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Biological action of angiopoietin-2 in a fibrin matrix model of angiogenesis is associated with activation of Tie2☆

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Abstract

The endothelial cell (EC) specific tyrosine kinase receptor, Tie2, interacts with at least two ligands, angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2). Ang1 stimulates Tie2 receptor autophosphorylation, while Ang2 has been reported to inhibit Ang1-induced Tie2 receptor autophosphorylation. We studied the effects of Ang1 and Ang2 in an in vitro model of angiogenesis. Human ECs (HUVEC), cultured on 3-D fibrin matrices, were treated with conditioned media (CM) from stably transfected cells expressing human Ang1 or Ang2, or with purified recombinant proteins. EC tube formation was measured as a differentiation index (DI), calculated as the ratio of total tube length over residual of EC monolayer. CM from Ang1 overexpressing A10 SMC or HEK293T cells induced profound HUVEC differentiation, resulting in the formation of extensive capillary-like tubes within 48 h (DI: 24.58±5.91 and 19.13±7.86, respectively) vs. control (DI: 2.73±1.68 and 2.15±1.45, respectively, both $P<0.001$). Interestingly, CM from two independent cell lines overexpressing Ang2 also produced a significant increase in EC differentiation (DI: 9.22±3.00 and 9.72±4.84, both $P<0.005$ vs. control) although the degree of angiogenesis was significantly less then that seen with Ang1. Addition of Ang1* (a genetically engineered variant of naturally occurring Ang1) or Ang2 also resulted in dose dependent increases in DI, which were blocked by an excess of soluble Tie2 receptor (20 μg/ml). Both Ang1* and Ang2 induced modest increases in [^{3}H]thymidine incorporation into HUVECs (20 and 26%, respectively), which were inhibited by excess soluble Tie2. Although Ang2 was unable to induce significant Tie2 receptor phosphorylation during a 5-min exposure, a 24-h pretreatment with Ang2, followed by brief re-exposure, produced Tie2 phosphorylation in HUVEC comparable to that produced by Ang1*. These results demonstrate for the first time that Ang2 may have a direct role in stimulating Tie2 receptor signaling and inducing in vitro angiogenesis. Our findings suggest that the physiological role of Ang2 is more complex than previously recognized: acting alternately to promote or blunt Tie2 receptor signaling in endothelial cells, depending on local conditions.

Keywords: Angiogenesis; Cell culture/isolation; Endothelial receptors; Extracellular matrix

1. Introduction

Angiogenesis is regulated by a delicate balance of stimulating and inhibiting factors which bind specific receptors on endothelial cells (ECs) [1–4]. Central to the angiogenic cascade are the endothelial-specific receptor tyrosine kinases (RTK) and their respective ligands. Two major subfamilies of endothelial-specific RTKs have been described. The vascular endothelial growth factor receptors (VEGFR) were the first to be identified and their role in vasculogenesis and angiogenesis has been well established [1,2]. The Tie/Tek receptors represent the other EC-spe-

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cific RTK subfamily consisting of two members, Tie1 and Tie2 (or Tek) [5–18]. The ligand(s) for Tie-1 are still unknown, however, at least two ligands have recently been identified for Tie2, angiopoietin-1 (Ang1) [19] and angiopoietin-2 (Ang2) [20].

Tie2 receptors have been implicated in the embryonic blood vessel formation. Targeted disruption of the Tie2 gene resulted in an embryonic lethal phenotype between E9.5 and E10.5. [9,13]. The most prominent defects in these mice included underdevelopment of the endothelial lining of the heart, the lack of remodeling of the primary capillary plexus to form vessels with higher degrees of organization and complexity, and the failure of capillary sprouts to invade the developing neuroectoderm. Ang1-deficient embryos also exhibited vascular defects, which were similar but less marked than those of the Tie2 knockout animals, with lethality at E12.5, consistent with a critical role of Ang1–Tie2 interaction in vascular development [21]. In contrast, no direct angiogenic role for Ang1 was demonstrated initially in models of postnatal vascular tube formation in vivo or in vitro [19,21].

Although Ang1 and Ang2 bind Tie2 with equal affinity, Ang2 was reported to competitively inhibit receptor phosphorylation induced by Ang1 [20]. In contrast, when Tie2 was expressed in nonendothelial cell lines, both Ang1 and Ang2 demonstrated a similar ability to induce receptor autophosphorylation, pointing to an EC-specific inhibitory mechanism preventing Ang2 from activating its receptor in ECs [20]. Interestingly, animals overexpressing Ang2 under the control of the Tie2 promoter demonstrated a phenotype similar to Ang1 or Tie2 knockout mice [20], consistent with the concept that Ang2 may function as an endogenous antagonist of Ang1. However, it is possible that an important physiological role for Ang2 might be masked by an abnormal spatial or temporal regulation of expression in these models. In support of this, both Ang1 and Ang2 potentiated VEGF-induced angiogenesis in vivo in the mouse corneal micropocket assay, although neither ligand alone was capable of initiating neovascularization in this model [22]. Moreover, Ang2 has been shown to express at the forefront of invading neovessels and is upregulated focally in the endothelium of developing blood vessels in certain tumours [23–25] and in myocardial ischemia [26].

Although, it has been initially reported that Ang1 failed to induce capillary-like tube formation in collagen matrices [19,27], Hayes et al. [28] reported that treatment of bovine aortic endothelial cells grown on collagen gels with Ang1 (100 μg/ml) caused the cells to migrate into the collagen and form capillary-like tubules. Furthermore, it was recently shown that Ang1 produces sprouting angiogenesis in a fibrin matrix model of in vitro angiogenesis [29], while the activity of Ang2 was not studied. Given the similarity in structure and sequence homology between the angiopoietins and fibrinogen, the precursor of fibrin, it is possible that specific interactions between this matrix and Ang1 may be important in modulating the ability of this ligand to activate its receptor and induce its biological effects. Alternatively, ECs may be sensitised to respond to angiopoietins in the presence of fibrin matrix, by an alteration in their state of activation or by changes in the expression of other regulatory genes.

In the present study, we tested the ability of Ang1 and Ang2 to cause EC differentiation in a 3-dimensional fibrin matrix model of angiogenesis. We now report that Ang2, like Ang1 induced extensive capillary-like tube formation in this model, comparable to that observed with VEGF. Moreover, the angiogenic action of both Ang1 and Ang2 was accompanied by increased Tie2 autophosphorylation, which was not modified by growth on fibrin matrices, although this was only observed with Ang2 after prolonged exposure.

2. Methods

2.1. Cell lines and culture

Human umbilical vein endothelial cells (HUVEC), rat aortic A10 smooth muscle cell (A10 SMC) and human embryonic kidney 293T (HEK293T) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The EA.hy926 endothelial cell line was a gift from Cora Edgell, University of North Carolina. HUVEC were maintained in culture in Ham's 12 medium, supplemented with 15% fetal bovine serum (FBS), penicillin (500 U/ml), streptomycin (50 μg/ml) and heparin (100 μg/ml) (all from Gibco/BRL, Burlington, ON, Canada), and endothelial cell growth factor (ECGF 20 μg/ml, Boehringer Mannheim, Laval, PQ, Canada) and equilibrated with 95% air and 5% CO_2 at 37°C. Cells were used between passages 13 and 18. A10 SMC and HEK293T cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS and antibiotics as above. EA.hy926 cells were maintained in DMEM with 10% FBS, as described above, in the presence of 1× HAT (Gibco/BRL).

