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## INTRODUCTION

The goal of the proposed research is to determine whether an endothelial cell-specific receptor tyrosine kinase, Tie2, can be used as a target for the development of anti-breast cancer therapy. Angiopoietin-1 (Ang1) has been shown to act as an angiogenic promoter in embryonic angiogenesis by promoting vascular branching, pericyte recruitment and endothelial survival. We have investigated the role of Ang1 in tumour neo-vascularisation under clinical conditions and in animal models. The expression of Ang1 in clinical breast cancer specimens was analysed by using laser capture microdissection and reverse transcriptase-linked polymerase chain reaction (RT-PCR) on RNA isolated from the samples. Despite the expression of Ang1 in many human breast cancer cell lines, the gene was expressed in only 3 out of 21 breast cancer clinical specimens, even though its receptor, Tie2, is abundant in the vasculature of all of these tumours. Ang1 was then over-expressed in a human breast cancer cell line (MCF-7) on its own and in conjunction with FGF-1, an angiogenic factor shown to be able to increase the tumourigenicity of MCF-7 cells. High concentrations of Ang1 were produced in the conditioned media of the transfected cells (range 156-820 ng.ml<sup>-1</sup>). However, in contrast to its physiological role as promoter of angiogenesis, overexpression of Ang1 did not enhance tumour growth, but instead caused up to a 3-fold retardation of tumour growth ( $p = 0.003$ ). These data support the view that Ang-1 may function physiologically to promote angiogenesis by inducing vessel maturation and stabilization, but this may inhibit angiogenesis in tumor where the vasculature is highly immature and unstable.

## BODY

### A. BACKGROUND

Tumours undergo a critical transition from an avascular to a vascular stage by invoking an angiogenic response from host vasculature (Folkman, 1971). To do this the cancer cells elaborate growth factors and cytokines that act upon the surrounding endothelial cells (Hanahan & Folkman, 1996) which induce vascular sprouting and new blood vessel formation. Certain of these factors, such as fibroblast growth factor-1 (FGF1), may have both paracrine and autocrine effects on tumour growth because of the distribution of their receptors on tumour cells, other stromal cells, as well as endothelial cells (Lehtola et al., 1992). Other factors such as vascular endothelial cell growth factor (VEGF), target only endothelial cells because the expression of their receptors is restricted to endothelial cells (de Vries et al., 1992; Terman et al., 1992).

Angiopoietin-1 (Ang1) is a recently described growth factor whose target cells are also endothelial cells, because the expression of the receptor, Tie2 (Tek), is restricted essentially to endothelial cells (Dumont et al., 1993; Maisonpierre et al., 1993). Transgenic animal studies indicate that Ang1 is central to embryonic vascular development (Maisonpierre et al., 1997; Sato et al., 1995; Suri et al., 1996; Suri et al., 1998; Thurston et al., 1999). Ang1 knock-out animals die in utero because of specific vascular deficits. These include failure of the primitive capillary plexus to branch appropriately and failure of the endothelial cells to form stable associations with surrounding basement membranes and pericytes. Knock-out of the Tie2 gene gave rise to essentially the same phenotype. Overexpression of Ang1 in the skin of transgenic animals causes an increase in the number, size and branching complexity of dermal vessels. *In vitro*, Ang1 protects against endothelial cell apoptosis, stabilizes endothelial tubules and induces vascular sprouting and tubule formation in collagen (Hayes et al., 1999; Holash et al., 1999; Koblizek et al., 1998; Papapetropoulos et al., 1999; Thurston et al., 1999). *In vivo* Ang1 synergises with VEGF to promote corneal neo-vascularisation (Asahara et al., 1998). Together these findings all suggest that Ang1 acts physiologically as an angiogenic promoter in the developing vascular system, in a coordinated process involving other angiogenic factors such as VEGF (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995).

We investigated whether Ang1 would serve an analogous function in tumours by promoting vascularisation. Ang1 expression levels were assessed in breast cancer cell lines and in breast cancer specimens. The gene was then over-expressed in a breast cancer cell line (MCF-7) by itself or together with FGF1. Our data indicate that Ang1 is rarely expressed by breast cancer cells in clinical samples. Moreover, Ang1 not only fails to enhance xenograft tumour growth in the MCF-7 tumour model, but appears to act in inhibitory capacity in this model. Possible reasons for the apparently contradictory roles for Ang1 in physiological and tumour angiogenesis are discussed.

### B. METHODS

Cell Culture: The MCPX cell line is a sub-line of ML-20 cells which were derived from MCF-7 cells by transfection with  $\beta$ -galactosidase (lacZ). (Kurebayashi et al., 1993; McLeskey et al., 1993; McLeskey et al., 1998). The  $\alpha$ 18 cells, referred to as Clone 18 previously, were derived from ML-20 cells by transfection with FGF1 (Zhang et al., 1997). Cell lines were cultured in 5% FBS/ IMEM/ 4 mM glutamine (Biofluids, Rockville, MA).

Immunohistochemistry for Tie2 and vWF. Frozen sections (5 µm) were immuno-stained with monoclonal antibodies to Tie2 (a gift from Dr. Kevin Peters, Procter and Gamble, Cincinnati, OH), and to von Willebrand Factor (Boehringer Mannheim, Indianapolis, IN). Sections were fixed in acetone at -20°C for 10 minutes, incubated in 0.3% hydrogen peroxide in methanol, blocked in 5% BSA (Sigma, Milwaukee, WI) 2.5% normal horse serum (Vector Labs, Burlingame, CA) in PBS then incubated with antibody in blocking solution. Sections were washed in PBS, then incubated for 1 hour with a 1:5000 dilution of a biotinylated polyclonal antibody against mouse IgG and developed using a Vector ABC kit (Vector Labs), then counter-staining with haematoxylin.

Laser capture micro-dissection: Frozen tumour samples and adjacent normal breast tissue were used for laser capture microdissection using previously described methods (Emmert-Buck et al., 1996), utilizing a Pixcell laser capture microdissection system (Arcturus Engineering, Mountain View, CA). Clinical sections, immuno-stained for Tie2 and vWF, were examined to identify areas of high microvessel density. Microvessel counting was performed using standard techniques (Weidner, 1995). Un-mounted serial sections stained with eosin, or with hematoxylin and eosin, were then examined to locate parallel areas. A Capsure Cap (Arcturus Engineering) was then placed onto the section over a 30 µm diameter capture area comprising either tumour cells in areas of high microvessel density, or epithelial cells in normal breast acini. Upon laser capture, cells in 50 areas were transferred into a 0.5 ml Eppendorf vial, yielding between 500 and 1000 cells per sample. Total RNA was retrieved using a Micro RNA Isolation kit (Stratagene, La Jolla, CA) and RNA corresponding to that collected from approximately 250 cells was used as a template for subsequent RT-PCR.

Reverse Transcriptase-linked Polymerase Chain Reaction (RT-PCR): The primer sequences for Ang1 are: 5'-CAACTGGAGC TGATGGACAC A-3' (sense) and 5'-ACTGCCTCTG ACTGGTAATG G-3' (antisense) and span base pairs 1060 to 1420 of Ang1 mRNA. The primer sequences for VEGF are 5'-GCCTTGCCCTT GCTGCTCTAC-3' (sense) and 5'-AATGCTTTCT CCGCTCTGA-3' (antisense) spanning base pairs 48 to 473. The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are 5'-AAGGTGAAGG TCGGAGTCAA CG-3' (sense) and 5'-TGGTGGTGCA GGAGGCATTG C-3' (antisense) spanning base pairs 40 to 496. The GAPDH primers span introns 1-5 in the genomic sequence. Total RNA collected from a panel of breast cancer cell lines was supplied by Dr Anke Schultke, Lombardi Cancer Center and 0.1 µg was used as a template. RT-PCR analysis was performed using reagents supplied in a GeneAmp RNA PCR core kit (Perkin Elmer, Wellesley, MA), with a Perkin Elmer GeneAmp 2400 PCR system. The DNA was transferred onto a Nytran nylon membrane (Schleicher & Schuell, Keene, NH). Southern hybridization was performed using oligonucleotide nested primers end-labeled with <sup>32</sup>P-dATP (Amersham, Piscataway, NJ). The sequences of the nested primers were: for Ang1, 5'-AGACTGTGCA TGTATATC-3'; for VEGF, 5'-CAATGACGAG GGCCTGGAGT-3'; and for GAPDH, 5'-GTCTTCACCA CCATGGAGAA-3'.

Transfection of the cancer cells with Ang1: The plasmid, jFE14/Ang1 is derived from the pSR plasmid which contains an HTLV-1 promoter (Takebe et al., 1988). Full length human Ang1 cDNA was cloned into this plasmid at two BstX 1 sites. Co-transfection was performed with the pcDNA3.1 Zeo plasmid (Invitrogen) which confers resistance to the antibiotic Zeocin (250 µg/ml), using Lipofectamine Plus reagent (GibcoBRL, Rockville, MD).

Western slot blot analysis: Conditioned media were mixed (1:1) with DPBS containing 0.01% CHAPS (Sigma) and loaded in duplicate onto a Bio-dot SF slot blot apparatus (Bio-Rad,

Hercules, CA), with Hybond ECL nitrocellulose membrane. After blocking, the membrane was incubated a rabbit polyclonal antibody against Ang1, developed at Regeneron Pharmaceuticals (Tarrytown, NY), in TBS, 0.1% Tween-20, 1.25% BSA, washed, then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody and developed using ECL chemiluminescence (Amersham)

Tie2 phosphorylation assay: Tie2-transfected fibroblasts were treated for 5 minutes with conditioned media then immunoprecipitated for phosphorylated Tie-2 as described previously (Maisonpierre et al., 1997). Cells were lysed in RIPA buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS plus protease- and phosphatase-inhibitors. Tie2 was immunoprecipitated using an anti-myc monoclonal antibody, 9E10 (Sigma) and protein G-Sepharose beads. Samples were analysed by standard Western blotting protocols, including resolution by reducing SDS-PAGE and electrotransfer to PVDF membranes. The amount of total Tie2 receptor and auto-phosphorylated Tie2 receptor was detected, respectively, by incubating replicate membranes with 9E10 or with the anti-phosphotyrosine-specific monoclonal antibody, 4G10 (Upstate Biotechnology, Lake Placid, NY) visualized with a HRP-conjugated secondary antibody.

BIAcore Analysis: Conditioned media were analysed for Ang1 with a BIAcore biosenser (Pharmacia, Piscataway, NJ) utilizing a CM5 BIAcore chip surface that had been covalently coupled with recombinant soluble Tie2-Fc as described previously (Davis et al., 1996). To control for the specificity of ligand binding, media were pre-incubated with 20 ug/ml of excess soluble Tie2-Fc or two irrelevant soluble receptors. Only soluble Tie2-Fc competed for Ang1 binding to the chip surface.

Northern blotting analysis for FGF1 expression: An FGF1 cDNA probe was labeled with <sup>32</sup>P-dCTP (Amersham) using a Random-Primers DNA labeling system (GibcoBRL) and used in Northern analyses of total RNA as described previously (Zhang et al., 1997).

Tumourigenicity assays: NCR (nu/nu) athymic female 4-6 week old mice (Taconic Farms Inc., Germantown, NY) were supplemented with subcutaneously embedded 0.72 mg oestrogen pellets then inoculated subcutaneously in the mammary fat pads with 5 x 10<sup>6</sup> cells in 0.2 ml IMEM. Tumour volumes (length x width x height) were measured two times a week in a blinded manner.

Statistical analyses: Generalized linear mixed effects models were used to estimate tumour growth rates. The analysis was performed using SAS PROC MIXED procedure according to SAS/STAT User's Guide (SAS Institute Inc., Cary, NC). Plots of tumour sizes versus time for the MPCX tumour growth data revealed an exponential growth pattern. Plots of tumour size versus time for the 18 tumour growth data indicated the growth rate in some cell lines was negative to some time points and then became positive, suggesting that an overall growth rate was not appropriate to summarize the growth pattern. For these data, we described the growth pattern for each cell line at certain time points and compared the growth rates at these time points using repeated analysis of variance.

## C. RESULTS

Ang1 expression in breast cancer cell lines: We analysed Ang1 expression in a panel of 19 breast cancer cell lines by RT-PCR analysis (Fig. 1). Ang1 transfected CHO cells were used as a positive control. PCR products were subjected to agarose gel electrophoresis and visualized with ethidium bromide. To exclude non-specific PCR amplifications, the PCR products were then

subjected to Southern blotting analysis with a radio-labeled "nested" Ang1 primer that corresponded to a sequence of Ang1 cDNA between PCR primers. A positive signal for Ang1 was identified from RNA isolated from 9 out of the 19 breast cancer cell lines.

Low Ang1 expression in human breast cancer clinical specimens: We then determined whether breast cancer cells express Ang1 under clinical conditions. We utilized laser capture microdissection and subsequent RT-PCR on the RNA isolated from microdissected specimens for this analysis. This technique permitted the isolation of a relatively homogeneous population of cancer cells adjacent to tumour vessels, which expressed a high level of the receptor Tie2 (Fig. 2). It also involved amplification of the mRNA signal, as our previous efforts to determine Ang1 expression with in situ hybridization experiments did not reveal any Ang1 signals in breast cancer specimens despite ample signals in control samples of Ang1-transfected cancer cells (data not shown). After reverse transcription of the mRNA from the microdissected cells, the cDNA samples were analysed for Ang1 by Southern blotting with a "nested" oligonucleotide probe. VEGF mRNA, which is abundant in tumours, was analysed as a positive control. Figure 3 shows the results of an analysis of six tumour specimens. Control cells that expressed Ang1 display a clear signal for Ang1 which is approximately equal to that seen for GAPDH, an internal control, irrespective of whether the RNA was collected directly from cells growing in tissue culture or from frozen sections of cell pellets. In the majority of microdissection experiments no Ang1 signal was seen even after prolonged exposure, while the GAPDH signal was clearly demonstrated (Fig. 3A). In contrast, similar analysis of VEGF expression in the same six tumour samples yielded a strong signal in 5 out of 6 cases, confirming the abundance of this angiogenic factor in tumours and the suitability of microdissection as a method of analysis (Fig. 3B).

Table 1 shows a summary of the results of Tie2 and Ang1 expression analysis of 11 normal and 23 malignant specimens. Samples were informative for Ang1 expression only if a GAPDH signal was visible after Southern hybridization. Although Tie2 is clearly expressed on microvessels of tumours and correlates closely to the expression of vWF (correlation coefficient 0.91), a detectable Ang1 signal was identified only in 3 out of 21 tumour cases analysed and none of 9 normal cases.

Transfection of Ang1 cDNA into breast cancer cell lines results in the expression of high levels of biologically active Ang1 in conditioned media: In order to determine the effect of elevated Ang1 levels on tumour growth, we stably transfected Ang1 into two cell lines derived from the oestrogen dependent, poorly angiogenic and weakly tumorigenic MCF-7 cells: the MPCX cells and the  $\alpha$ 18 cells. The MPCX cells, which were transfected with the  $\beta$ -galactosidase gene (*lacZ*) for convenient detection, maintains essentially the same growth characteristics to the MCF-7 cells, while the  $\alpha$ 18 cells, which were further transfected with FGF1, exhibited a greatly increased tumorigenicity and an extreme form of the dysfunctional vascular phenotype seen in xenograft tumours, with abundant vessels that are dilated (Zhang et al., 1997).

Ang1 protein in the conditioned media of the transfected cells was detected by Western slot blot analysis using a polyclonal antibody against human Ang1 (Fig. 4A). An estimation of the amount of Ang1 was made by comparison with signals from an Ang1 preparation of known concentration. The ability of the Ang1 protein to bind to Tie2 was determined by using a BIAcore analyzer, which also allowed a quantitative determination of the concentration of Ang1 (Table 2). The Ang1 concentrations determined by these methods correlated closely.

