, we analysed the phosphorylation status of a number of intracellular signalling molecules known to be activated by either FGF and/or VEGF. We were interested in specific proteins such as PLC-γ which has been implicated in FGF-mediated cytoskeletal reorganisation [18] and VEGF-mediated mitogenicity [19], Akt/PKB which is known to regulate cell survival, p44/42 MAPK which regulates mitogenicity [1], and p38 MAPK implicated in FGF-mediated differentiation [20] and VEGF-mediated migration [21]. Fig. 3B shows that neither AS nor ES affected the phosphorylation status of PLC-γ, Akt, p44/42 MAPK and p38 MAPK in response to FGF and VEGF in HDMECs, suggesting that these anti-angiogenic agents did not affect pathways regulating cell survival and mitogenicity in endothelial cells. In an attempt to study AS and ES action under identical conditions as during migration, we also plated HDMECs onto collagen type I-coated plates and incubated them with either AS or ES for 2 and 24 h. Analysis of the intracellular phosphorylation status of PLC-γ, Akt, p44/42 MAPK and p38 MAPK following FGF or VEGF stimulation revealed that AS and ES treatment showed no effect (data not shown).

We next determined the direct effect of AS and ES on HDMEC mitogenicity by measuring [^{3}H]thymidine incorporation in response to FGF and VEGF. However, only FGF-2 was able to evoke a mitogenic response in these cells (Fig. 4). Neither AS nor ES affected FGF-2-stimulated mitogenicity, further supporting their lack of effect on pathways such as MAPK (Fig. 3). Both AS and ES were originally reported to inhibit the growth of bovine capillary endothelial cells [3,9]. However, recent data have shown that AS does not affect FGF- or VEGF-mediated mitogenicity in human umbilical vein cells [22]. Taken together with our data, it is pos-

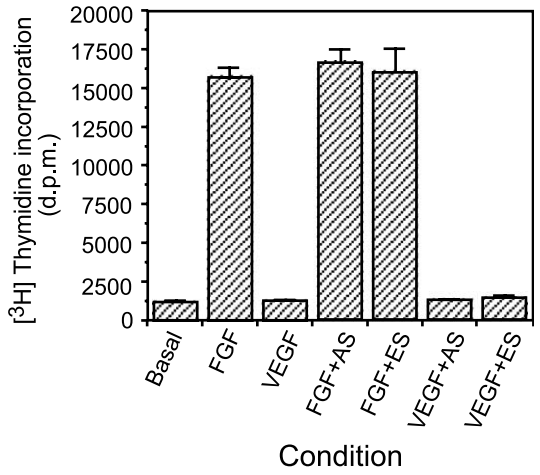

Fig. 4. Effect of AS and ES on mitogenicity. HDMECs were stimulated with either FGF (10 ng/ml) or VEGF (30 ng/ml) for 20 h in the presence of either AS (3 μg/ml) or ES (3 μg/ml). Mitogenicity was measured by [^{3}H]thymidine incorporation. Results are expressed as [^{3}H]thymidine incorporation (mean ± S.D., dpm $n = 3$) from a single experiment representative of three.

sible that the effect of AS and ES on proliferation is cell type specific.

3.4. AS and ES do not affect Rac and PAK activity in response to VEGF and PDGF

Cellular migration is a complex process involving lamellipodium extension, formation of new adhesions, cell body contraction and tail detachment [14]. The small molecular weight GTPase Rac is responsible for regulating lamellipodium formation, and focal complex assembly, in response to growth factors which stimulate cell migration [23]. Rac activity is spatially and temporally regulated by the lipid products of phosphoinositide 3-kinase (PI3-kinase) activity [24,25]. We analysed the GTP status of cellular Rac in response to growth factors by using a PAK-Crib-GST fusion protein pull-down assay to specifically isolate GTP-bound Rac. We found that in HDMECs, Rac was transiently activated by both VEGF and PDGF. In contrast, Rac did not appear to be activated by FGF in these cells (data not shown). Preincubation with both AS and ES did not affect the activation status of Rac in response to VEGF and PDGF (Fig. 5A). Addition of wortmannin, a known PI3-kinase inhibitor, prevented activation of Rac in response to both VEGF and PDGF. Taken together, these data suggest that AS and ES do not affect growth factor-stimulated PI3-kinase/Rac activity, and therefore have a unique action in inhibiting cellular migration.