2.2. Generation and use of Ang1 and Ang2 expressing cells

Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) cDNAs were subcloned into the mammalian expression vector pSecTagB/Myc-His(+) (Invitrogen, Mississauga, ON) to generate Myc-Histidine epitope-tagged constructs (Ang1MH and Ang2MH). A portion of Ang1 lacking the leader signal sequence was cloned into SignalpIg-plus (Novagen, Madison, WI), to generate Ang1Fc as previously reported [30]. To produce stable cell lines expressing Ang1MH and Ang2MH, HEK293T and A10 SMC were transfected with 10 μg of pSecTagB-Ang1MH or with pSecTagB-Ang2MH plasmids. Transfection of HEK293T

cells was performed using Lipofectin reagent and OPTI-MEM medium (both from Gibco/BRL) according to the manufacture's instructions. A10 SMC were transfected using SuperFect Transfection reagent (Qiagen, Mississauga, ON) according to the manufacturer's recommendation. Transfectants were selected in 1 mg/ml zeocin (Invitrogen) and individual colonies isolated by ring cloning, pooled and expanded. To produce Ang1Fc expressing stable cell lines, HEK293T cells were transfected with 10 μg of SignalpIg-plus-Ang1 using Lipofectin reagent. Transfectants were selected in 1.5 mg/ml G418 (Gibco/BRL) and individual colonies were pooled and expanded as above. Conditioned medium (CM) was collected during 24 h incubation of the stably transfected cells in DMEM supplemented in 0.1 or 10% FBS. Ligand production was confirmed by RT-PCR and immunoprecipitation with c-Myc antibody (Invitrogen), following Western analysis. HUVEC were treated for 48 h with CM from either mock-transfected, Ang1MH, Ang1Fc, Ang1Fc-depleted or Ang2MH. To deplete Ang1Fc from HEK293T CM, 10 ml of media was incubated with 500 μl 20% protein A-Sepharose beads that had been coated with Tie2-Fc rocked for 12 h. The beads were spun down and the remaining supernatant was applied to HUVEC.

Human Ang1*, Ang2 and solubleTie2- (s Tie2-Fc) were kindly provided by Regeneron Pharmaceuticals, Inc., Tarrytown, NY [19,20]. Ang1* and Ang2 recombinant proteins were produced as described previously [19]. Ang1* is a genetically engineered variant that retains properties similar to wild type Ang1. In Ang1*, the non-conserved cysteine at residue 245 has been mutated to the corresponding serine residue of Ang2, and the first 77 amino acids of human Ang1 have been replaced with the first 73 residues of Ang2 [20,31]. The recombinant proteins were prepared in buffer containing 0.05 mol/l Tris–HCl pH 7.5, 150 mmol/l NaCl and 0.05% Chaps. Soluble Tie2-Fc is a recombinant fusion protein consisting of the ectodomain of the Tie2 receptor fused to the Fc portion of human IgG, which was constructed, produced and purified as described [19]. Ang1* and Ang2 were used at concentrations from 0 to 2000 ng/ml for 24–48 h.

2.3. Preparation of fibrin gels

Endotoxin and plasminogen free human fibrinogen (10 mg/ml, Calbiochem NOVA Biochem Corp., La Jolla, CA) was dissolved in serum free medium and filtered through a 0.22-μm filter (Millex G.S Millipore, Mississauga, ON). Fibrin matrices were prepared by polymerizing the fibrinogen solution using a low concentration of α-thrombin (2.5 U/ml, Sigma) as described before [32]. After polymerization, gels were soaked in cultured medium containing 15% FBS for 2 h at 37°C to inactivate the thrombin. Confluent endothelial cell cultures were washed with Hank's buffered saline solution (HBSS) and harvested using 0.05% trypsin/0.53 mmol/l EDTA, and counted using a hemocytometer. HUVEC were resuspended in F12 medium with 15% FBS and plated at a density 5×10^5 cells/well in six-well plates, either directly on plastic, or on the surface of the three-dimensional matrix and cultured for periods up to 48 h in the presence or absence of study agents as described above. VEGF (10 ng/ml), purchased from R&D was used as positive control.

2.4. Co-culture experiments

Wild type or stably transfected A10 SMC overexpressing Ang1 were trypsinized and resuspended in 1% fibrinogen solution. The fibrinogen–cell mixture was pipetted into six-well tissue culture plates, then α-thrombin was added to polymerize the fibrin, as described above. After polymerization, cells embedded in the gel were soaked in cultured medium (DMEM) containing 10% FBS for 2 h. HUVEC were plated on the surface of the fibrin gel and cultured for periods up to 48 h, then examined for endothelial cell-tube formation, and photographed.

2.5. Quantitation of endothelial differentiation

In vitro angiogenesis on fibrin matrices was quantified as previously described [32]. In brief, culture plates containing HUVEC were assessed after 24–48 h incubation under study conditions using an Olympus BX50 inverted microscope (100×). Images were digitized using a Sony CCD-IRIS/RGB camera (Cohu Inc, Tokyo, Japan) and analyzed using a computer-assisted morphometric analysis system (C Imaging, Compix Inc., Cranberry Township, PA) by observers unaware of the culture conditions. Tube-like structures (>30 μm in length) were identified and total tube length was derived for each of six randomly pre-selected fields. At the same time, the total area of the culture covered by ECs was determined in the same fields. The differentiation index (DI) was calculated as the ratio of total tube length over cell area for each field and a mean DI value was obtained for each culture well.

2.6. Quantitation of endothelial cell proliferation

DNA synthesis was determined by quantifying the incorporation of [methyl-^{3}H]thymidine into trichloroacetic acid-insoluble macromolecules as described previously [32]. Endothelial cells were seeded at a density $1–2\times10^4$ cells/well in 24-well plates directly on plastic or in dishes coated with fibrin, in F12 medium supplemented with 15% FCS and were left to adhere overnight. Cells were then incubated for 24 h in low serum media (2% FBS) prior to the addition of pure recombinant protein Ang1* or Ang2. After a pre-incubation period of 12 h, a tracer amount of [^{3}H]thymidine (1 μCi per well; specific activity at 1.85 TBq/mmol; Amersham Pharmacia Biotech, Quebec, Canada) was added and the cells were incubated for an additional 12 h. Subsequently the cells were washed three

times with phosphate-buffered saline (PBS) and DNA was precipitated with 10% ice-cold trichloroacetic acid (TCA) for 20 min. The resulting precipitate was washed with ice-cold 10% TCA, 95% ethanol, and ethanol/ether (3:1 v/v), then air dried. DNA was extracted and solubilized with 0.3 mol/l NaOH at room temperature for 20 min, followed by neutralization with 0.3 mol/l HCl. Radioactivity was measured in a liquid scintillation counter (Liquid Scintillation System, Beckman Instrument Inc. Mississauga, ON). Data are expressed as recovered cpm/well.