Furthermore, the biological activity of Ang1 in the conditioned media was determined by measuring the ability of the conditioned media to induce Tie2 tyrosine phosphorylation (Fig. 4B). The extent of phosphorylation induced by conditioned media from the highest expressing clones was equivalent to that produced by 200 ng of recombinant Ang1, which gave rise to a maximum extent of phosphorylation of Tie2 on the cells. The  $\alpha$ 18-derived cell lines were further analysed for continued expression of FGF1 mRNA by Northern analysis (Fig. 4C). All of the FGF1 transfected cell lines expressed high levels and equivalent amounts of FGF1 mRNA. In vitro mitogenesis assays were performed on all transfected and parental cell lines prior to animal inoculation to ensure that the transfection procedure or the expression of Ang1 had not altered the in vitro growth characteristics (data not shown). As expected, Ang1 over-expression had no effect on the growth rates of all the selected clones in cultures since MCF-7 cells do not express the Tie2 receptor.

Inhibition of MCF-7 xenograft tumour growth by Ang1 over-expression: Three Ang1 expressing clones, as well as a pooled population of empty vector transfected cells and the parental cells, were inoculated into the mammary fat pads of athymic nude mice. The growth rates of the xenograft tumours were monitored. The tumours were retrieved at the end of the experiment and the expression of Ang1 confirmed by Northern blotting analysis (data not shown). Although Ang1 over-expressing MPCX cells were able to grow xenograft tumours, the growth rates of the transfected cells were decreased as compared with the parental or vector control. A dramatic inhibitory effect ( $p=0.003$ ) was observed with the clone MAng 184 that expressed the most Ang1 (Fig. 5A). The extent of rate decrease correlated reasonably well with the amount of Ang1 produced by the transfected cells (Fig. 5B and Table 2).

Similar experiments were carried out with the FGF1 and Ang1 co-transfected  $\alpha$ 18 cell lines. The growth of the xenograft tumours of the Ang1 over-expressing cells was again found to be much slower than that of the parental cells and the vector mock transfected cells (Fig. 6A). A statistically significant inhibition of tumour growth was observed with clones  $\alpha$ Ang 18 and  $\alpha$ Ang 29 ( $p=0.03$ ). The dimensions of the tumours produced by clone  $\alpha$ Ang 14 were not statistically different from the parental or vector controls. However for clone  $\alpha$ Ang 14 the recorded tumour volume did not represent the actual volume of tumour cells. This is because the majority of the volume of the xenografts formed by this clone at the end of the assay was caused by blood in a haemangectasic sac. In contrast, while the parental and vector cells produced a haemangectasic sac initially, this was replaced by a solid mass of tumour cells as the tumour growth progressed (Zhang et al., 1997). We demonstrated this at the end of the experiment by staining all of the xenografts with a  $\beta$ -galactosidase substrate. This allowed for easy identification of the tumour as blue cells (Fig. 6B). A solid mass of cancer cells had replaced the haemangectasic sac in the tumours produced by the parental  $\alpha$ 18 cells (Fig. 6B, right). In contrast, the haemangectasic sac responsible for the large dimensions of the  $\alpha$ Ang 14 tumours was not replaced by a tumour mass at the end of the assay still but consisted principally of blood (Fig. 6B, left). On transection, it was demonstrated that the cancer cells occupied a relatively small proportion of the tumour volume (Fig. 6B, center) implying that the rate of tumour cell growth was very much smaller than that recorded by measurement of xenograft dimensions.

FIGURES

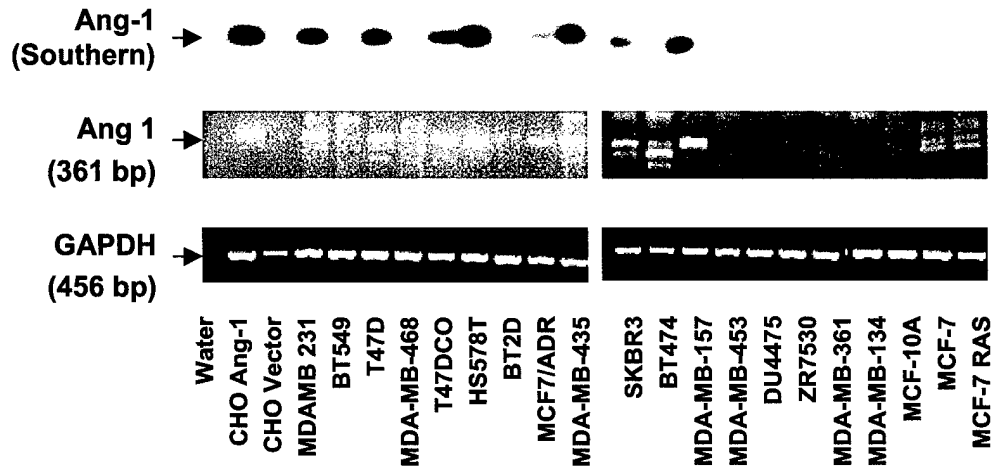


Figure 1. RT-PCR analysis of breast cancer cell lines for Ang1 mRNA. Positive and negative control cells were CHO cells transfected with Ang1 (CHO Ang1) or an empty vector (CHO Vector). The lower two panels show RT-PCR products after agarose gel electrophoresis, visualized by ethidium bromide staining. The top row is a Southern hybridization of the Ang1 RT-PCR products with a <sup>32</sup>P-labelled nested primer.

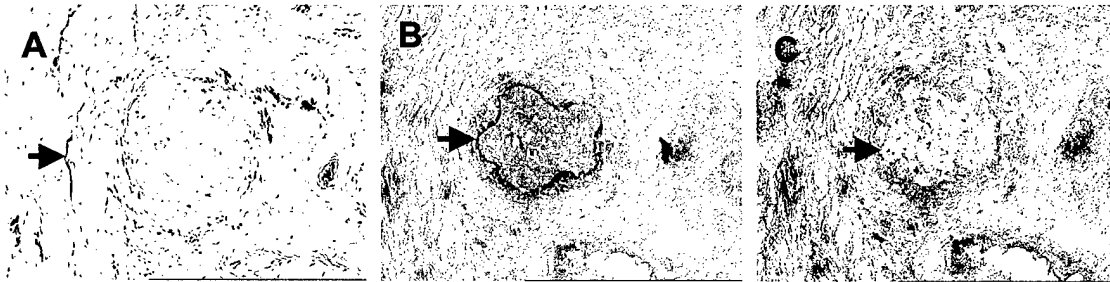


Figure 2. Human breast cancer specimens before and after laser capture microdissection. (A) A frozen section of ductal carcinoma was immuno-stained with a monoclonal antibody against Tie2: brown staining (arrow) indicates blood vessels that express Tie-2. (B) A serial section (eosin staining) showing the same ductule filled with breast cancer cells in which the cancer cells have been outlined by a laser field (arrow) prior to micro-dissection. (C) The same specimen as in B but subjected to micro-dissection; note the empty space left by the breast cancer cells now captured.

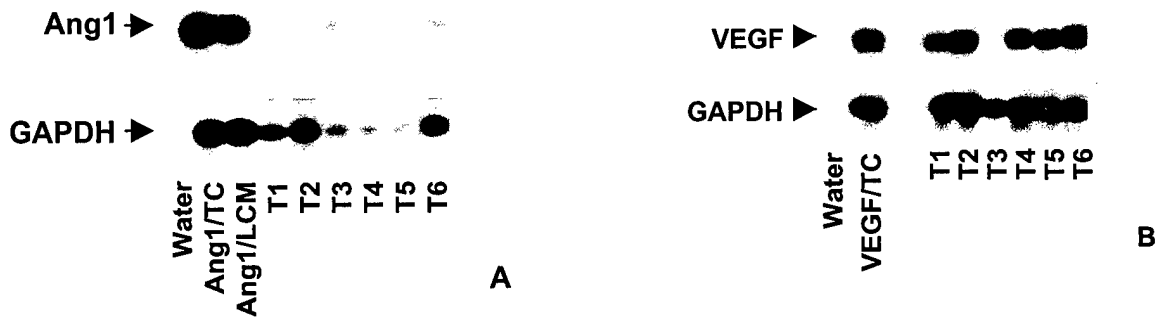


Figure 3. Expression of Ang1 and VEGF in tumour specimens determined by using laser-capture microdissection. (A) Detection of Ang1 expression in 6 tumour specimens, by RT-PCR and subsequent Southern analysis. Controls are RNA isolated from Ang1 transfected cells growing in culture (Ang1/TC) or microdissected from a section of a cell pellet (Ang1/LCM). (B) Detection of VEGF in the same cancer specimens by RT-PCR and Southern analysis.

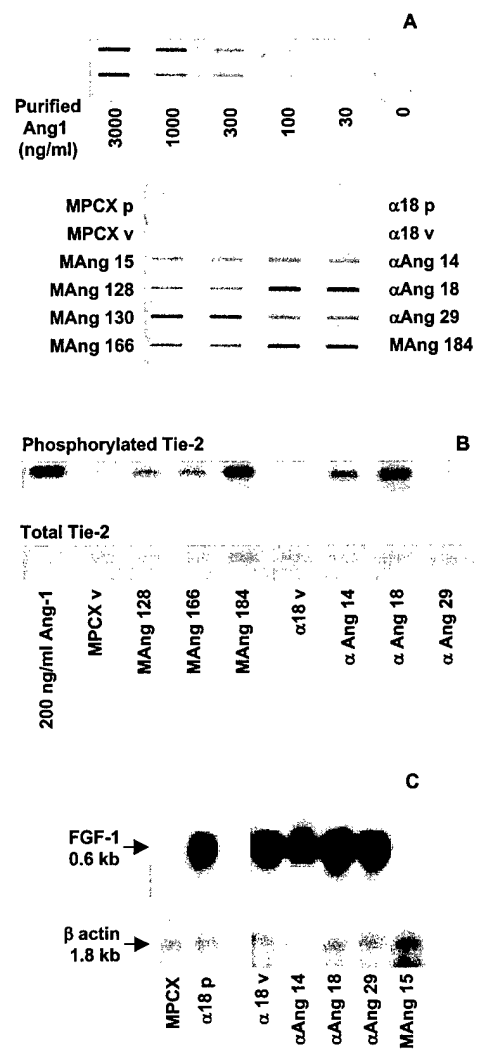


Figure 4. Production of Ang1 by Ang1-transfected MPCX and  $\alpha 18$  cells. (A) Western slot blot analysis of Ang1 in the conditioned media. Duplicate aliquots of COS cell conditioned media of known Ang1 concentration were used as standards (upper panel). Five clones of the Ang1-transfected MPCX cells and three clones of the Ang1-transfected  $\alpha 18$  cells were shown in duplicate (lower panel), in comparison with the parental (P) and empty vector transfected (V) cells. (B) Ang1 activity in the conditioned media was determined for the ability to induce Tie2 tyrosine phosphorylation. NIH 3T3 cells over-expressing myc-tagged Tie2 were treated with the conditioned media, then subjected to immunoprecipitation with an anti-myc antibody, and Western blotting analysis with an anti-phosphotyrosine antibody (upper panel). The cells were also treated with 200 ng/ml of Ang1 to provide a positive control. A Western analysis with an antibody against the myc-tag as a loading control (lower panel). (C) Northern blotting analysis of FGF1 in RNA collected from Ang1 transfected  $\alpha 18$  cells. The internal control was  $\beta$ -actin FGF1.

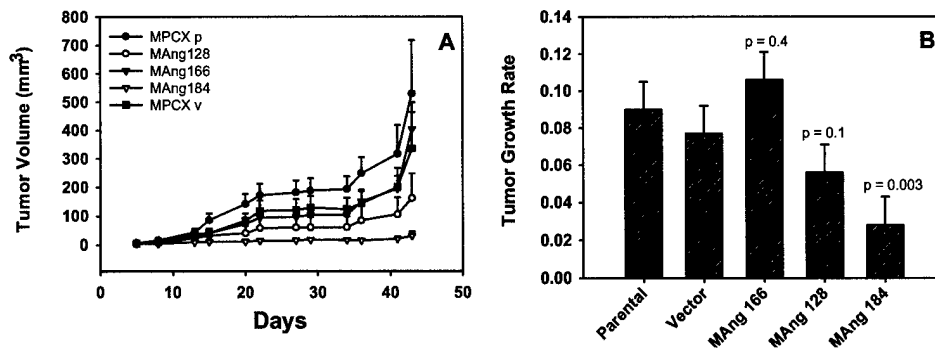


Figure 5. Inhibition of MCF-7 human breast cancer xenograft tumour growth by Ang1 over-expression. (A) Plots of tumour volumes of the xenograft tumours formed by the MPCX parental cell line (closed circles), empty vector transfected cells (closed squares), and three clones of Ang1 expressing transfectants: MAng 128 (open circles), MAng 166 (closed triangles), and MAng 184 (open triangles). There were five animals per group. The experiment was repeated and the results were reproducible. (B) The tumour sizes as a function of time were fitted with an exponential tumour growth model (see Methods) to determine the rate constants of the xenograft tumour growth. Growth rates for the Ang1-over-expressing MPCX clones were compared to that of the vector mock transfected cells (ANOVA).

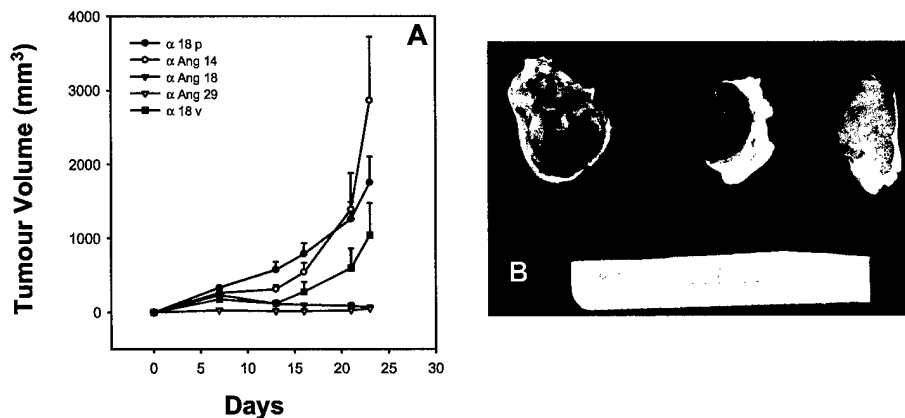


Figure 6. Inhibition of FGF1 transfected MCF-7 breast cancer xenograft tumour growth by Ang1 over-expression. (A) Plots of tumour volumes of the xenograft tumours formed by the α18 parental cell line (closed circles), empty vector transfected cells (closed squares), and three clones of Ang1 expressing transfectants: αAng 14 (open circles), αAng 18 (closed triangles), and αAng 29 (open triangles). The overall statistical significance at 5% level was  $p = 0.03$  (ANOVA) for αAng 18 and αAng 29. The experiment was repeated and the results were reproducible. (B) Photographs of the xenograft tumours: a tumour formed by αAng 14 giving the typical appearance of a blood filled sac (left), cross-section of a tumour in the same group showing that the interior of the sac is empty once opened, and that there are only a few cancer cells which were stained blue (center), and the tumour formed by the parental α18 cells consists of blue cancer cells.

TABLES

Table 1. Summary of Ang1, Tie2, and vWF expression in clinical specimens. Clinical specimens (9 grade 3 tumours, 9 grade 2 tumours, 3 grade 1 tumours and 11 normal breast sections) were stained with monoclonal antibodies to Tie2 and vWF. Positively staining vessels were counted manually using standard protocols (Weidner, 1995). Densities ( $\pm$  SEM) refer to number of vessels per 0.74 mm<sup>2</sup> microscopy field at 200x magnification. Ang1 expression was analysed by laser capture microdissection from cells adjacent to the immuno-stained vessels. Specimens were considered informative for Ang1 expression only if GAPDH was detectable.