Active Rac is known to bind to the serine/threonine kinase PAK resulting in the phosphorylation of PAK and the activation of a number of signalling cascades [26]. Using a phosphospecific antibody, we determined the effect of AS and ES on the activation status of PAK. Fig. 5B shows that neither AS nor ES affected the phosphorylation of PAK in response to VEGF, further confirming their inability to affect the Rac cascade.

In this study, we have demonstrated that AS and ES do not affect the key intracellular signalling cascades implicated in cellular migration and proliferation. Recent data have suggested that ES may disturb endothelial cell–matrix interactions by transiently blocking the association of β-catenin and paxillin [27] and by binding to integrin $\alpha_5\beta_1$ and caveolin-1 [28]. Therefore, it would seem probable that AS and ES disrupt cellular migration via subtle effects on the cell matrix. The inability of AS and ES to affect intracellular signalling pathways probably reflects the low toxicity of these agents in the clinic, where anti-angiogenic effects have been observed in the absence of systemic toxicity in the vasculature. Whilst both AS and ES have the ability to affect cellular migration, their ability to differentially affect the vasculature in the embryoid body model suggests they have different mechanisms of action.

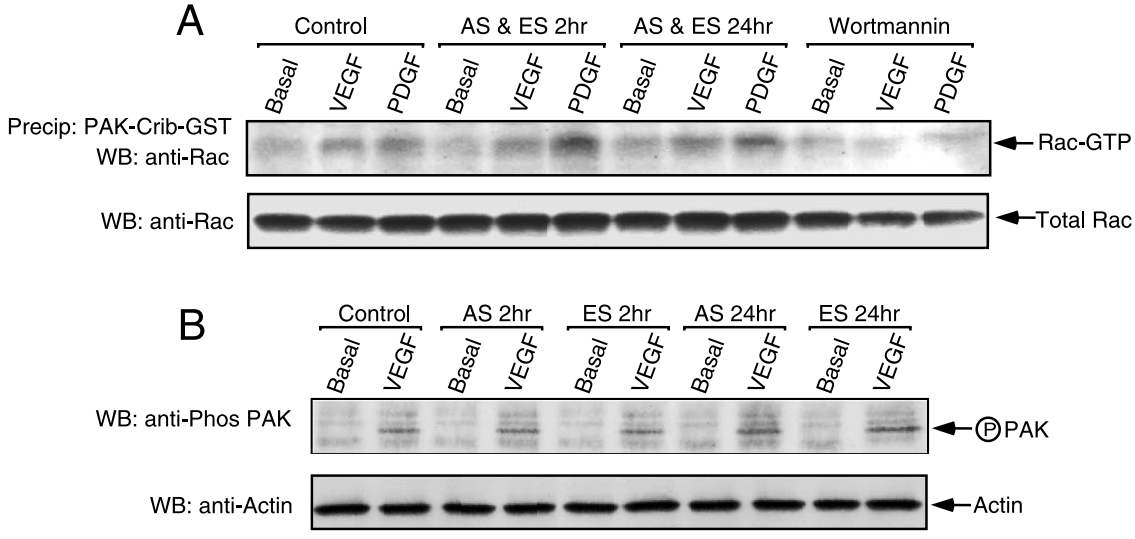

Fig. 5. Effect of AS and ES on Rac and PAK activation. A: HDMECs were preincubated with both AS (3 μg/ml) and ES (3 μg/ml) for the time periods shown. Cells were also preincubated with wortmannin (500 nM) for 30 min. Cells were stimulated with either VEGF (30 ng/ml) or PDGF (30 ng/ml), lysed and Rac activity determined by use of a GST-PAK-Crib domain pull-down as described in Section 2. B: HDMECs were preincubated with either AS (3 μg/ml) or ES (3 μg/ml) for the periods shown. Cells were stimulated with VEGF (30 ng/ml), lysed and proteins separated by SDS–PAGE followed by Western blotting. Actin is used as a loading control. Results shown are representative of two separate experiments.

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