2.7. Detection of angiopoietins in conditioned media

Conditioned media was clarified by centrifugation prior to immunoprecipitation. Ang1MH and Ang2MH were precipitated from 10 ml of CM with 2 μg of anti-Myc antibody (Invitrogen) and 2 μg of purified rabbit anti-mouse IgG and recovered using protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden). The precipitated proteins were eluted in twice-concentrated sodium dodecyl sulfate (SDS) sample buffer and boiled for 10 min, separated using SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane (Novex, Helixx Technologies, Scarborough, ON) using a semi-dry transfer apparatus (BioRad), according to manufacturer' s instructions. Filters were blocked in 5% non-fat milk in TBST buffer (10 mmol/l Tris (pH 7.5), 150 mmol/l NaCl and 0.1% Tween-20) prior to immunoblotting with anti-Myc antibodies at 1:5000 dilutions according to the supplier's instruction (Invitrogen). An HRP conjugated anti-mouse antibody coupled with ECL reagent (Amersham) was used to visualize angiopoietin-specific band (70 kDa). Ang1Fc was detected with HRP-conjugated donkey anti-human IgG H+L (Jackson ImmunoResearch, West Grove, PA) as 97 kDa protein.

2.8. Tie2 autophosphorylation

For stimulation of EA.hy926 (an endothelial cell line used previously to study Tie2 activation) [30] by Ang1 and Ang2 CM, cells were serum-starved for 6 h, pretreated with 1 mmol/l sodium orthovanadate (phosphatase inhibitor), pH 8, for 10 min at 37°C, and stimulated with 5 ml of conditioned medium in the presence of sodium orthovanadate for 10 min. Alternatively HUVEC were maintained overnight in MCDB 131 medium (Gibco/BRL) with 1% FBS, and treated with Ang1* or Ang2 recombinant proteins (300 ng/ml each) for 5 min, 1 or 24 h. In some experiments, cells were pre-treated for 24 h in the presence of factors, medium was removed, cells were then serum-starved in MCDB 131 medium for 2 h, followed by 5 min rechallenge with Ang1* or Ang2. After the stimulation, the cells were solubilized with RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mmol/l Tris, pH 7.6, 50 mmol/l sodium fluoride, 150 mmol/l sodium chloride, 1 mmol/l EDTA, 5 mmol/l benzamidine, 1 mmol/l sodium orthovanadate, 10 μg/ml aprotinin, 1 mmol/l PMSF, 10 μg/ml leupeptin, and 1 μg/ml pepstatin). The lysates were immunoprecipitated with an anti-Tie2 antibodies. Immunocomplexes were recovered on Protein G-Sepharose and separated by SDS–PAGE, transferred to blotting membrane as described above and then probed with anti-Tie2, and then with anti-phosphotyrosine antibodies 4G10 (Upstate Biotechnology, Inc, Lake Placid, NY). Monoclonal and polyclonal anti-Tek/Tie2 antibodies specific to the extracellular domain were a kind gift of Fu-Kuen Lin (Amgen, Thousand Oaks, CA). In experiments with pure proteins, rabbit polyclonal antibody against Tie2 (RG133, Regeneron Pharmaceutical) or commercially available polyclonal antibody against Tie2, (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) has been used. Specific bands were visualized using the ECL system (Amersham Pharmacia Biotech). Densitometry was performed by scanning the immunoblots (Imaging Densitometer, BioRad) and the intensity of each band was analyzed using the Molecular Analyst software (BioRad).

2.9. Statistical analysis

Statistical analysis was performed using the SYS-STAT software. Significance of differences between groups was performed using a two-tailed Student's *t*-test or a one-way ANOVA for dose–response experiments, with Bonferonni correction for multiple comparisons where appropriate. Values are presented as mean±S.D. A P value of less than 0.05 was interpreted as statistically significant.

3. Results

3.1. Detection of angiopoietins in conditioned media by Western analysis

Western blot analysis of CM from stably transfected HEK293T and A10 SMC cells confirmed similar expression levels of Ang1, Ang2 and Ang1Fc. Fig. 1 shows the presence of Ang1 and Ang2 in CM from angiopoietin MH overexpressing cell lines, detected as a 70-kDa band with αMyc antibodies, whereas Ang1Fc migrated as 97 kDa protein in CM from stably transfected HEK293Tcells.

3.2. Effect of angiopoietin-1 overexpressing cells and conditioned media on EC differentiation

HUVEC grown on fibrin gels in the presence of CM from mock-transfected cells or in co-culture on gels containing wild type A10 SMCs showed no significant angiogenic response (Fig. 2a and c). However, when co-cultured with Ang1 overexpressing cells, there was a marked phenotypic change in the endothelial cell monolayer with the appearance of a network of capillary-like

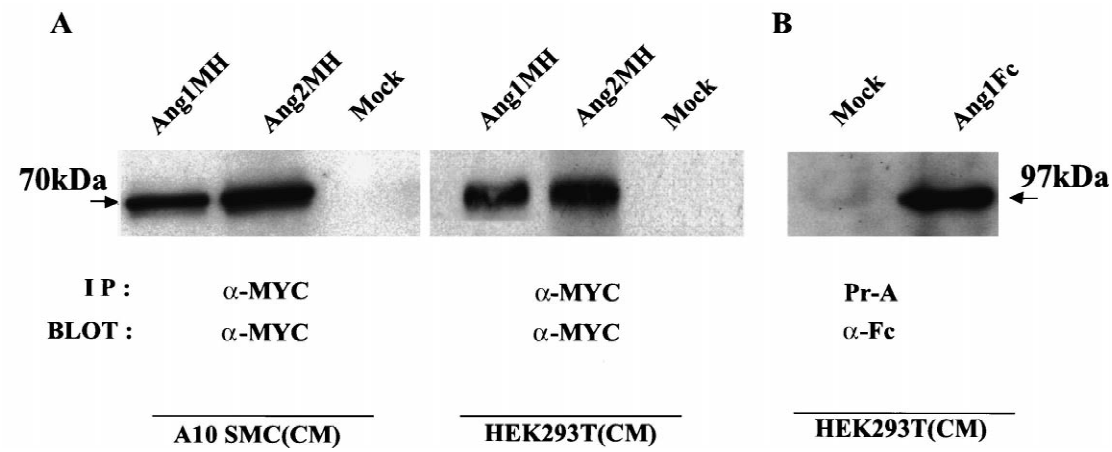

Fig. 1. Detection of angiopoietins in conditioned media (CM) by Western analysis. Conditioned medium were obtained from mock-transfected A10 SMC and HEK293T cells (Mock) or cells expressing either Ang1MH, Ang2MH, or Ang1Fc. CM were immunoprecipitated (IP) with anti-Myc (panel A) antibodies or precipitated with protein A-Sepharose (Pr.A) (panel B), followed by immunobloting with either anti-Myc or donkey anti-human IgG (αFc), respectively. Ang1MH, Ang2MH (panel A) and Ang1Fc (panel B) were detected in CM collected from transfected cells but not from mock medium. Rainbow molecular marker (BioRad) was used as estimation of molecular size, as indicated.

tubes (Fig. 2d). As well, media conditioned by 24 h exposure to smooth muscle cells overexpressing Ang1 induced profound endothelial cell differentiation (panel b). Similar responses were observed with Ang1Fc CM and Ang2 CM (photomicrographs not shown).