Sample	Cases stained for Tie-2 and vWF	Number of Tie-2 expressing micro-vessels per sample	Number of vWF expressing micro-vessels per sample	Cases informative for Ang1 expression	Cases with Ang1 signal
Normal	11	12 ( $\pm$ 2.7)	20 ( $\pm$ 4.6)	9	0
Tumour	23	35 ( $\pm$ 3.7)	44 ( $\pm$ 3.9)	21	3

Table 2. Ang1 concentrations in the conditioned media of either vector-transfected or Ang1 transfected cells (BIAcore analysis).

Cell line	Ang1 (ng.ml <sup>-1</sup> )
MPCX Vector	0
MAng 128	240
MAng 166	160
MAng 184	820
$\alpha$ 18 Vector	0
$\alpha$ Ang 14	172
$\alpha$ Ang 18	612
$\alpha$ Ang 29	156

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## KEY RESEARCH ACCOMPLISHMENT

- Discovered that Ang1, a physiological promoter of angiogenesis, may be down-regulated in human breast cancer.
- Discovered that overexpression of Ang1 may result in the inhibition of breast cancer xenograft tumor growth
- The findings led to a provocative hypothesis that Ang-1 may function physiologically to promote angiogenesis by inducing vessel maturation and stabilization, but this may inhibit angiogenesis in tumor where the vasculature is highly immature and unstable.

## REPORTABLE OUTCOMES

Hayes, AJ, Huang, WQ, McLeskey, S, Kern, F, Lippman, ME, Maisonpierre, P, and Li, LY. Function of angiopoietin-1 in human breast cancer. *Brit. J. Cancer*, in press, 2000

Hayes, A, Huang, W, McLeskey, SW, Maisonpierre, P, Li, LY: Overexpression of angiopoietin-1 in human breast cancer cells inhibits xenograft tumor growth. *Proc. Am. Assoc. Cancer Res.* 41:489, 2000

Over-expression of angiopoietin-1 in human breast cancer cells inhibits xenograft tumor growth. Lu-Yuan Li, Andrew J. Hayes, Wei-Qun Huang, Sandra W. McLeskey, and Peter Maisonpierre. Keystone Symposium on Angiogenesis, Salt Lake City, UT, 2000

## CONCLUSIONS

We tested the hypothesis whether Ang1 would serve a pro-angiogenic role in the context of tumours, similar to its role suggested for physiological neovascularisation. We found that Ang1 expression is lacking from areas adjacent to tumour blood vessels in human breast cancer despite abundant expression of Tie2 in these vessels. In addition, over-expression of Ang1 did not enhance xenograft growth and the majority of Ang1 transfected clones demonstrated decreased growth rates. Our data demonstrate that Ang1 may not function as a promoter of angiogenesis in breast tumours. Moreover the data suggests that it may act in an inhibitory capacity in this model.

These findings initially appears to be in contrast to transgenic studies in which Ang1 was over-expressed locally in the skin of developing mice. This over-expression of Ang1 induced remarkable increases in vascularity in the skin of these transgenic animals (Thurston, 1999). These seemingly contrary findings for Ang1 over-expression in transgenic animals and in our tumour models suggest that the process of tumour vascularisation may be dissimilar to that seen in physiological settings. Physiological angiogenesis occurs in the context of a number of angiogenic factors and the expression of these factors is precisely coordinated both temporally and spatially (Dumont et al., 1995). Tumours elaborate a variety of angiogenic factors (Relf et al., 1997) and as a consequence of the abundance of angiogenic influences in the tumour micro-environment, the microvessel density in a tumour may be very high although many vessels are dysfunctional. This dysfunction is manifested by areas of tumour necrosis and tumour hypoxia seen near to areas of increased microvessel density. The failure to identify Ang1 expression in the breast cancer epithelial cells suggests that Ang1, which is hypothesized to promote the ordered expansion of the vascular tree physiologically, may not be a pertinent angiogenic factor to the highly disordered tumour vasculature.

The data from overexpression of Ang1 in a xenograft model suggest that Ang1 may inhibit tumour growth, presumably via an effect on tumour angiogenesis, as Ang1 had no effect on the *in vitro* growth of the tumour cells. This is in keeping with the hypothesized roles of Ang1 and its functional antagonist Ang2 (Maisonpierre et al., 1997) on vessel stability and receptivity to other angiogenic influences. Ang1 stabilizes the association between the endothelial cell and pericyte (Suri et al., 1996; Suri et al., 1998; Thurston et al., 1999). Ang2 by antagonizing this effect can result in vessel disassembly and subsequent vessel regression, but may, in the presence of other suitable angiogenic factors, facilitate new vessel sprouting (Maisonpierre et al., 1997). It has recently been observed that Ang2 is focally up-regulated in the immediate vicinity of tumour vessels (Stratmann et al., 1998; Zagzag et al., 1999). Therefore in tumours, where a variety of other angiogenic factors exist, this stabilizing effect of Ang1 might in fact inhibit the intense continuous new vessel sprouting that is typical of tumour vascularisation. That Ang1 is able to inhibit tumour growth even in the presence of FGF1 supports the view that Ang1 retards tumour growth by vascular stabilization. Additionally, our findings that Ang1 is expressed in many breast cancer cell lines *in vitro*, but in very few clinical specimens, suggest that expression of Ang1 may be down-regulated in tumours because of its negative selective effect on the developing tumour.

To identify the mechanism by which Ang1 may lead to retardation of tumour growth requires a variety of further experimental approaches. These may include a detailed histological characterization of vessel branching and endothelial pericyte relations in the Ang1 overexpressing tumours and a direct *in situ* assessment of cellular proliferation rates within the

xenografts expressing Ang1. It will be informative to assess the expression patterns of other ligands to Tie-2 in clinical specimens, in particular Ang-2. Such studies will further elucidate the role played by this complex vascular signaling pathway in the process of tumour vascularisation.

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APPENDICES

Reprints:

1. Hayes, A, Huang, WQ, Mallah, J, Yang, D, Lippman, ME, and Li, LY. Angiopoietin-1 and its receptor Tie-2 participate in the regulation and survival of endothelial cells. *Microvascular Research* 58:224-237, 1999
2. Hayes, AJ, Huang, WQ, McLeskey, S, Kern, F, Lippman, ME, Maisonpierre, P, and Li, LY. Function of angiopoietin-1 in human breast cancer. *Brit. J. Cancer*, in press, 2000

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3. Hayes, A.J., Huang, W., Mallah, J., Yang, D., Lippman, M.E., and Li, L.Y. Angiopoietin-1 and its receptor Tie-2 are involved in endothelial cell survival and tubule formation. PROC. AMER. ASSOC. CANCER RES. 40, March 1999
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7. Lu-Yuan Li, Andrew J. Hayes, Wei-Qun Huang, Sandra W. McLeskey, and Peter Maisonpierre. Over-expression of angiopoietin-1 in human breast cancer cells inhibits xenograft tumor growth. Keystone Symposium on Angiogenesis, Salt Lake City, UT, 2000

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# Angiopoietin-1 and Its Receptor Tie-2 Participate in the Regulation of Capillary-like Tubule Formation and Survival of Endothelial Cells

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Angiopoietin-1 (Ang-1) and its receptor Tie-2, a transmembrane tyrosine kinase uniquely expressed by endothelial cells, are shown by null mutation studies to be essential to developmental angiogenesis. The phenotypic abnormalities in these knockout animals suggest that Tie-2 signaling is necessary for the maintenance and expansion of the primitive capillary network. We present *in vitro* evidence indicating that the Ang-1/Tie-2 system participates in the regulation of capillary tubule formation and is necessary for the survival of confluent endothelial cells. Although recombinant Ang-1, which induces Tie-2 phosphorylation, has no effect on the proliferation of endothelial cells, treatment of confluent adult bovine aortic endothelial cells (ABAE) cells grown on collagen gels with Ang-1 (100 ng/ml) causes the cells to migrate into the collagen gel and form capillary-like tubules. The tubule-forming effect of Ang-1 is similar to the effect caused by FGF-2. A soluble form of the Tie-2 extracellular domain, in fivefold molar excess, blocks Ang-1-induced tubule formation. Specific elimination of Tie-2 protein expression in cultured ABAE cells as a result of transfection with an antisense oligonucleotide causes cell death in a dose-dependent manner ( $IC_{50} = 50$  nM). The antisense treatment has no effect on cells that do not express Tie-2. Cells treated with antisense oligonucleotide show a six-fold increase in the rate of apoptosis as assessed by *in situ* end labeling of fragmented DNA. These findings are con-

sistent with the view that Ang-1/Tie-2 signaling is essential for both angiogenesis and endothelial cell survival. © 1999 Academic Press

**Key Words:** angiogenesis; endothelial cell; angiopoietin-1, Tie-2.

## INTRODUCTION

Endothelial cell organization into functioning vessels is an essential early developmental process and is central to pathological processes such as tumor formation. Endothelial cell proliferation and organization are under regulation, at least in part, of ligands signaling through endothelial-cell-specific, transmembrane receptor tyrosine kinases, in particular the vascular endothelial growth factor receptors 1 and 2 (VEGFR-1 and -2) (Fong *et al.*, 1995; Shalaby *et al.*, 1995) and the Tie receptors (Tie-1 and Tie-2) (Dumont *et al.*, 1994; Sato *et al.*, 1995). The ephrin family of factors may be responsible for further differentiation of the arterial and venous systems. Studies in transgenic animals deficient for VEGFR-1 and -2 indicate that VEGF signaling is crucial to the initial expansion of the endothelial cell population and its formation into capillary tubules (Fong *et al.*, 1995; Shalaby *et al.*, 1995). VEGFR-1 and VEGFR-2 (also known as Flk-1

and KDR) (Terman *et al.*, 1992; Quinn *et al.*, 1993) are endothelial cell specific and are also up regulated in pathological angiogenesis (Ferrara, 1995), whereas VEGFR-3 may have non-endothelial-cell functions (Soker *et al.*, 1998).

The phenotypes of transgenic mice deficient for Tie-1 and Tie-2 indicate functions for these receptors in the organization of the expanding capillary network (Dumont *et al.*, 1994; Sato *et al.*, 1995). Two ligands have been identified for Tie-2 which are called angiopoietin-1 and angiopoietin-2 (Ang-1 and Ang-2). The former activates the receptor and the latter antagonizes this effect by competitive inhibition at the receptor-binding domain (Davis *et al.*, 1996; Maisonpierre *et al.*, 1997). No ligand for Tie-1 has been reported to date. The binding of Ang-1 to Tie-2 results in receptor autophosphorylation and subsequent binding to the downstream proteins GRB-2 and SH-PTP2, perhaps after receptor dimerization (Huang *et al.*, 1995). GRB-2 and SH-PTP2 are involved in signaling pathways that can result in alterations in cellular morphology and differentiation.

Tie-2 and Ang-1 knockout mice have a lethal phenotype *in utero* at days E10.5 and E12.5, respectively (Dumont *et al.*, 1994; Sato *et al.*, 1995; Suri *et al.*, 1996). Tie-2 is not expressed in the embryo until day E8.5, after the expression of the VEGF receptors (Dumont *et al.*, 1995), indicating that expression of Tie-2 may not be necessary for the initial expansion of the endothelial cell lineage but may be necessary for its later remodeling and maintenance. Dumont *et al.* (1994) suggest that absence of Tie-2 signaling at this later stage in the development of the circulatory system results in selective endothelial cell apoptosis that is responsible for the lethal phenotype of these transgenic animals. *In vitro* studies indicate that binding of Ang-1 to Tie-2 also results in binding of phosphatidylinositol 3-kinase and subsequent activation of protein kinase B/Akt, further supporting the hypothesis that Tie-2 signaling is antiapoptotic (Kontos *et al.*, 1998).

Morphological analysis of the Tie-2 and Ang-1 knockout animals also indicates a dynamic role in angiogenesis. Both transgenic animals demonstrate typical abnormalities in the formation of the vascular network. The vascular tree shows deficiencies in

vessel branching with considerably fewer, simpler vessels (Sato *et al.*, 1995; Patan, 1998). Furthermore, overexpressing Ang-1 in the transgenic animals under a keratin 14 promoter that directs gene expression only to the skin produces an increased number of highly branched vessels within the skin (Suri *et al.*, 1998). On an ultrastructural level the Tie-2 and Ang-1 knockouts both demonstrate a loss of adhesion between endothelial cells and associated pericytes and basement membranes, with the endothelial cells rounding up and separating from adjacent structures (Suri *et al.*, 1996). Taken together, these transgenic phenotypes imply a normal function for Tie-2 in the expansion of the primitive endothelial tubules to a mature capillary network either by inducing vessel branching or intussusceptive microvascular growth and also for recruitment of pericytes to the newly formed tubules.

Analysis of the expression of autophosphorylated Tie-2 in adult tissues is consistent with a dual role for Tie-2 signaling both in endothelial survival and in active angiogenesis. Although Tie-2 expression is up regulated in areas of angiogenesis, it is also present in an autophosphorylated state in the entire spectrum of adult quiescent vasculature (Wong *et al.*, 1997).

We analyzed the possible role for the Tie-2 signaling both in angiogenesis and in endothelial survival *in vitro* using two approaches. First, we analyzed the ability of recombinant Ang-1 protein to induce the migration of endothelial cells from a confluent monolayer into a collagen matrix and form tubule-like structures, an assay which is analogous to the expansion of the vascular network *in vivo*. Second, we eliminated Tie-2 mRNA using an antisense oligonucleotide in order to assess the functional role of Tie-2 in confluent endothelial cells that are analogous to the quiescent endothelium *in vivo*. We report both that recombinant protein can induce endothelial cells to migrate into collagen gels and form a primitive capillary-like network and that elimination of Tie-2 mRNA and protein by the antisense treatment results in death of endothelial cells and subsequent apoptosis. These findings are consistent with a role for Tie-2 signaling in both endothelial cell survival and angiogenesis.

## MATERIALS AND METHODS

**Cell culture.** Adult bovine aortic endothelial (ABAE) cells were a gift from Dr. Peter Bohlen (ImClone, New York). NIH 3T3 cells were obtained from the Lombardi Cancer Center Tissue Culture Core Facility (Georgetown University Medical Center, Washington, DC). Cells were maintained in IMEM (Biofluids) and 10% FCS and 5 mM glutamine. ABAE cells were supplemented with 1 ng/ml fibroblast growth factor-2 (FGF-2) (R&D Systems). Cells were cultured in 37°C/5% CO<sub>2</sub> and passaged by the addition of 0.05% trypsin/0.53 mM EDTA (Biofluids). All experiments were performed using ABAE cells between passages 8 and 14.

**Reagents.** Synthesis and purification of phosphorothioate oligodeoxynucleotides were performed by Genosys. Antisense (5'-GCTAAAGAATCCATGCT-TCCCC-3) and sense (5'-GGGGAAGCATGGAT-TCTTTAGC-3) oligonucleotides were constructed that corresponded to base pairs 318–337 of bovine Tie-2 mRNA, which spans the AUG translation initiation codon. Both sequences were compared to the NIH nucleotide database confirming the specificity of the antisense oligonucleotide to Tie-2 mRNA and also that the sense control oligonucleotide did not correspond to other known mRNA sequences.