Fig. 3 shows differentiation index as measured in six separate experiments using media conditioned by A10 SMC and HEK293T cells (panel A). Media conditioned by A10 SMCs or HEK293T cells overexpressing Ang1MH resulted in significant increases in EC differentiation index, compared with conditioned media from mock-transfected cells. Interestingly, CM from cells overexpressing

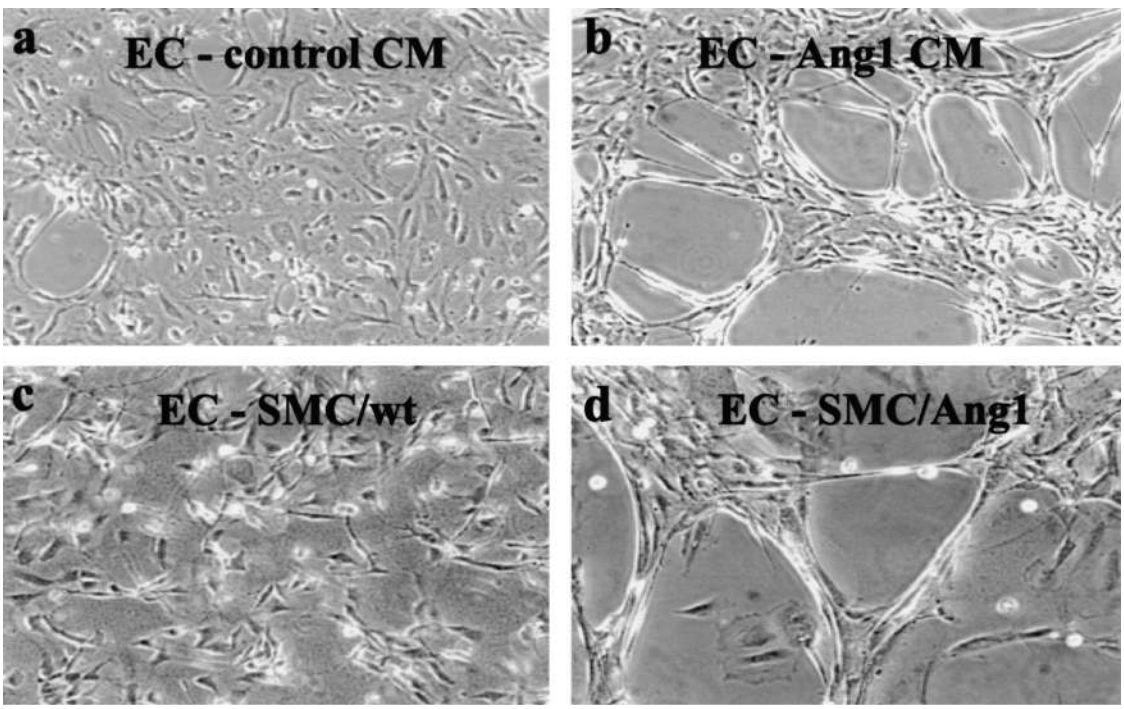

Fig. 2. Ang1 induces capillary-like tube formation in a fibrin-matrix model of angiogenesis. The overlay co-culture assay was performed as described in methods. Cultures were incubated for 48 h and photographed. HUVEC grown on fibrin gels in the presence of CM from mock-transfected cells (control), or co-cultured with wild type (wt) A10 SMC, showed no significant angiogenic response (a,c), respectively. In contrast, media conditioned by SMC overexpressing Ang1 induced EC capillary-like tube formation (b). Similarly, HUVEC co-cultured with Ang1 overexpressing cells showing marked changes in morphology, with the appearance of a network of capillary-like tubes (d).

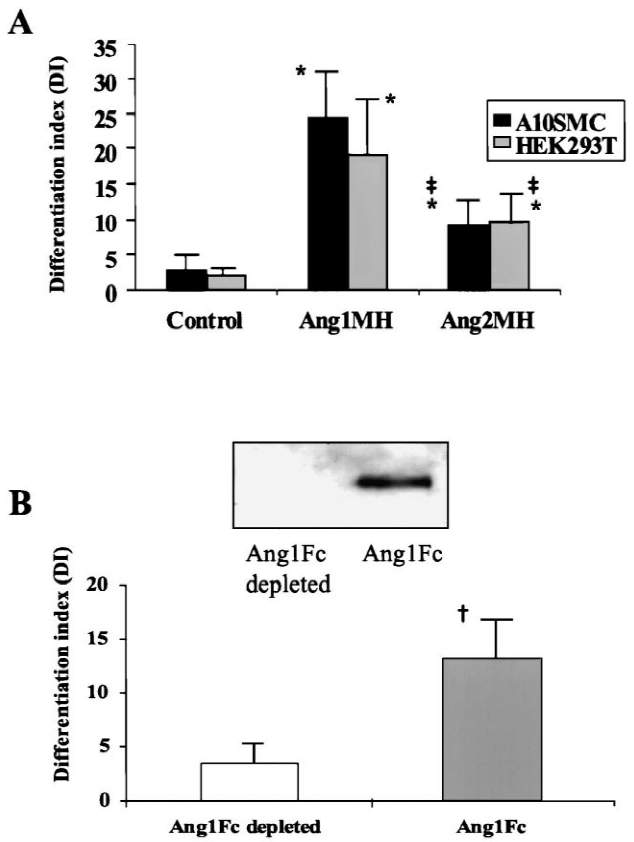

Fig. 3. Quantification of the EC differentiation. HUVEC were incubated in the presence of CM from A10 SMCs and media conditioned by HEK293T cells. Media conditioned by Ang1MH transfected cell lines induced a marked increase in EC differentiation index (DI) compared with media from mock-transfected cells (panel A). SMCs or HEK293T CM from cells overexpressing Ang2MH also produced significant increases in EC differentiation index, but less than seen with Ang1MH overexpressing cells (panel A). CM from Ang1Fc cells showed a response similar to Ang1MH (panel B), indicating that both ligands function in similar manner in vitro differentiation assay. Ang1Fc-CM depleted by protein A-Sepharose (insert) showed minimal angiogenic activity (panel B). Data are expressed as mean±S.D. of five to six experiments. Statistical comparisons were performed using one-way ANOVA followed by the Student's *t*-test. * $P<0.001$ vs. control; † $P<0.05$ vs. Ang1Fc depleted; ‡ $P<0.001$ vs. Ang1MH.

Ang2MH produced similar increases in EC differentiation, although the magnitude of angiogenic response was less than that seen with Ang1 CM. Media conditioned by Ang1Fc transfected cells produced a response similar to that observed with Ang1MH (Fig. 3B). Moreover, depletion of the Ang1Fc from the CM by protein A-Sepharose, abolished the angiogenic effect.