**In vitro angiogenesis assay.** Routine media without basic FGF, freshly prepared 1.8% NaHCO<sub>3</sub>, and type 1 rat tail collagen (Collaborative Biomedical Products) were chilled individually on ice for 1 h and then mixed in the ratio 3:1:1 by volume. The mixture (0.5 ml) was pipetted into each well of a 24-well plate and incubated at room temperature for 3 h to allow the gels to solidify. At the end of this period ABAE cells were seeded in each well at density of 50,000 cells/well in 0.5 ml of medium containing FGF-2 (1 ng/ml). The plates were incubated routinely and when the cells reached 80% confluence, each well was washed twice with PBS and then 0.5 ml of medium without FGF-2 was added. After 48 h, the medium was replaced with medium supplemented with the various concentrations of test compounds and the plate was incubated for a further 48 h at 37°C/5% CO<sub>2</sub> to allow tubule formation. At the end of this period

the plate was chilled on ice for 15 min, each well was washed with cold PBS, and the gels were fixed with 0.5 ml of cold methanol (–20°C) for 15 min. Each well was washed three times with cold PBS and then the gels covered with 0.2 ml of 50% glycerol in PBS. The next day the glycerol was removed and the gels were transferred to microscope slides. Coverslips, supported by two thin pieces of wire (1-mm thickness), were placed onto the gels so as to produce an entirely flat gel surface while the wire supports prevented the gel from being crushed by atmospheric pressure. The gel was surrounded by 50% glycerol, and the gap between the coverslip and slide was sealed with Permount (Fischer Scientific). The following day the gels were visualized by phase contrast microscopy using a X6.3 or X16 objective on an inverted Zeiss microscope and images were recorded using a panasonic CCD 72SX digital camera (Panasonic). Each image was analyzed using Optimas 5.2 image analysis software (Optimas Corporation). FGF-2-treated gels for transmission electron microscopy were fixed in 3% glutaraldehyde, postfixed for 1 h in 1% osmium tetroxide in cacodylate buffer, and dehydrated with ethanol. Sections were imaged with a JEOL 100CX-11 transmission electron microscope.

**Treatment of cells with Tie-2 sense and antisense oligonucleotides.** Confluent cells were harvested by trypsinization and seeded in 12-well plates at a density of 50,000 cells per well in 1 ml of culture medium (IMEM/10% FCS/5 mM glutamine supplemented with 1 ng/ml FGF-2) and allowed to reach confluence (3 days). Each well was washed twice with Optimem medium (Gibco BRL) with no serum or FGF-2 supplementation. The appropriate oligonucleotides were added to the Optimem medium containing GC-30:DOPE lipid solution (kindly provided by Dr. Bob Brown, Genta Inc., San Diego, CA) at 1:9 ratio of oligonucleotide:lipid by weight and the cells were incubated for 4 h at 37°C/5% CO<sub>2</sub>. After incubation each well was washed twice with Optimem and then incubated for a further 20 h with 1 ml of standard culture medium after which time the treatment was repeated. Cells were assessed after 48 h from the start of the first treatment. At the end of 48 h the cells were washed twice with PBS and then collected by incubation with 0.5 ml per well of trypsin/EDTA at 37°C which was

neutralized with 1 ml of culture medium after all cells had detached. Aliquots from each well were counted on a Coulter counter.

**Western analysis.** Cell lysates were prepared as follows. After treatment was complete, the cells were washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 50 µg/ml aprotinin, 50 µg/ml leupeptin, and 2 mM sodium orthovanadate). Total protein concentration was determined with BCA protein assay reagent (Pierce). Equal amounts of proteins were subjected to SDS-PAGE on an 8–16% Tris glycine gradient gel (Novex). The proteins were electroblotted onto Hybond ECL nitrocellulose membrane (Amersham) and then blocked with 3% BSA in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.2% polyoxyethylenesorbitan monolaureate (Tween 20)). Tie-2 receptor was detected with a monoclonal antibody obtained from Dr. K. Peters (Duke University, NC) incubated with a horseradish-peroxidase-linked antimurine secondary antibody and then visualized by chemiluminescence (ECL Amersham). Equal loading of protein was confirmed by staining with Ponceau S (Sigma) according to the manufacturer's instructions.

**In situ end labeling (ISEL) staining.** Cells were stained for apoptotic bodies using a modification of an *in situ* end labeling technique described previously (Wijsman *et al.*, 1993). ABAE cells were treated with oligonucleotides as described above, all media changes being collected and stored at 4°C so as all detached cells were collected. At the end of the treatment period adherent cells were collected by trypsinization and these cells were pooled with the previously collected media from the same treatment group. The cells were centrifuged at 1000 rpm in a bench-top centrifuge and the pellet was washed with ice-cold PBS. This procedure was repeated after which the cells were fixed in 10% paraformaldehyde for 10 min. The cells were centrifuged and washed in PBS two times and then resuspended in a small volume of PBS, aliquots of which were placed on microscope slides. These slides were air-dried, rehydrated with PBS for 10 min, and then immersed in 0.3% hydrogen peroxide for 30 min. The slides were incubated at room temperature for 5 min in Buffer A (containing

0.05 M Tris, pH 7.5, 0.005 M MgCl<sub>2</sub>, 0.001% 2-mercaptoethanesulfonic acid (Sigma), 0.005% BSA, fraction V (Sigma)) and then incubated at 37°C for 1 h with 400 µl of Buffer A per slide containing 0.2 mM dCTP, dATP, and dTTP (Promega), 0.02 mM biotin-16-UTP (Boehringer Mannheim), and 20 U/ml Klenow DNA polymerase (Boehringer Mannheim). The slides were washed twice in PBS and then incubated with an avidin-biotin-horseradish peroxidase conjugate prepared in PBS (Vectastain ABC, Vector Laboratories, Burlingame, CA). The slides were incubated with VIP substrate (Vector), lightly counterstained with methyl green (Vector), dehydrated, and mounted with Permount (Fisher Scientific).

## RESULTS

**Recombinant Ang-1 protein induces the formation of capillary-like tubules in collagen gels.** Previous reports indicated that Ang-1 does not induce endothelial cell proliferation *in vitro*, despite inducing receptor autophosphorylation (Davis *et al.*, 1996), and our experience with recombinant Ang-1 protein in endothelial cell mitogenesis assays confirmed this lack of mitogenic activity (data not shown). We therefore attempted to identify other phenotypic responses to Tie-2 activation by analyzing the effect of Ang-1 on the ability of confluent ABAE cells to form capillary-like structures in gels of collagen. Endothelial cells when seeded on the surface of gels of basement membrane extracts, such as collagen, will normally grow until they reach confluence. At this point they can be induced to form tubule-like structures by the addition of a variety of agents including phorbol esters and FGF-2 (Maciag *et al.*, 1982; Feder *et al.*, 1983; Montesano and Orci, 1985; Montesano and Orci, 1987; Goto *et al.*, 1993). Figure 1A demonstrates the typical cobblestone appearance of a confluent monolayer of endothelial cells cultured on collagen gels. The addition of a suitable angiogenic promoter, in this case FGF-2 (Fig. 1B), induces marked morphological changes. Endothelial cells invade the underlying gel and form a latticework of interconnecting cellular cords. Figure 1C demonstrates the appearance of ABAE cells on collagen gels

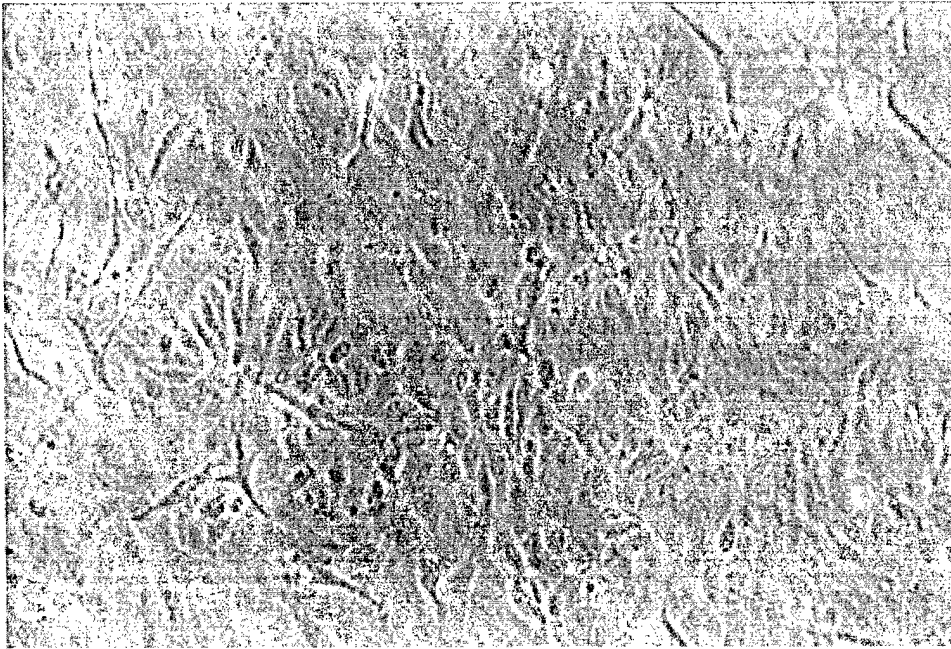
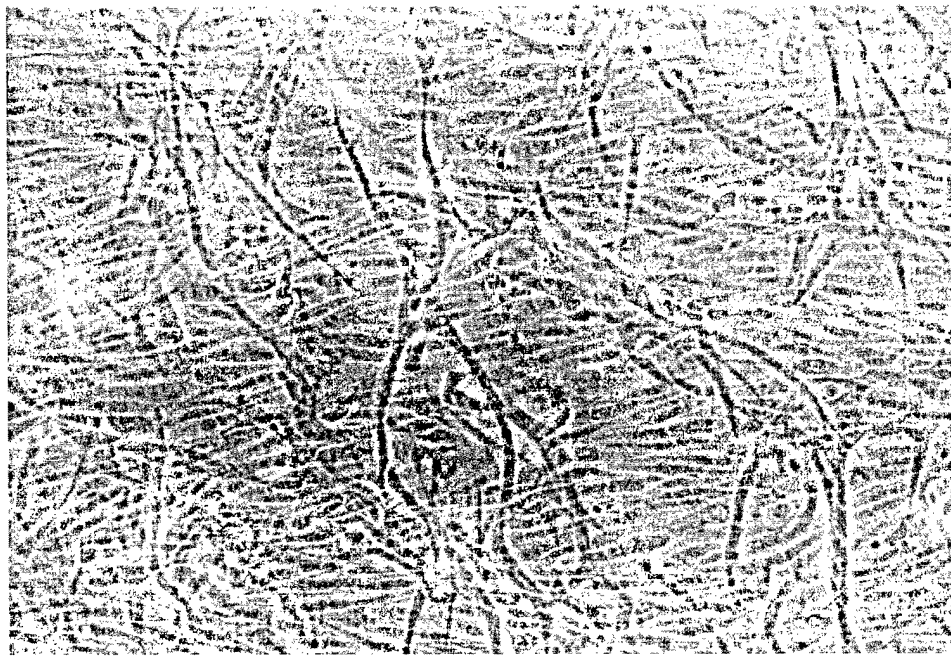
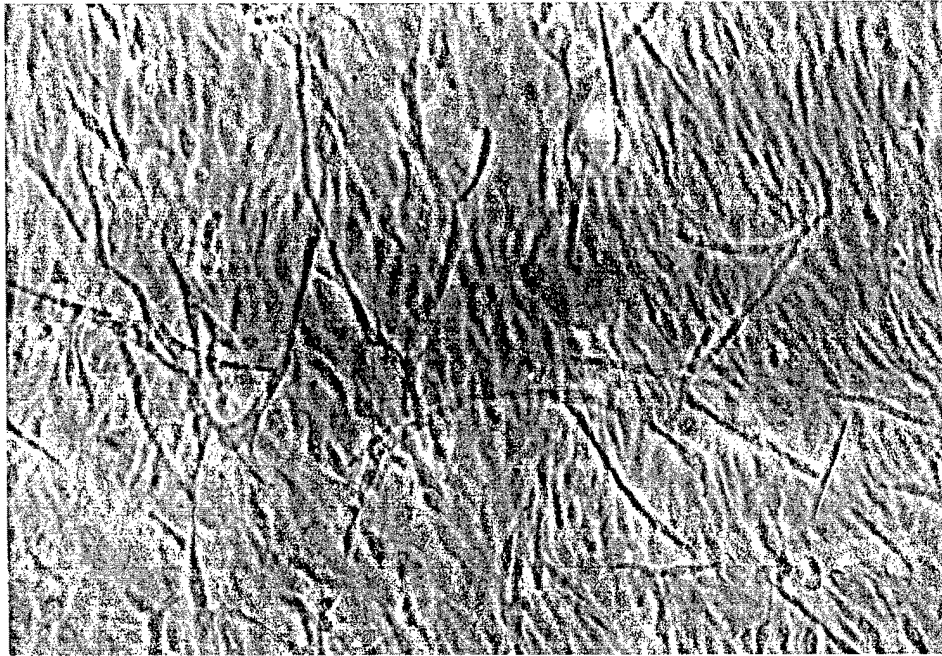
**A****B**

FIG. 1. Induction of adult bovine aortic endothelial (ABAE) cell capillary-like tubule formation by Ang-1. A. The typical cobblestone morphology of untreated cells with cells adopting a polygonal appearance. B. 50 ng/ml FGF-2. The cells form a lattice of interconnecting cords within the gel. C. Tubular structures induced by 100 ng/ml Ang-1. D. Inhibition of Ang-1 induced tubule formation by 25  $\mu$ g/ml of soluble Tie-2 extracellular domain.

after the addition of 100 ng/ml Ang-1. There is a moderately well-developed network of capillary-like structures that is similar to, although less extensive

than, those induced by FGF-2. This assay is viewed as representative of the process of capillary sprouting and tubule formation because the endothelial cell in-

C



D

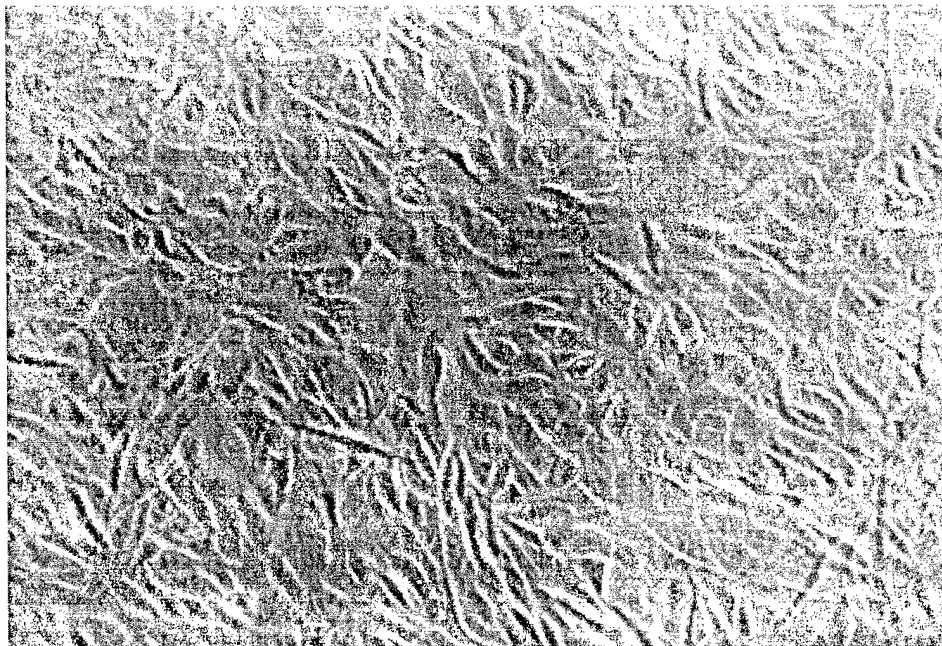


FIG. 1—Continued

vasion is associated with protease activity and collagen degradation, and the structures can be shown to constitute endothelial cell tubules surrounding a lumen on cross-sectional analysis (Montesano and Orci, 1985, and Fig. 2). In order to quantify the extent of tubule formation we utilized an image analysis system

that records the total length of the tubule-like structures in a given area (Figs. 2A and 2B). Ten images were recorded randomly from gels from each treatment group and subjected to image analysis using this system. Induction of tubule formation by Ang-1 is dose dependent (Fig. 2D). The maximum amount of