3.3. Effect of purified recombinant Ang1 and Ang2 on endothelial cell tube formation in fibrin gels*

Representative fields from HUVEC cultured on fibrin matrices in the presence and absence of Ang1* and Ang2 are shown in Fig. 4. Both Ang1* and Ang2 (220 ng/ml) resulted in the formation of extensive capillary-like structures (b and c) and the addition of the soluble Tie2 receptor (sTie2, 20 μg/ml) largely prevented EC differentiation in response to both angiopoietins (e and f).

The concentration–response relationships for Ang1* and Ang2 are shown in Fig. 5A and B. The recombinant proteins resulted in similar dose dependent increases in EC differentiation index after 24 h which was maximal for both Ang1* and Ang2 at 670 ng/ml, and the magnitude of differentiation was similar for both recombinant proteins. As well, addition of excess soluble Tie2 receptor (20 μg/ml) markedly inhibited endothelial cell differentiation in response to both Ang1* and Ang2.

3.4. [^{3}H]Thymidine incorporation in HUVEC

The effect of Ang1* and Ang2 on rates of proliferation of EC cultured on plastic (non-angiogenic) and fibrin (angiogenic condition) are shown in Fig. 6. In these studies, the proliferation rates of ECs on fibrin was greater than on plastic (23 641±1729 vs. 13 632±1819 cpm, respectively, $P<0.05$) In the presence of control medium with 2% FBS, HUVEC demonstrated a relatively high basal level of [^{3}H]thymidine incorporation (control). Both Ang1* and Ang2 produced modest but significant increases in [^{3}H]thymidine incorporation in HUVEC cultured on fibrin gels or plastic. Maximal [^{3}H]thymidine incorporation was reached at 74 ng/ml for Ang1* (panel A) and at 220–670 ng/ml for Ang2 (panel B). Addition of sTie2 receptor (20 μg/ml) significantly inhibited angiopoietin induced EC proliferation in response to both proteins.

3.5. Autophosphorylation of Tie2 receptor by angiopoietins

Tie2 receptor phosphorylation was studied using EA.hy926 cells, an endothelial cell line used previously to study Tie2 activation [20,30]. Tyrosine phosphorylation of Tie2 was not detected in EA.hy926 cells stimulated by CM from the mock-transfected cells either in the presence or absence of vanadate (Fig. 7); however, Ang1-CM stimulated robust tyrosine autophosphorylation of Tie2. These data are in agreement with previous findings establishing a role for Ang1 as Tie2 agonist. In contrast, CM from Ang2 transfected cells did not induce receptor activation at 5 min in EA.hy926 cells cultured on plastic (data not shown), as has previously been reported [20].

The effect of the purified recombinant Ang1* or Ang2 on Tie2 phosphorylation was studied in HUVEC grown on plastic or fibrin (Fig. 8). Consistent with previous reports, Ang1* produced a strong response but Ang2 was unable to induce Tie2 receptor phosphorylation at early time points (5 min) after ligand addition (panel A). A weak tyrosine phosphorylation of Tie2 was detected upon stimulation for 1 h with Ang2. Treatment of EC for 24 h with Ang2 resulted in a robust Ang2-induced phosphorylation of Tie2

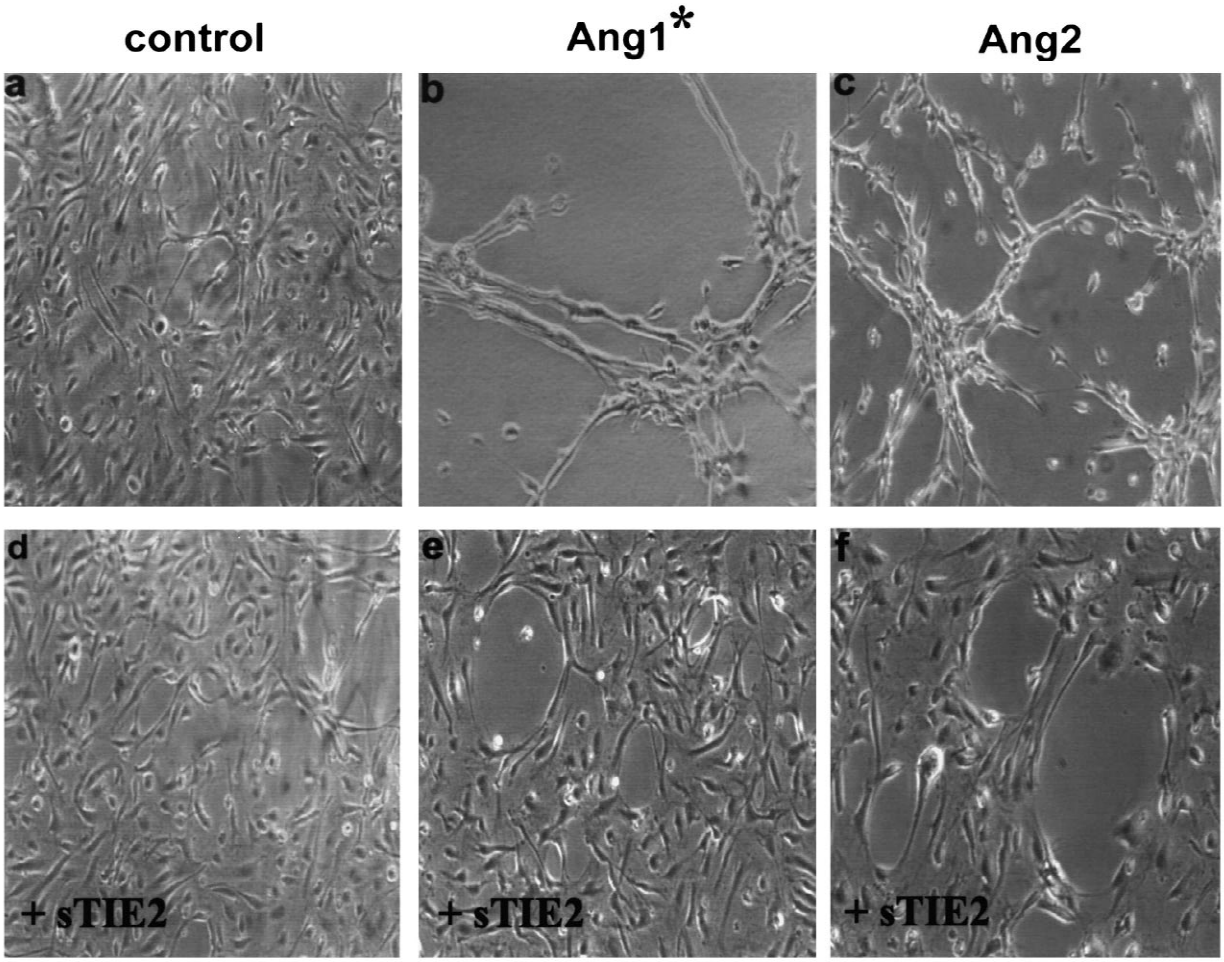

Fig. 4. Representative photomicrographs of HUVEC cultured on fibrin-matrix for 48 h in control media (only vehicle, PBS–0.01% CHAPS, a and d), in the presence of Ang1* (b and e) or Ang2 (c and f) (concentration 220 ng/ml each). Results were similar in six independent experiments. A significant increase in DI was observed with both ligands, to a similar degree as that in the CM experiments. * $P<0.05$ vs. control. The activity of both angiopoietins was blocked by the addition of excess (20 μg/ml) soluble Tie2-Fc (panel e and f). Original magnification: ×100.

(panel C), comparable to that seen in response to Ang1*. Similar results were obtained if Tie2 receptor activity was determined after 24 h of pretreatment with Ang2 followed by washout (2 h) and readdition of Ang2 for 5 min (panel D). Quantitative analysis of Tie2 phosphorylation induced by Ang1* or Ang2 is provide in Fig. 9. Both Ang1* and Ang2 revealed significant increase in Tie2 phosphorylation either on fibrin, or plastic ($P<0.05$ vs. control; by *t*-test). These results clearly demonstrate the ability of Ang2 to phosphorylate Tie2 in Tie2-expressing endothelial cells after prolonged exposure.

4. Discussion

The present study shows that angiopoietin-2 as well angiopoietin-1 can promote substantial capillary-like tube formation in an in vitro model of angiogenesis. Consistent with these findings, we observed that both ligands induce Tie2 receptor autophosphorylation, although robust receptor activation by Ang2 required prolonged exposure to the ligand.

The angiopoietins were originally identified by secretion-trap and subsequent homology expression cloning, and shown to be specific ligands for the Tie2 receptor tyrosine kinase [19,20]. Targeted disruption of Tie2 in mice resulted in an embryonic lethal phenotype marked by abnormalities in vascular remodeling [9,14]. Unlike the targeted disruption of VEGF or Flk-1, in which there was failure of endothelial cell differentiation, Tie2-deficient embryos displayed defects in the later events, including vascular remodeling and stabilization. Disruption of the Ang1 gene produced a nearly identical phenotype as that of Tie2 receptor knockouts, with embryos exhibiting large vascular channels lacking normal complexity of branching and impaired recruitment of pericytes and basement membrane formation [21]. The Ang2 null phenotype has not yet been described, however, transgenic-overexpression of Ang2 produced an embryonic lethal phenotype generally similar to that of Tie2 and Ang1 knockouts [20]. In in vitro

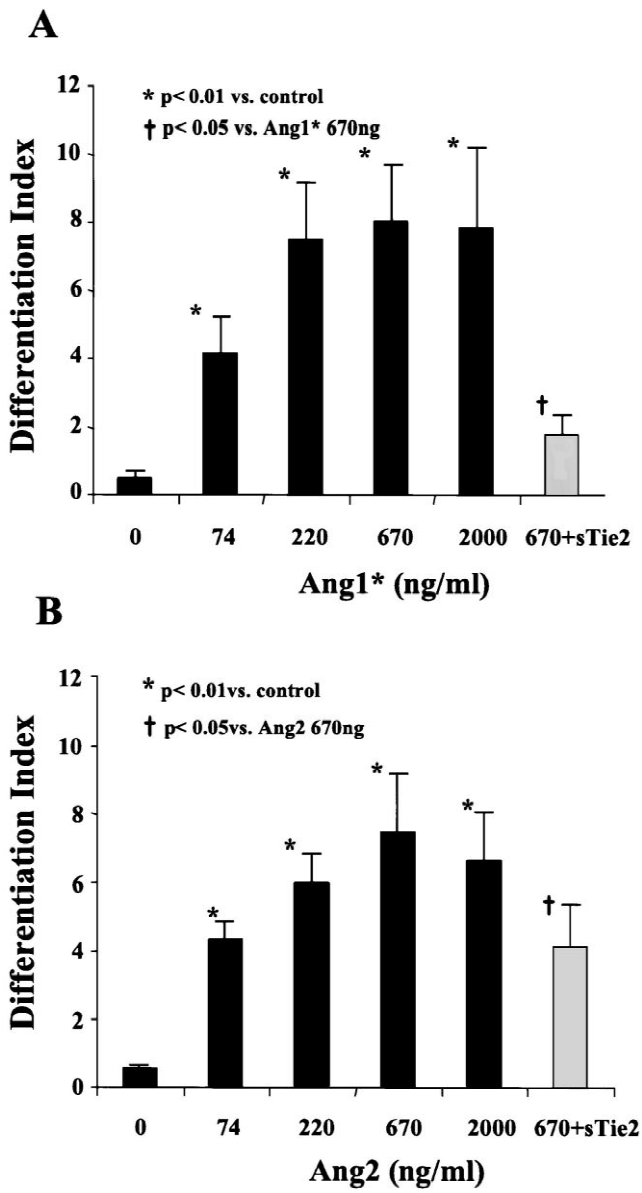

Fig. 5. Recombinant Ang1* (panel A) and Ang2 (panel B) induced a concentration-dependent increase in EC differentiation. Recombinant Tie2-Fc receptor (20 μg/ml) was used to inhibit Ang1* and Ang2 activity. Each bar represents the mean±S.D. of six experiments. Difference among groups were analyzed by one-way ANOVA followed by the Student's *t*-test.

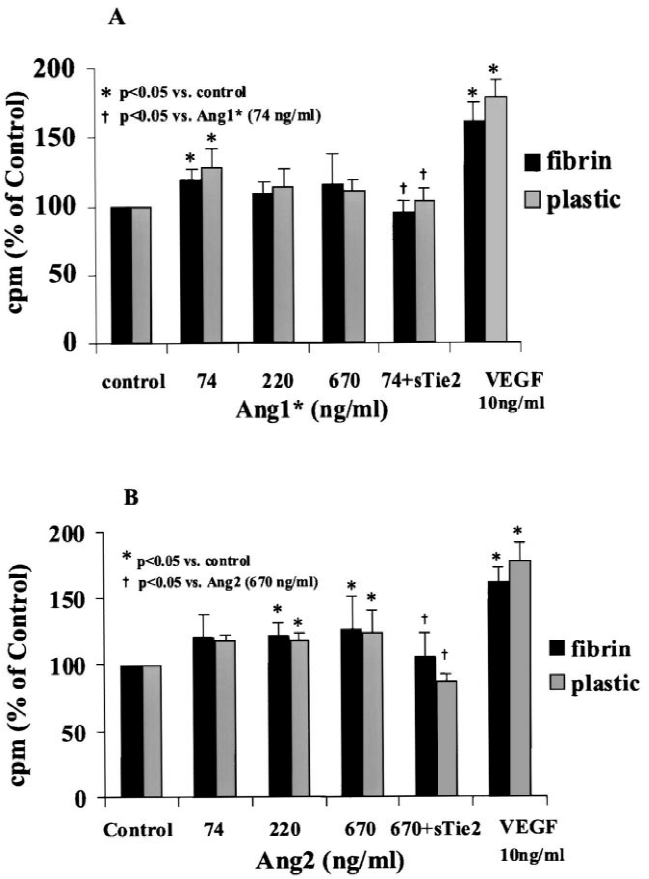

Fig. 6. [^{3}H]Thymidine incorporation in HUVEC after treatment with Ang1* and Ang2. HUVEC were seeded (20 000 cells/well) into 24-well plates. The following day, medium containing 2% FBS with the indicated amounts of Ang1*, Ang2 or VEGF was added and the cells were incubated for 24 h. Cells were pulsed for the last 6 h with [^{3}H]thymidine (1 μCi per well). Ang1* (panel A) and Ang2 (panel B) produced a weak mitogenic effect, which was blocked by the addition of excess soluble Tie2-Fc (20 μg/ml). VEGF (10 ng/ml) was used as positive control. Each data bar represents the mean±S.D. of four separate experiments, performed in quadruplicates. Significance was determined by the Student's *t*-test.

assays, Ang2 has been shown to inhibit Ang1-induced Tie2 receptor autophosphorylation, while showing no direct stimulation of receptor phosphorylation by itself. These results suggest that Ang2 may be an endogenous inhibitor of Ang1-induced Tie2 receptor activation [20].