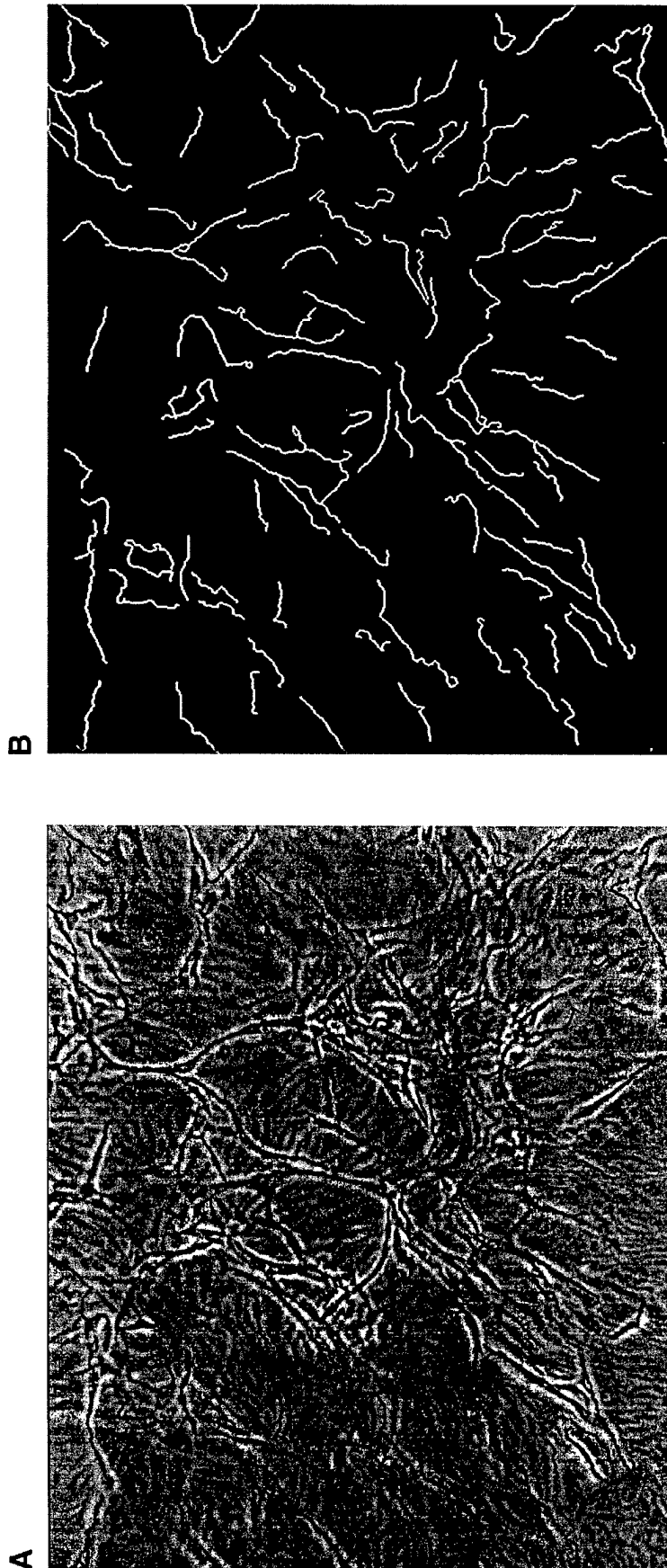
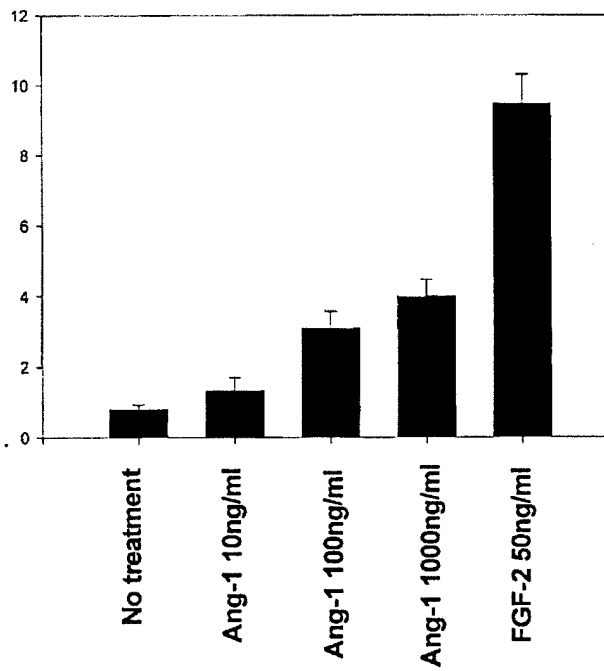


FIG. 2. Quantification of Ang-1 induced tubule formation. A and B illustrate the quantification of the total length of tubules formed in a gel by a computer-assisted image analysis system. C. Transmission electron micrograph demonstrating the tubular phenotype on cross-sectional analysis of a capillary like structure induced by FGF-2, with ABAE cells surrounding a primitive lumen (magnification  $\times 6500$ ). D. The total length of Ang-1-induced tubule formation is dose dependent. E. Inhibition of Ang-1-induced tubule formation by an excess of soluble Tie-2 extra-cellular domain (error bars = SEM).

C



D  
Tubule length (mm/mm<sup>2</sup>gel)



E  
Tubule length (mm/mm<sup>2</sup>gel)

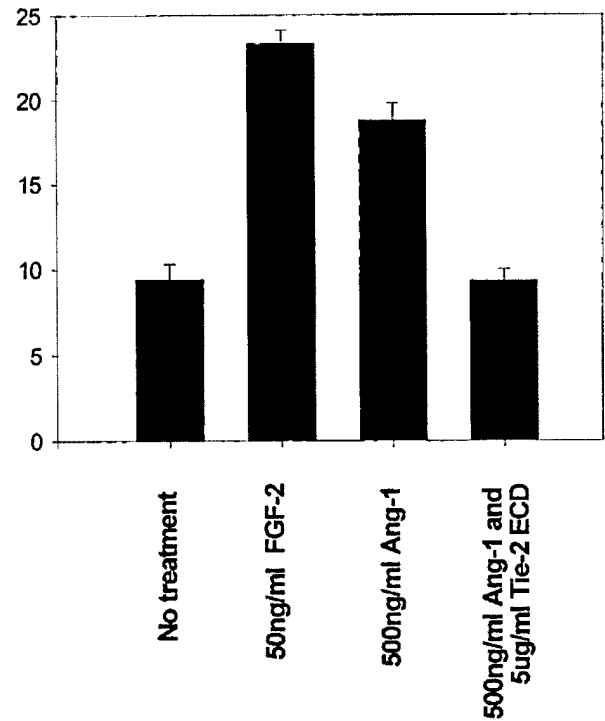


FIG. 2—Continued

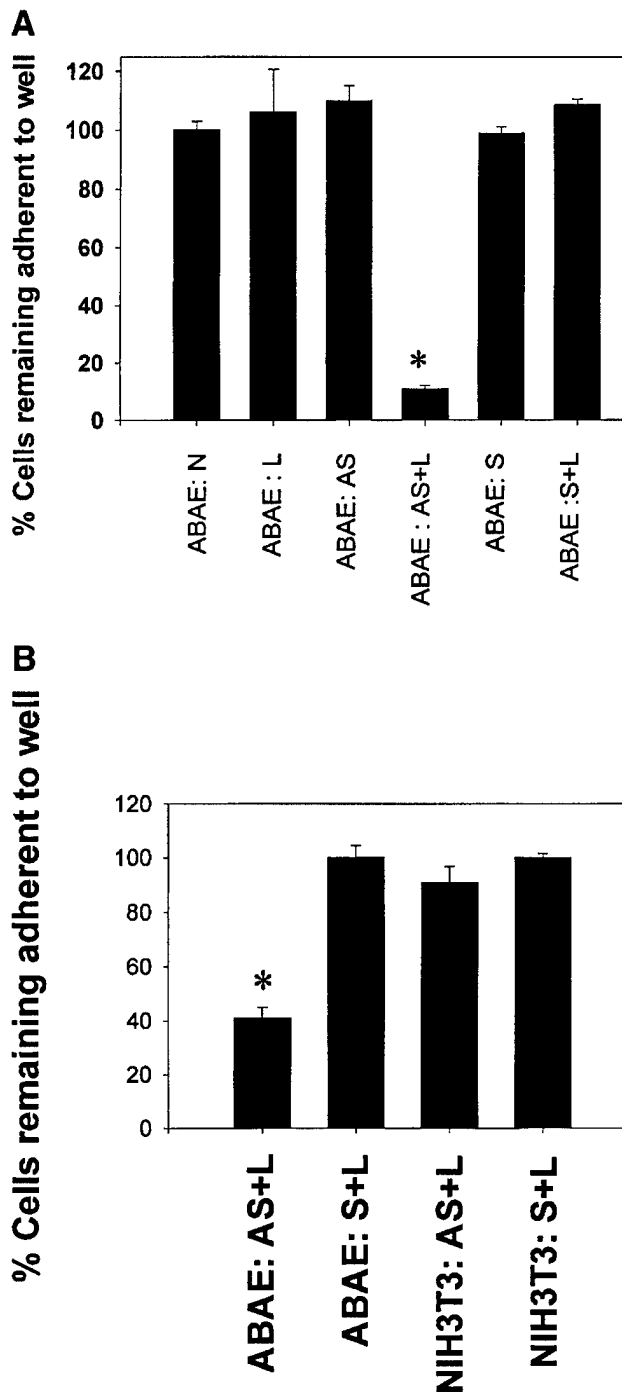


FIG. 3. Detachment of confluent endothelial cells caused by Tie-2 antisense oligonucleotide. A. Effect of 150 nM Tie-2 antisense on ABAE cell adhesion (AS, antisense; S, sense; L, lipid). Quantification of cell death was carried out by trypsinization and counting cells on a Coulter counter or by measuring total protein concentration of the cells that remained attached after treatment. Results are expressed as a percentage of values for untreated cells (error bars = SD). Only treatment with antisense oligonucleotide in the presence of lipid resulted in cell death (\* = Student's *t* test,  $P < 0.001$ ). B. 300 nM Tie-2 antisense had no significant effect on NIH 3T3 cells while resulting

tubules induced by Ang-1 (1000 ng/ml) is about 50% of that induced by FGF-2 (50 ng/ml). The tubule-forming effect can be entirely abrogated by the addition of an excess of soluble Tie-2 extracellular domain that functions effectively as a blocking antibody (Figs. 1D and 2E). To validate the accuracy of the image analysis system as a means of quantitating the extent of capillary-like tubule formation, we also assessed the tubule formation induced by FGF-2 and Ang-1 in Fig. 2D manually. The same 10 randomly selected images per data point were ranked, in a blinded fashion, from 1 to 10 for extent of tubule formation compared with a panel of 10 standard images by three independent observers. The extent of tubule formation by Ang-1 at the  $IC_{50}$  of 100ng/ml was within 6% of that scored by the image analysis system when normalized to the positive control (data not shown).

**Elimination of Tie-2 by incorporation of a Tie-2 antisense oligonucleotide into confluent endothelial cells results in cell death.** To find out whether functional Tie-2 was necessary for the survival of confluent endothelial cells we eliminated Tie-2 protein by transfecting confluent endothelial cells with an antisense oligonucleotide against Tie-2 mRNA. An antisense phosphorothioate oligodeoxynucleotide complementary to the start codon region of the Tie-2 mRNA was constructed, previous studies having demonstrated this site as likely to result in successful hybridization (Stein and Cheng, 1993). This oligonucleotide was introduced into cultured ABAE cells by mixing with a lipid transfection reagent. A mixture of the lipids and the oligonucleotide at a final concentration of 150 nM oligonucleotide caused death of about 90% of the confluent ABAE cells, as determined by the number of cells that remained attached to the culture dish and the amount of total protein in the cell lysate of cells that remained attached. Neither the lipids nor the oligonucleotide alone had any adverse effect on the cells. Additionally a corresponding sense

in endothelial cell death. Results are expressed as a percentage of sense-treated control (error bars = SD, \* = Student's *t* test,  $P < 0.001$ ). C. Cell death in response to decreasing doses of antisense oligonucleotide demonstrating a dose dependent effect (error bars = SD). D. Western blotting analysis for Tie-2 protein prepared from cells treated in B confirming a dose-dependent loss of Tie-2 protein that correlates to cell death.