The mechanism by which Ang2 inhibits Ang1-induced Tie2 receptor autophosphorylation remains poorly understood. It does not appear to be due to an inability of this ligand to associate with its receptor since Ang2 binds Tie2 with an affinity equal to that of Ang1, and can produce phosphorylation of Tie2 when this receptor is expressed in non-endothelial cell lines [20]. Thus, it has been suggested that there is an inhibitory mechanism which is EC-specific [20]. Indeed, it is likely that this mechanism also modulates the basal level of Tie2 receptor autophosphorylation, as well as the degree of Ang1-induced phosphorylation [20].

The remarkable similarity in angiogenic response to Ang1 and Ang2 in the present in vitro assay is therefore surprising. Pro-angiogenic actions of Ang2 were seen not only with the conditioned media from two different lines of Ang2 overexpressing cells, but also with a purified recombinant Ang2 protein. Moreover, the inhibition of EC capillary like tube formation by excess soluble Tie2 receptor provides further evidence that Ang2 was acting as a specific agonist of Tie2 in this assay.

The results of the present study could be explained by a differential ability of Ang1 and Ang2 to produce homo-

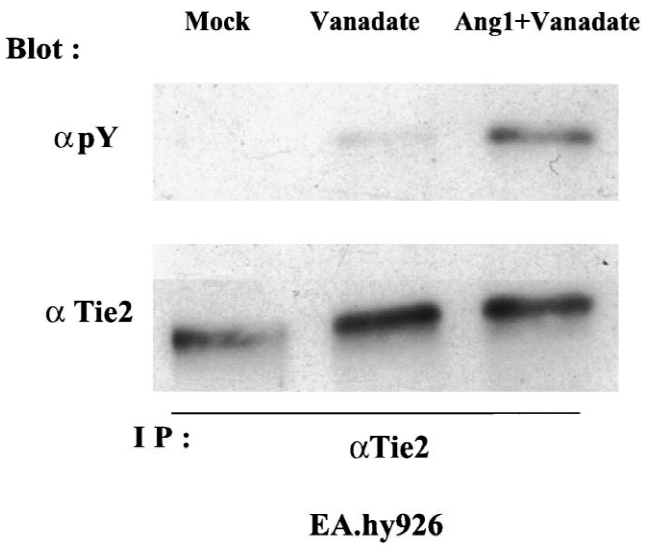

Fig. 7. Activation of Tie2 receptor by Ang1CM in human ECs. EA.hy926 cells (immortalized hybrid of human umbilical vein endothelial cells) were serum-starved, and stimulated for 5 min with either Mock or Ang1MH conditioned medium from HEK293T cells in the presence of orthovanadate. Lysates of cells were immunoprecipitated (IP) with anti-Tie2 polyclonal antibodies. Tie2 receptor tyrosine phosphorylation was visualized by immunoblot analysis with mouse monoclonal anti-phosphotyrosine antibodies (upper panel), and then stripped and re-probed with monoclonal anti TEK/Tie2 antibodies (lower panel). Phosphorylation of Tie2 could be detected following stimulation with Ang1MH, whereas no phosphorylation was seen following stimulation of cells with Mock conditioned medium.

dimerization of the ligand-receptor, possibly based on differences in the molecular characteristics of the angiopoietins. The C-terminal portion of these molecules bears a high degree of homology with fibrinogen and other fibrinogen-like molecules [19,20,31], whereas N-terminal portion consists of a coiled-coil domain, which is known to be important in oligomerization in a wide variety of molecular systems [33]. Thus, it is possible that the coiled-coil domains of the angiopoietins are critical for self-association of these ligands, whereas the fibrinogen-like domain appears to be involved in binding to Tie2 [34]. Interactions between the angiopoietins and the fibrin matrix of the present model system may be important in facilitating angiopoietin self-association in a manner analogous to the role of heparin in facilitating the response to bFGF [35]. Indeed, an another report demonstrating in vitro angiogenic actions of Ang1 also utilised fibrin matrix as the substrate for EC capillary-like tube formation [29] and conflicting results exist about Ang1-induced angiogenesis in collagen gels [19,27,28]. However, culturing ECs on fibrin matrix alone induced only weak basal Tie2 autophosphorylation, and there was no potentiation of phosphorylation in response to the angiopoietin ligands in the presence, compared to the absence, of fibrin. Indeed, Ang2 was unable to activate Tie2 in HUVEC grown either on fibrin matrix or plastic at an early time point (5 min).

Central to an understanding of the mechanism of Ang2-induced effect on EC growth and differentiation is the apparent discrepancy between the biological effects of Ang2 observed in the present study and the previously reported lack of ability of Ang2 to induce Tie2 autophosphorylation [20]. However, Tie2 phosphorylation at early time points after ligand addition may not accurately reflect the activity of this receptor at the later time points, at which EC growth and differentiation are determined. Therefore, we studied the effect of 24-h pre-treatment with either Ang1 or Ang2 on Tie2 phosphorylation under the same conditions used to produce EC differentiation. Prolonged treatment of EC with Ang2 under these conditions resulted in a robust phosphorylation of Tie2 at 24 h. Ang2-induced Tie2 activation could still be observed when the cells were rechallenged with Ang2 2 h after this ligand had been washed out. This novel observation is important since it suggests that the interaction of Ang2 with Tie2 is more complex then previously recognized. Experiments using Tie2 ectopically expressed in various non-EC cell lines have demonstrated that this inhibitory mechanism is EC-specific. Our data now suggest that inhibition is also subject to regulation during prolonged exposure to Ang2. The physiological significance of the time-dependent modulation of the Ang2–Tie2 interaction remains to be explored. A brief time-limited up-regulation of Ang2 expression is likely to result predominantly in inhibition of Tie2 activation, which might release the vascular endothelium from the constitutive homeostatic influence of Ang1 and thus facilitate EC activation, as has previously been suggested [20,23,36–38]. However, during sustained exposure, the effects of Ang2 may progressively shift from that of an antagonist to an agonist, and at later time points it may contribute directly to vascular tube formation and neovessel stabilization, in a manner analogous to Ang1.

Several recent studies have provided support for a permissive role for Ang2 in the angiogenic response [24]. Holash et al. [37]and others [23,36] have shown that Ang2 is upregulated in developing glioblastomas in a highly focal manner, specifically localised to newly formed blood vessels. As well, Ang2 is expressed at the leading edge of vessels invading the corpus luteum [20]. We have recently shown that Ang2 is upregulated following myocardial infarction in the rat, particularly in the periinfarct region [26]. Moreover, Asahara et al. [22], have recently demonstrated that both Ang1 and Ang2 significantly potentiated the effect of VEGF in a mouse corneal micropocket assay of in vivo angiogenesis, consistent with the synergi between VEGF and angiopoietins in vascular development [39]. In this corneal model, both Ang1 and Ang2 induced the recruitment of perivascular cells (pericytes and SMC), and Ang1 produced greater capillary density, whereas, Ang2 resulted in greater vessel length. However, direct intramuscular injection of plasmid DNA encoding Ang1 but not Ang2 augmented revascularization in the rabbit ischemic hindlimb [40]. The difference in the biological

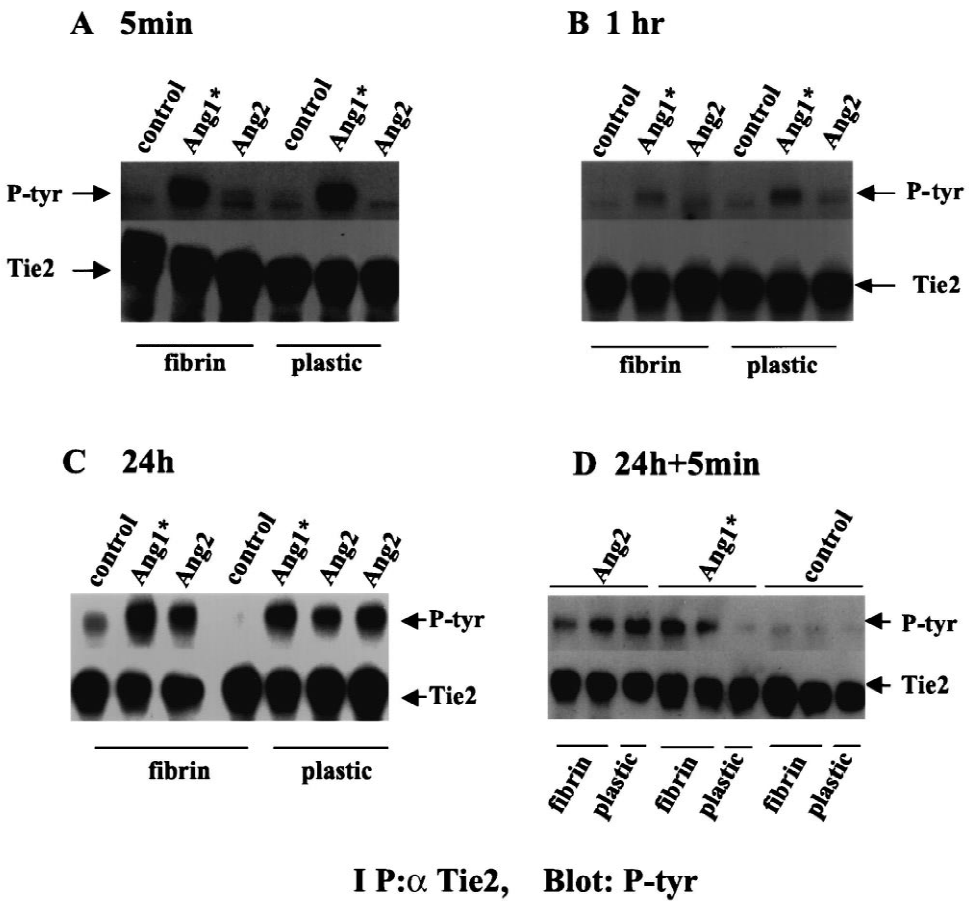

Fig. 8. Phosphorylation of Tie2 receptor by Ang1* and Ang2. HUVEC grown on plastic, or on fibrin matrix, were serum-starved, as described in Methods, and incubated for 5 min (panel A), 1 h (panel B), 24 h (panel C), with pure recombinant proteins (300 ng/ml) or incubated for 24 h with these factors followed by wash out, and incubation with serum free medium for 2 h, and 5 min rechallenge with Ang1* or Ang2 (panel D). Lysates of cells were immunoprecipitated (IP) with anti-Tie2 antibodies and analysed for levels of tyrosine-phosphorylation by immunoblot analysis using anti-phosphotyrosine antibodies as described in Methods. Specific bands were visualized using the ECL system (Amersham Pharmacia Biotech). Similar results were obtained in three experiments.

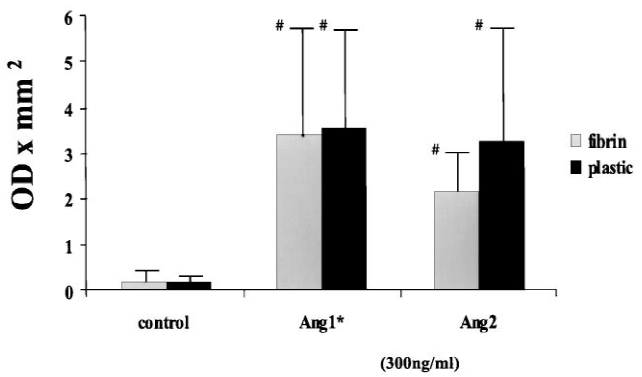

Fig. 9. Quantitative analysis of Tie2 phosphorylation induced by Ang1* or Ang2. Experiments were performed as described in Fig. 8 (panel D). Densitometry was performed by scanning the autoradiographs, and the intensity of each band was analyzed using Molecular Analyst (BioRad). Bars represent mean±S.D. from three separate experiments. The difference between each individual group were evaluated by Student's *t*-test. Differences were considered significant at $^{\#}P<0.05$.

effects between Ang1 and Ang2 need to be further defined at the molecular and cellular level.

Clearly the effects of angiopoietins on proliferation were relatively modest. It is quite possible that there were competing proliferative (i.e. activation) and antiproliferative (i.e. differentiating) actions, and that higher concentrations the latter may have predominated. For example, Tie2 activation has been reported to inhibit EC apoptosis via Akt/PI3 kinase [41,42]. This same pathway is known to activate eNOS by serine phosphorylation [43], and we have previously shown that NO can inhibit HUVEC proliferation [32]. This might also explain the difference between Ang1 and Ang2, since the delay in receptor activation may have slightly favoured proliferation at higher concentrations. It is unlikely that these proliferative actions of angiopoietins would play much of a role, rather their much stronger effects of EC differentiation, and vascular maturation would be of much greater importance.

Thus, these findings provide the first demonstration that Ang2 can induce angiogenesis in vitro and suggest that the

previously described EC-specific inhibition of Tie2 phosphorylation by Ang2 is subject to time-dependent modulation. This observation may provide insight into the mechanism of inhibition of Tie2 by Ang2 and may have important physiological implications regarding the role of Ang2 in the regulation of vascular homeostasis and the angiogenic response.

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