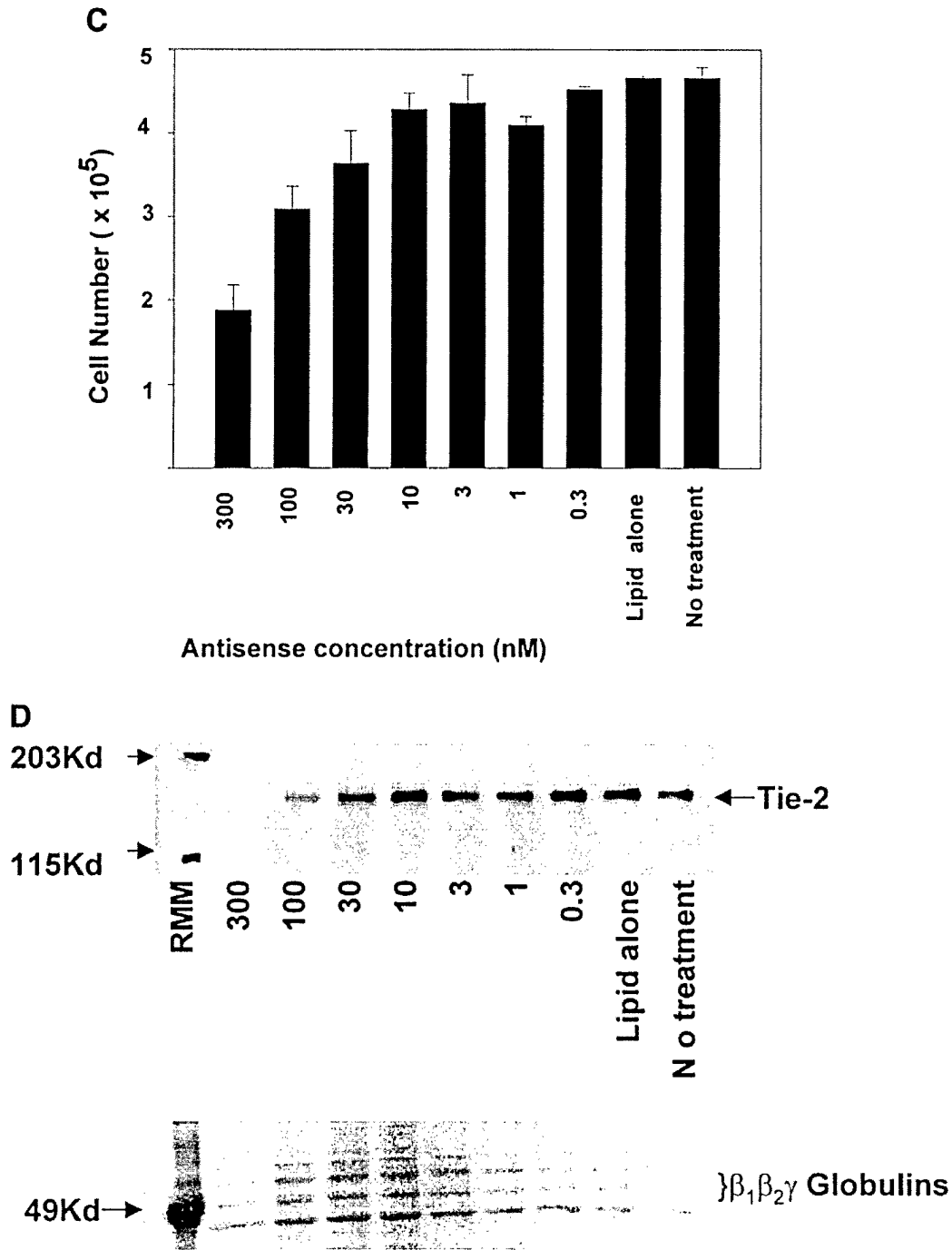


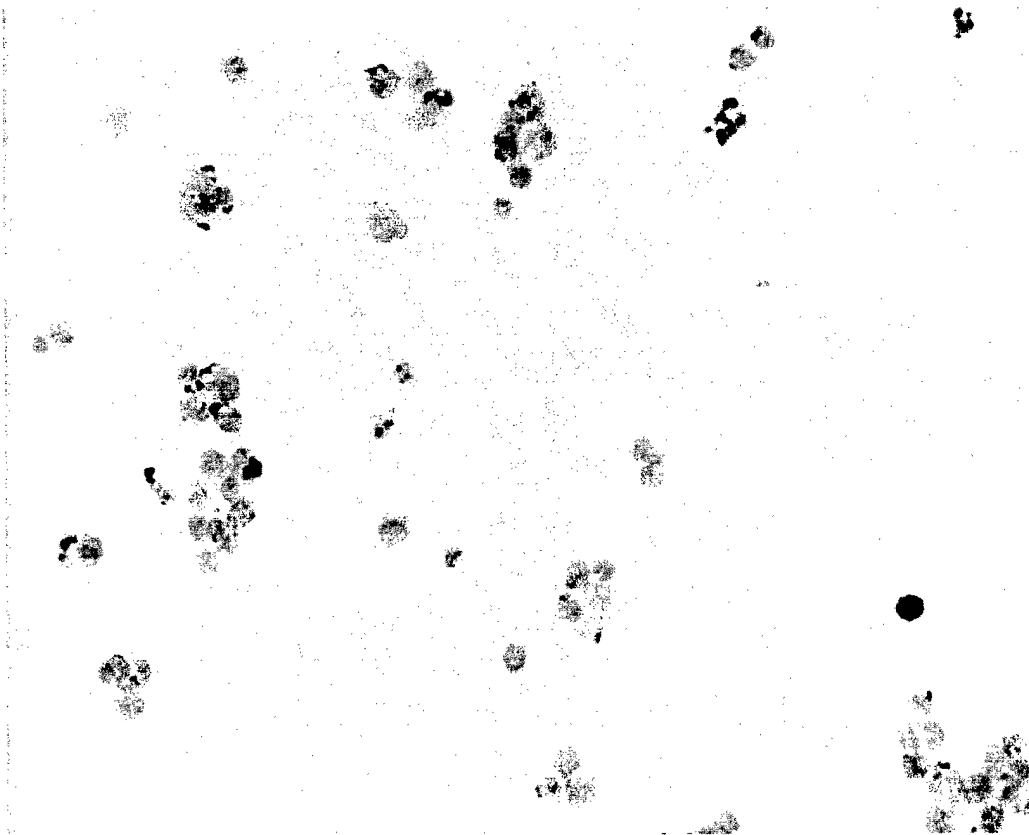
FIG. 3—Continued

oligonucleotide had no effect on ABAE attachment. A fibroblast cell line (NIH 3T3) that does not express Tie-2 was also not affected by the antisense oligonucleotide treatment (Fig. 3B). We analyzed both cell detachment and Tie-2 protein expression in response to various concentrations of antisense oligonucleotide and could demonstrate dose-dependent cell detachment and a corre-

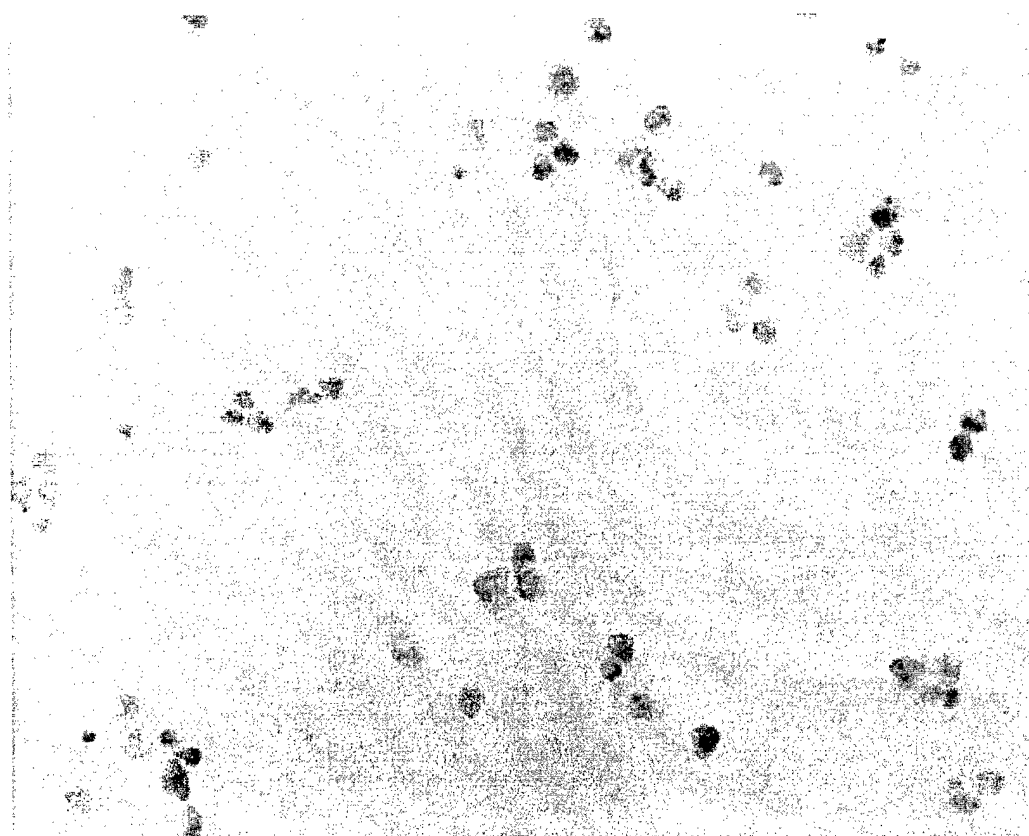
sponding dose-dependent loss of Tie-2 protein expression (Figs. 3C and 3D). Equal loading of protein was confirmed by staining total protein with Ponceau S (Sigma) to visualize β<sub>1</sub>β<sub>2</sub> and γ globulins.

**Treatment with antisense oligonucleotide to Tie-2 results in apoptosis.** We then assessed the rate of apoptosis in ABAE cells treated by antisense or sense

A



B



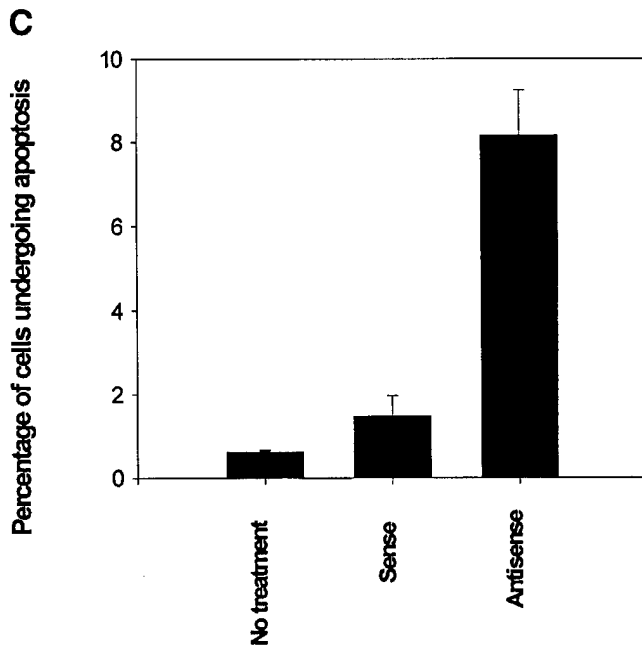


FIG. 4—Continued

oligonucleotides by *in situ* end labeling. ABAE cells were treated with oligonucleotide on two occasions for 4 h each in serum-free medium, and then returned to normal medium for a further 20-h incubation. At the end of the total 48-h incubation period, remaining adherent cells were collected and pooled with cells that had already detached. These cells were stained by ISEL for fragmented DNA. Intact DNA does not incorporate any labeled nucleotides, whereas fragmented DNA, as occurs in apoptosis, incorporates biotinylated nucleotides. This fragmented DNA can be visualized as darkly staining discreet apoptotic bodies and these morphological appearances allow distinction between cells undergoing apoptosis and simple necrosis, where the staining is amorphous. Controls were in the form of treatment with the sense oligonucleotide or changes of medium to serum-free medium that contained no oligonucleotide. Figures 4A and 4B demonstrate the appearance of ABAE cells staining positively for fragmented DNA with numerous purple apoptotic bodies which are not seen with

the sense control. Figure 4C shows results of a representative experiment in which the rate of apoptosis is increased sixfold or greater over controls. The rates of apoptosis identified using this technique were very similar to those seen after 20 h of treatment with 3  $\mu\text{g}/\text{ml}$  cycloheximide, a protein synthesis inhibitor that effectively induces apoptosis in endothelial cells (data not shown).

## DISCUSSION

Developmental angiogenesis is a coordinated process requiring integrated signaling through a number of ligand-activated receptor tyrosine kinases that are specifically expressed on endothelial cells in a temporally coordinated manner (Dumont *et al.*, 1995). The functions of the Tie-2 /Ang-1 signaling pathway in this process, as suggested by the phenotypes of the transgenic mice, include maintenance of the endothelial cell population and vascular expansion by vessel branching, intussusceptive vessel division, and pericyte recruitment (Dumont *et al.*, 1994; Sato *et al.*, 1995; Suri *et al.*, 1996, 1998; Patan, 1998).

We have presented evidence at a cellular level that supports these putative roles for the Tie-2 signaling pathway both in vascular expansion and in endothelial cell survival. Our data from the collagen gel assays suggest an active role in Tie-2 signaling in tubule formation. The phenotypic analysis of both Ang-1 and Tie-2 knockout animals shows a decrease in the amount and complexity of capillary branches while overexpression of Ang-1 increases the number and branching complexity of vessels, suggesting that Tie-2 signaling is necessary for the expansion of primitive tubules. *In vitro*, Ang-1 is able to induce migration of ABAE cells (Witzenbichler *et al.*, 1998) and both Ang-1 and Ang-2 appear to be able to synergize with VEGF to induce angiogenesis in a mouse

FIG. 4. Tie-2 antisense oligonucleotide treatment causes apoptosis in endothelial cells. A and B. ISEL staining for apoptotic bodies in cells treated with antisense (A) and sense (B) oligonucleotides. Note the darkly staining purple peripheral apoptotic bodies representing condensations of fragmented chromatin. C. Quantification of apoptosis. C represents the percentage of apoptotic cells as identified by ISEL staining from 1000 cells counted by a "blinded" observer (error bars = SEM).

corneal pocket assay (Asahara *et al.*, 1998). The collagen gel assay that we utilized exhibits many of the elemental endothelial cell activities during angiogenesis. The endothelial cells migrate from a confluent monolayer into a substratum and undergo proliferation, migration associated with proteolytic degradation of collagen, and ultimately organization into tubular structures that possess lumens (Maciag *et al.*, 1982; Feder *et al.*, 1983; Montesano and Orci, 1985; Montesano and Orci, 1987; Goto *et al.*, 1993). Soluble Ang-1 induces approximately 50% of the tubule-like structures that are induced by FGF-2. This effect can be entirely abolished by the addition of an excess of soluble Tie-2 extracellular domain. This evidence is in keeping with existing evidence for a dynamic role for Ang-1 in angiogenesis, particularly in the more complex morphological transitions that are involved in the expansion of an existing vascular system that may not involve mitogenesis. Such angiogenic processes may include capillary sprouting, which is known to occur under conditions where endothelial cell mitogenesis has been prevented (Sholley *et al.*, 1984), and the expansion of preexistent collateral vasculature as is seen in response to ischemia. Both of these processes can be induced by Ang-1 (Koblizek *et al.*, 1998; Shyu *et al.*, 1998).

The hypothesis that Tie-2 signaling is a necessary endothelial cell survival factor is suggested by the phenotype of Tie-2 knockout mice and also from the observation that Tie-2 phosphorylation results in downstream activation of protein kinase B/Akt, a protein that is central to cellular antiapoptotic signaling pathways (Kontos *et al.*, 1998). Elimination of Tie-2 protein expression in confluent endothelial cells with an anti-Tie-2 antisense oligonucleotide is extremely detrimental to the cells and this effect is specific to endothelial cells, as no effect was observed in cells that do not express Tie-2. The most striking characteristic of this treatment is the almost complete cellular detachment induced by antisense treatment. Subsequent endothelial cell apoptosis may be a direct consequence of the loss of attachment, a phenomenon referred to as "anoikis." The ultrastructural abnormalities in transgenic animals that are deficient for Ang-1 do suggest a role for Tie-2 signaling in endothelial cell attachment to adjacent cellular structures or basement membranes (Suri *et al.*, 1996), but as yet the mechanism by which such a cellular effect is mediated remains unclear. Our antisense data, where elimination of

Tie-2 protein results both in endothelial cell detachment from the substratum and in apoptosis, lends support to the hypothesis that this pathway is necessary for the stable association between the endothelial cell and surrounding structures.

In summary we have presented *in vitro* evidence that is highly supportive of a central physiological role for the Ang-1/Tie-2 signaling pathway in the expansion of the endothelial cell population into a network of capillary tubules and also in the survival and stabilization of the quiescent endothelial monolayer. Further investigation will be directed toward the elucidation of Ang-1/Tie-2 signaling pathways involved in these two seemingly separate functions. This elucidation of the physiological role for the Tie-2 signaling pathway further emphasizes potential therapeutic avenues in a number of diseases manifesting altered angiogenesis, either by preventing signaling as an attempt to prevent pathological angiogenesis as seen in tumors or by augmenting signaling as an approach to ischemia (Baumgartner *et al.*, 1998; Lin *et al.*, 1998).

## ACKNOWLEDGMENTS

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Expression and Function of Angiopoietin-1 in Breast Cancer

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**ABSTRACT**

Angiopoietin-1 (Ang1) has been shown to act as an angiogenic promoter in embryonic angiogenesis by promoting vascular branching, pericyte recruitment and endothelial survival. We have investigated the role of Ang1 in tumour neo-vascularisation under clinical conditions and in animal models. The expression of Ang1 in clinical breast cancer specimens was analysed by using laser capture microdissection and reverse transcriptase-linked polymerase chain reaction (RT-PCR) on RNA isolated from the samples. Despite the expression of Ang1 in many human breast cancer cell lines, the gene was expressed in only 3 out of 21 breast cancer clinical specimens, even though its receptor, Tie2, is abundant in the vasculature of all of these tumours. Ang1 was then over-expressed in a human breast cancer cell line (MCF-7) on its own and in conjunction with FGF-1, an angiogenic factor shown to be able to increase the tumourigenicity of MCF-7 cells. High concentrations of Ang1 were produced in the conditioned media of the transfected cells (range 156-820 ng.ml<sup>-1</sup>). However, in contrast to its physiological role as promoter of angiogenesis, overexpression of Ang1 did not enhance tumour growth, but instead caused up to a 3-fold retardation of tumour growth ( $p = 0.003$ ).

**KEYWORDS:** angiogenesis, angiopoietin, neo-vascularisation, breast cancer, gene expression, tumourigenesis

## INTRODUCTION

Tumours undergo a critical transition from an avascular to a vascular stage by invoking an angiogenic response from host vasculature (Folkman, 1971). To do this the cancer cells elaborate growth factors and cytokines that act upon the surrounding endothelial cells (Hanahan & Folkman, 1996) which induce vascular sprouting and new blood vessel formation. Certain of these factors, such as fibroblast growth factor-1 (FGF1), may have both paracrine and autocrine effects on tumour growth because of the distribution of their receptors on tumour cells, other stromal cells, as well as endothelial cells (Lehtola *et al.*, 1992). Other factors such as vascular endothelial cell growth factor (VEGF), target only endothelial cells because the expression of their receptors is restricted to endothelial cells (de Vries *et al.*, 1992; Terman *et al.*, 1992).

Angiopoietin-1 (Ang1) is a recently described growth factor whose target cells are also endothelial cells, because the expression of the receptor, Tie2 (Tek), is restricted essentially to endothelial cells (Dumont *et al.*, 1993; Maisonpierre *et al.*, 1993). Transgenic animal studies indicate that Ang1 is central to embryonic vascular development (Maisonpierre *et al.*, 1997; Sato *et al.*, 1995; Suri *et al.*, 1996; Suri *et al.*, 1998; Thurston *et al.*, 1999). Ang1 knock-out animals die *in utero* because of specific vascular deficits. These include failure of the primitive capillary plexus to branch appropriately and failure of the endothelial cells to form stable associations with surrounding basement membranes and pericytes. Knock-out of the Tie2 gene gave rise to essentially the same phenotype. Overexpression of Ang1 in the skin of transgenic animals causes an increase in the number, size and branching complexity of dermal vessels. *In*

*vitro*, Ang1 protects against endothelial cell apoptosis, stabilises endothelial tubules and induces vascular sprouting and tubule formation in collagen (Hayes *et al.*, 1999; Holash *et al.*, 1999; Koblizek *et al.*, 1998; Papapetropoulos *et al.*, 1999; Thurston *et al.*, 1999). *In vivo* Ang1 synergises with VEGF to promote corneal neo-vascularisation (Asahara *et al.*, 1998). Together these findings all suggest that Ang1 acts physiologically as an angiogenic promoter in the developing vascular system, in a co-ordinated process involving other angiogenic factors such as VEGF (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Shalaby *et al.*, 1995).

We investigated whether Ang1 would serve an analogous function in tumours by promoting vascularisation. Ang1 expression levels were assessed in breast cancer cell lines and in breast cancer specimens. The gene was then over-expressed in a breast cancer cell line (MCF-7) by itself or together with FGF1. Our data indicate that Ang1 is rarely expressed by breast cancer cells in clinical samples. Moreover, Ang1 not only fails to enhance xenograft tumour growth in the MCF-7 tumour model, but appears to act in inhibitory capacity in this model. Possible reasons for the apparently contradictory roles for Ang1 in physiological and tumour angiogenesis are discussed.

## METHODS

Cell Culture: The MCPX cell line is a sub-line of ML-20 cells which were derived from MCF-7 cells by transfection with  $\beta$ -galactosidase (*lacZ*). (Kurebayashi *et al.*, 1993; McLeskey *et al.*, 1993; McLeskey *et al.*, 1998). The  $\alpha$ 18 cells, referred to as Clone 18 previously, were derived from ML-20 cells by transfection with FGF1 (Zhang *et al.*, 1997). Cell lines were cultured in 5% FBS/ IMEM/ 4 mM glutamine (Biofluids, Rockville, MA).

Immuno-histochemistry for Tie2 and vWF. Frozen sections (5  $\mu$ m) were immuno-stained with monoclonal antibodies to Tie2 (a gift from Dr. Kevin Peters, Procter and Gamble, Cincinnati, OH), and to von Willebrand Factor (Boehringer Mannheim, Indianapolis, IN). Sections were fixed in acetone at -20°C for 10 minutes, incubated in 0.3% hydrogen peroxide in methanol, blocked in 5% BSA (Sigma, Milwaukee, WI) 2.5% normal horse serum (Vector Labs, Burlingame, CA) in PBS then incubated with antibody in blocking solution. Sections were washed in PBS, then incubated for 1 hour with a 1:5000 dilution of a biotinylated polyclonal antibody against mouse IgG and developed using a Vector ABC kit (Vector Labs), then counter-staining with haematoxylin.

Laser capture micro-dissection: Frozen tumour samples and adjacent normal breast tissue were used for laser capture microdissection using previously described methods (Emmert-Buck *et al.*, 1996), utilising a Pixcell laser capture microdissection system (Arcturus Engineering, Mountain View, CA). Clinical sections, immuno-stained for Tie2 and vWF, were examined to identify areas of high microvessel density. Microvessel counting was performed using standard techniques (Weidner, 1995). Un-mounted serial

sections stained with eosin, or with hematoxylin and eosin, were then examined to locate parallel areas. A Capsure Cap (Arcturus Engineering) was then placed onto the section over a 30  $\mu\text{m}$  diameter capture area comprising either tumour cells in areas of high microvessel density, or epithelial cells in normal breast acini. Upon laser capture, cells in 50 areas were transferred into a 0.5 ml Eppendorf vial, yielding between 500 and 1000 cells per sample. Total RNA was retrieved using a Micro RNA Isolation kit (Stratagene, La Jolla, CA) and RNA corresponding to that collected from approximately 250 cells was used as a template for subsequent RT-PCR.

Reverse Transcriptase-linked Polymerase Chain Reaction (RT-PCR): The primer sequences for Ang1 are: 5'-CAACTGGAGC TGATGGACAC A-3' (sense) and 5'-ACTGCCTCTG ACTGGTAATG G-3' (antisense) and span base pairs 1060 to 1420 of Ang1 mRNA. The primer sequences for VEGF are 5'-GCCTTGCCTT GCTGCTCTAC-3' (sense) and 5'-AATGCTTTCT CCGCTCTGA-3' (antisense) spanning base pairs 48 to 473. The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are 5'-AAGGTGAAGG TCGGAGTCAA CG-3' (sense) and 5'-TGGTGGTGCA GGAGGCATTG C-3' (antisense) spanning base pairs 40 to 496. The GAPDH primers span introns 1-5 in the genomic sequence. Total RNA collected from a panel of breast cancer cell lines was supplied by Dr Anke Schultke, Lombardi Cancer Center and 0.1  $\mu\text{g}$  was used as a template. RT-PCR analysis was performed using reagents supplied in a GeneAmp RNA PCR core kit (Perkin Elmer, Wellesley, MA), with a Perkin Elmer GeneAmp 2400 PCR system. The DNA was transferred onto a Nytran nylon membrane (Schleicher & Schuell, Keene, NH). Southern hybridisation was performed using oligonucleotide nested primers end-labelled with  $\gamma$ - $^{32}\text{P}$ -dATP (Amersham, Piscataway,

NJ). The sequences of the nested primers were: for Ang1, 5'-AGACTGTGCA TGTATATC-3'; for VEGF, 5'-CAATGACGAG GGCCTGGAGT-3'; and for GAPDH, 5'-GTCTTCACCA CCATGGAGAA-3'.

Transfection of the cancer cells with Ang1: The plasmid, jFE14/Ang1 is derived from the pSR $\alpha$  plasmid which contains an HTLV-1 promoter (Takebe *et al.*, 1988). Full length human Ang1 cDNA was cloned into this plasmid at two BstX 1 sites. Co-transfection was performed with the pcDNA3.1 Zeo plasmid (Invitrogen) which confers resistance to the antibiotic Zeocin (250 mg/ml), using Lipofectamine Plus reagent (GibcoBRL, Rockville, MD).

Western slot blot analysis: Conditioned media were mixed (1:1) with DPBS containing 0.01% CHAPS (Sigma) and loaded in duplicate onto a Bio-dot SF slot blot apparatus (Bio-Rad, Hercules, CA), with Hybond ECL nitrocellulose membrane. After blocking, the membrane was incubated a rabbit polyclonal antibody against Ang1, developed at Regeneron Pharmaceuticals (Tarrytown, NY), in TBS, 0.1% Tween-20, 1.25% BSA, washed, then incubated with a horseradish peroxidase-conjugated anti-rabbit IgG antibody and developed using ECL chemiluminescence (Amersham)

Tie2 phosphorylation assay: Tie2-transfected fibroblasts were treated for 5 minutes with conditioned media then immunoprecipitated for phosphorylated Tie-2 as described previously (Maisonpierre *et al.*, 1997). Cells were lysed in RIPA buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS plus protease- and phosphatase-inhibitors. Tie2 was immunoprecipitated using an anti-myc monoclonal antibody, 9E10 (Sigma) and protein G-Sepharose beads. Samples were analysed by standard Western blotting protocols, including resolution by reducing SDS-PAGE and electrotransfer to

PVDF membranes. The amount of total Tie2 receptor and auto-phosphorylated Tie2 receptor was detected, respectively, by incubating replicate membranes with 9E10 or with the anti-phosphotyrosine-specific monoclonal antibody, 4G10 (Upstate Biotechnology, Lake Placid, NY) visualised with a HRP-conjugated secondary antibody.

BIAcore Analysis: Conditioned media were analysed for Ang1 with a BIAcore biosensor (Pharmacia, Piscataway, NJ) utilising a CM5 BIAcore chip surface that had been covalently coupled with recombinant soluble Tie2-Fc as described previously (Davis *et al.*, 1996). To control for the specificity of ligand binding, media were pre-incubated with 20 ug/ml of excess soluble Tie2-Fc or two irrelevant soluble receptors. Only soluble Tie2-Fc competed for Ang1 binding to the chip surface.

Northern blotting analysis for FGF1 expression: An FGF1 cDNA probe was labelled with  $\alpha^{32}\text{P}$ -dCTP (Amersham) using a Random-Primers DNA labelling system (GibcoBRL) and used in Northern analyses of total RNA as described previously (Zhang *et al.*, 1997).

Tumourigenicity assays: NCR (nu/nu) athymic female 4-6 week old mice (Taconic Farms Inc., Germantown, NY) were supplemented with subcutaneously embedded 0.72 mg oestrogen pellets then inoculated subcutaneously in the mammary fat pads with  $5 \times 10^6$  cells in 0.2 ml IMEM. Tumour volumes (length x width x height) were measured two times a week in a blinded manner.

Statistical analyses: Generalised linear mixed effects models were used to estimate tumour growth rates. The analysis was performed using SAS PROC MIXED procedure according to SAS/STAT User's Guide (SAS Institute Inc., Cary, NC). Plots of tumour sizes versus time for the MPCX tumour growth data revealed an exponential growth pattern. Plots of tumour size versus time for the  $\alpha 18$  tumour growth data indicated the

growth rate in some cell lines was negative to some time points and then became positive, suggesting that an overall growth rate was not appropriate to summarise the growth pattern. For these data, we described the growth pattern for each cell line at certain time points and compared the growth rates at these time points using repeated analysis of variance.

## RESULTS

*Ang1 expression in breast cancer cell lines:* We analysed Ang1 expression in a panel of 19 breast cancer cell lines by RT-PCR analysis (Fig. 1). Ang1 transfected CHO cells were used as a positive control. PCR products were subjected to agarose gel electrophoresis and visualised with ethidium bromide. To exclude non-specific PCR amplifications, the PCR products were then subjected to Southern blotting analysis with a radio-labelled “nested” Ang1 primer that corresponded to a sequence of Ang1 cDNA between PCR primers. A positive signal for Ang1 was identified from RNA isolated from 9 out of the 19 breast cancer cell lines.

*Low Ang1 expression in human breast cancer clinical specimens:* We then determined whether breast cancer cells express Ang1 under clinical conditions. We utilised laser capture microdissection and subsequent RT-PCR on the RNA isolated from microdissected specimens for this analysis. This technique permitted the isolation of a relatively homogeneous population of cancer cells adjacent to tumour vessels, which expressed a high level of the receptor Tie2 (Fig. 2). It also involved amplification of the mRNA signal, as our previous efforts to determine Ang1 expression with *in situ* hybridisation experiments did not reveal any Ang1 signals in breast cancer specimens despite ample signals in control samples of Ang1-transfected cancer cells (data not shown). After reverse transcription of the mRNA from the microdissected cells, the cDNA samples were analysed for Ang1 by Southern blotting with a “nested” oligonucleotide probe. VEGF mRNA, which is abundant in tumours, was analysed as a

positive control. Figure 3 shows the results of an analysis of six tumour specimens. Control cells that expressed Ang1 display a clear signal for Ang1 which is approximately equal to that seen for GAPDH, an internal control, irrespective of whether the RNA was collected directly from cells growing in tissue culture or from frozen sections of cell pellets. In the majority of microdissection experiments no Ang1 signal was seen even after prolonged exposure, while the GAPDH signal was clearly demonstrated (Fig. 3A). In contrast, similar analysis of VEGF expression in the same six tumour samples yielded a strong signal in 5 out of 6 cases, confirming the abundance of this angiogenic factor in tumours and the suitability of microdissection as a method of analysis (Fig. 3B).

Table 1 shows a summary of the results of Tie2 and Ang1 expression analysis of 11 normal and 23 malignant specimens. Samples were informative for Ang1 expression only if a GAPDH signal was visible after Southern hybridisation. Although Tie2 is clearly expressed on microvessels of tumours and correlates closely to the expression of vWF (correlation coefficient 0.91), a detectable Ang1 signal was identified only in 3 out of 21 tumour cases analysed and none of 9 normal cases.

*Transfection of Ang1 cDNA into breast cancer cell lines results in the expression of high levels of biologically active Ang1 in conditioned media:* In order to determine the effect of elevated Ang1 levels on tumour growth, we stably transfected Ang1 into two cell lines derived from the oestrogen dependent, poorly angiogenic and weakly tumourigenic MCF-7 cells: the MPCX cells and the  $\alpha$ 18 cells. The MPCX cells, which were transfected with the  $\beta$ -galactosidase gene (*lacZ*) for convenient detection, maintains essentially the same growth characteristics to the MCF-7 cells, while the  $\alpha$ 18 cells, which

were further transfected with FGF1, exhibited a greatly increased tumourigenicity and an extreme form of the dysfunctional vascular phenotype seen in xenograft tumours, with abundant vessels that are dilated (Zhang *et al.*, 1997).

Ang1 protein in the conditioned media of the transfected cells was detected by Western slot blot analysis using a polyclonal antibody against human Ang1 (Fig. 4A). An estimation of the amount of Ang1 was made by comparison with signals from an Ang1 preparation of known concentration. The ability of the Ang1 protein to bind to Tie2 was determined by using a BIAcore analyser, which also allowed a quantitative determination of the concentration of Ang1 (Table 2). The Ang1 concentrations determined by these methods correlated closely. Furthermore, the biological activity of Ang1 in the conditioned media was determined by measuring the ability of the conditioned media to induce Tie2 tyrosine phosphorylation (Fig. 4B). The extent of phosphorylation induced by conditioned media from the highest expressing clones was equivalent to that produced by 200 ng of recombinant Ang1, which gave rise to a maximum extent of phosphorylation of Tie2 on the cells. The  $\alpha$ 18-derived cell lines were further analysed for continued expression of FGF1 mRNA by Northern analysis (Fig. 4C). All of the FGF1 transfected cell lines expressed high levels and equivalent amounts of FGF1 mRNA. *In vitro* mitogenesis assays were performed on all transfected and parental cell lines prior to animal inoculation to ensure that the transfection procedure or the expression of Ang1 had not altered the *in vitro* growth characteristics (data not shown). As expected, Ang1 over-expression had no effect on the growth rates of all the selected clones in cultures since MCF-7 cells do not express the Tie2 receptor.

*Inhibition of MCF-7 xenograft tumour growth by Ang1 over-expression:* Three Ang1 expressing clones, as well as a pooled population of empty vector transfected cells and the parental cells, were inoculated into the mammary fat pads of athymic nude mice. The growth rates of the xenograft tumours were monitored. The tumours were retrieved at the end of the experiment and the expression of Ang1 confirmed by Northern blotting analysis (data not shown). Although Ang1 over-expressing MPCX cells were able to grow xenograft tumours, the growth rates of the transfected cells were decreased as compared with the parental or vector control. A dramatic inhibitory effect ( $p=0.003$ ) was observed with the clone MAng 184 that expressed the most Ang1 (Fig. 5A). The extent of rate decrease correlated reasonably well with the amount of Ang1 produced by the transfected cells (Fig. 5B and Table 2).

Similar experiments were carried out with the FGF1 and Ang1 co-transfected  $\alpha 18$  cell lines. The growth of the xenograft tumours of the Ang1 over-expressing cells was again found to be much slower than that of the parental cells and the vector mock transfected cells (Fig. 6A). A statistically significant inhibition of tumour growth was observed with clones  $\alpha$ Ang 18 and  $\alpha$ Ang 29 ( $p=0.03$ ). The dimensions of the tumours produced by clone  $\alpha$ Ang 14 were not statistically different from the parental or vector controls. However for clone  $\alpha$ Ang 14 the recorded tumour volume did not represent the actual volume of tumour cells. This is because the majority of the volume of the xenografts formed by this clone at the end of the assay was caused by blood in a haemangectasic sac. In contrast, while the parental and vector cells produced a haemangectasic sac initially, this was replaced by a solid mass of tumour cells as the tumour growth progressed (Zhang *et al.*, 1997). We demonstrated this at the end of the

experiment by staining all of the xenografts with a  $\beta$ -galactosidase substrate. This allowed for easy identification of the tumour as blue cells (Fig. 6B). A solid mass of cancer cells had replaced the haemangectasic sac in the tumours produced by the parental  $\alpha$ 18 cells (Fig. 6B, right). In contrast, the haemangectasic sac responsible for the large dimensions of the  $\alpha$ Ang 14 tumours was not replaced by a tumour mass at the end of the assay still but consisted principally of blood (Fig. 6B, left). On transection, it was demonstrated that the cancer cells occupied a relatively small proportion of the tumour volume (Fig. 6B, centre) implying that the rate of tumour cell growth was very much smaller than that recorded by measurement of xenograft dimensions.

## DISCUSSION

We tested the hypothesis whether Ang1 would serve a pro-angiogenic role in the context of tumours, similar to its role suggested for physiological neovascularisation. We found that Ang1 expression is lacking from areas adjacent to tumour blood vessels in human breast cancer despite abundant expression of Tie2 in these vessels. In addition, over-expression of Ang1 did not enhance xenograft growth and the majority of Ang1 transfected clones demonstrated decreased growth rates. Our data demonstrate that Ang1 may not function as a promoter of angiogenesis in breast tumours. Moreover the data suggests that it may act in an inhibitory capacity in this model.

These findings initially appears to be in contrast to transgenic studies in which Ang1 was over-expressed locally in the skin of developing mice. This over-expression of Ang1 induced remarkable increases in vascularity in the skin of these transgenic animals (Thurston, 1999). These seemingly contrary findings for Ang1 over-expression in transgenic animals and in our tumour models suggest that the process of tumour vascularisation may be dissimilar to that seen in physiological settings. Physiological angiogenesis occurs in the context of a number of angiogenic factors and the expression of these factors is precisely co-ordinated both temporally and spatially (Dumont *et al.*, 1995). Tumours elaborate a variety of angiogenic factors (Relf *et al.*, 1997) and as a consequence of the abundance of angiogenic influences in the tumour micro-environment, the microvessel density in a tumour may be very high although many vessels are dysfunctional. This dysfunction is manifested by areas of tumour necrosis and

tumour hypoxia seen near to areas of increased microvessel density. The failure to identify Ang1 expression in the breast cancer epithelial cells suggests that Ang1, which is hypothesised to promote the ordered expansion of the vascular tree physiologically, may not be a pertinent angiogenic factor to the highly disordered tumour vasculature.

The data from overexpression of Ang1 in a xenograft model suggest that Ang1 may inhibit tumour growth, presumably via an effect on tumour angiogenesis, as Ang1 had no effect on the *in vitro* growth of the tumour cells. This is in keeping with the hypothesised roles of Ang1 and its functional antagonist Ang2 (Maisonpierre *et al.*, 1997) on vessel stability and receptivity to other angiogenic influences. Ang1 stabilises the association between the endothelial cell and pericyte (Suri *et al.*, 1996; Suri *et al.*, 1998; Thurston *et al.*, 1999). Ang2 by antagonising this effect can result in vessel disassembly and subsequent vessel regression, but may, in the presence of other suitable angiogenic factors, facilitate new vessel sprouting (Maisonpierre *et al.*, 1997). It has recently been observed that Ang2 is focally up-regulated in the immediate vicinity of tumour vessels (Stratmann *et al.*, 1998; Zagzag *et al.*, 1999). Therefore in tumours, where a variety of other angiogenic factors exist, this stabilising effect of Ang1 might in fact inhibit the intense continuous new vessel sprouting that is typical of tumour vascularisation. That Ang1 is able to inhibit tumour growth even in the presence of FGF1 supports the view that Ang1 retards tumour growth by vascular stabilisation. Additionally, our findings that Ang1 is expressed in many breast cancer cell lines *in vitro*, but in very few clinical specimens, suggest that expression of Ang1 may be down-regulated in tumours because of its negative selective effect on the developing tumour.

To identify the mechanism by which Ang1 may lead to retardation of tumour growth requires a variety of further experimental approaches. These may include a detailed histological characterisation of vessel branching and endothelial pericyte relations in the Ang1 overexpressing tumours and a direct *in situ* assessment of cellular proliferation rates within the xenografts expressing Ang1. It will be informative to assess the expression patterns of other ligands to Tie-2 in clinical specimens, in particular Ang-2. Such studies will further elucidate the role played by this complex vascular signalling pathway in the process of tumour vascularisation.

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## FIGURE LEGENDS

Figure 1. RT-PCR analysis of breast cancer cell lines for Ang1 mRNA. Positive and negative control cells were CHO cells transfected with Ang1 (CHO Ang1) or an empty vector (CHO Vector). The lower two panels show RT-PCR products after agarose gel electrophoresis, visualised by ethidium bromide staining. The top row is a Southern hybridisation of the Ang1 RT-PCR products with a  $^{32}\text{P}$ -labelled nested primer.

Figure 2. Human breast cancer specimens before and after laser capture microdissection. (A) A frozen section of ductal carcinoma was immuno-stained with a monoclonal antibody against Tie2: brown staining (arrow) indicates blood vessels that express Tie-2. (B) A serial section (eosin staining) showing the same ductule filled with breast cancer cells in which the cancer cells have been outlined by a laser field (arrow) prior to microdissection. (C) The same specimen as in B but subjected to micro-dissection; note the empty space left by the breast cancer cells now captured.

Figure 3. Expression of Ang1 and VEGF in tumour specimens determined by using laser-capture microdissection. (A) Detection of Ang1 expression in 6 tumour specimens by RT-PCR and subsequent Southern analysis. Controls are RNA isolated from Ang1 transfected cells growing in culture (Ang1/TC) or microdissected from a section of a cell pellet (Ang1/LCM). (B) Detection of VEGF in the same cancer specimens by RT-PCR and Southern analysis.

Figure 4. Production of Ang1 by Ang1-transfected MPCX and  $\alpha$ 18 cells. (A) Western slot blot analysis of Ang1 in the conditioned media. Duplicate aliquots of COS cell conditioned media of known Ang1 concentration were used as standards (upper panel). Five clones of the Ang1-transfected MPCX cells and three clones of the Ang1-transfected  $\alpha$ 18 cells were shown in duplicate (lower panel), in comparison with the parental (P) and empty vector transfected (V) cells. (B) Ang1 activity in the conditioned media was determined for the ability to induce Tie2 tyrosine phosphorylation. NIH 3T3 cells over-expressing myc-tagged Tie2 were treated with the conditioned media, then subjected to immuno-precipitation with an anti-myc antibody, and Western blotting analysis with an anti-phosphotyrosine antibody (upper panel). The cells were also treated with 200 ng/ml of Ang1 to provide a positive control. A Western analysis with an antibody against the myc-tag as a loading control (lower panel). (C) Northern blotting analysis of FGF1 in RNA collected from Ang1 transfected  $\alpha$ 18 cells. The internal control was  $\beta$ -actin FGF1.

Figure 5. Inhibition of MCF-7 human breast cancer xenograft tumour growth by Ang1 over-expression. (A) Plots of tumour volumes of the xenograft tumours formed by the MPCX parental cell line (closed circles), empty vector transfected cells (closed squares), and three clones of Ang1 expressing transfectants: MAng 128 (open circles), MAng 166 (closed triangles), and MAng 184 (open triangles). There were five animals per group. The experiment was repeated and the results were reproducible. (B) The tumour sizes as a function of time were fitted with an exponential tumour growth model (see Methods) to determine the rate constants of the xenograft tumour growth. Growth rates for the Ang1-

over-expressing MPCX clones were compared to that of the vector mock transfected cells (ANOVA).

Figure 6. Inhibition of FGF1 transfected MCF-7 breast cancer xenograft tumour growth by Ang1 over-expression. (A) Plots of tumour volumes of the xenograft tumours formed by the  $\alpha 18$  parental cell line (closed circles), empty vector transfected cells (closed squares), and three clones of Ang1 expressing transfectants:  $\alpha$ Ang 14 (open circles),  $\alpha$ Ang 18 (closed triangles), and  $\alpha$ Ang 29 (open triangles). The overall statistical significance at 5% level was  $p = 0.03$  (ANOVA) for  $\alpha$ Ang 18 and  $\alpha$ Ang 29. The experiment was repeated and the results were reproducible. (B) Photographs of the xenograft tumours: a tumour formed by  $\alpha$ Ang 14 giving the typical appearance of a blood filled sac (left), cross-section of a tumour in the same group showing that the interior of the sac is empty once opened, and that there are only a few cancer cells which were stained blue (center), and the tumour formed by the parental  $\alpha 18$  cells consists of blue cancer cells.

Table 1. Summary of Ang1, Tie2, and vWF expression in clinical specimens. Clinical specimens (9 grade 3 tumours, 9 grade 2 tumours, 3 grade 1 tumours and 11 normal breast sections) were stained with monoclonal antibodies to Tie2 and vWF. Positively staining vessels were counted manually using standard protocols (Weidner, 1995). Densities ( $\pm$  SEM) refer to number of vessels per  $0.74 \text{ mm}^2$  microscopy field at 200x magnification. Ang1 expression was analysed by laser capture microdissection from cells adjacent to the immuno-stained vessels. Specimens were considered informative for Ang1 expression only if GAPDH was detectable.

Sample	Cases stained for Tie-2 and vWF	Number of Tie-2 expressing micro-vessels per sample	Number of vWF expressing micro-vessels per sample	Cases informative for Ang1 expression	Cases with Ang1 signal
Normal	11	12 ( $\pm$ 2.7)	20 ( $\pm$ 4.6)	9	0
Tumour	23	35 ( $\pm$ 3.7)	44 ( $\pm$ 3.9)	21	3

Table 2. Ang1 concentrations in the conditioned media of either vector-transfected or Ang1 transfected cells (BIAcore analysis).

Cell line	Ang1 (ng.ml <sup>-1</sup> )
MPCX Vector	0
MAng 128	240
MAng 166	160
MAng 184	820
$\alpha$ 18 Vector	0
$\alpha$ Ang 14	172
$\alpha$ Ang 18	612
$\alpha$ Ang 29	156

FIGURE 1

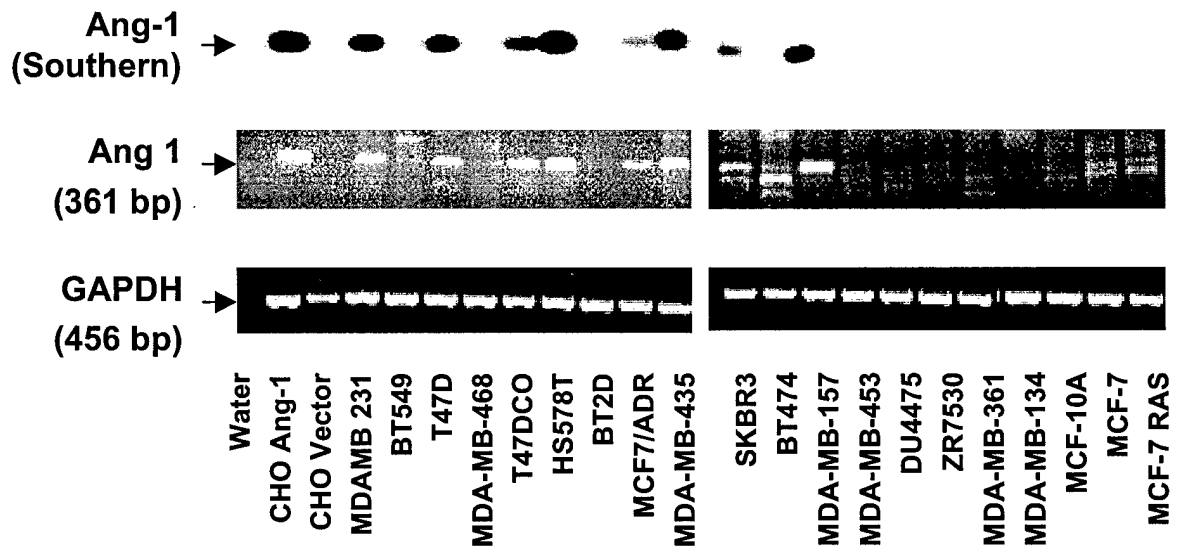


FIGURE 2

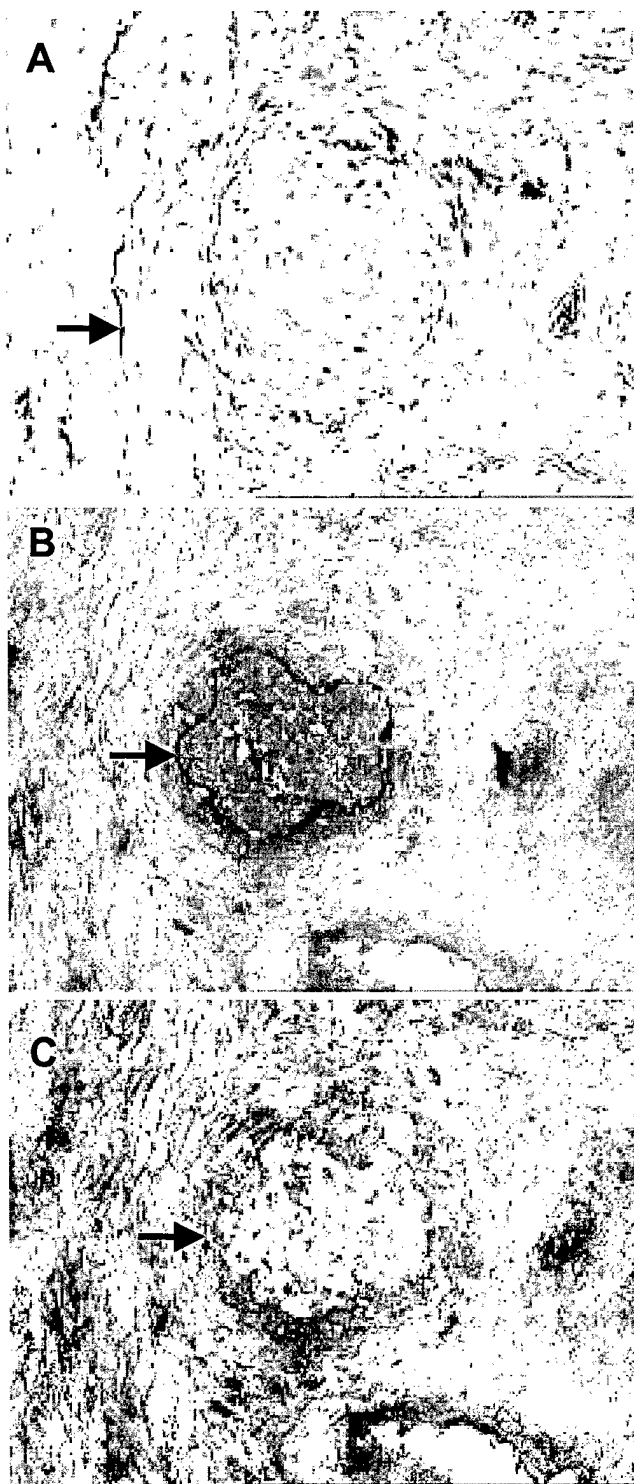


FIGURE 3

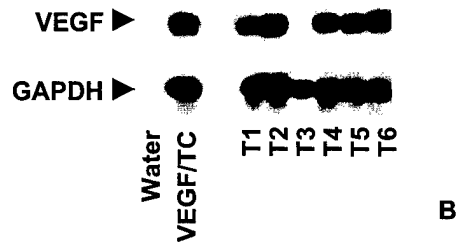
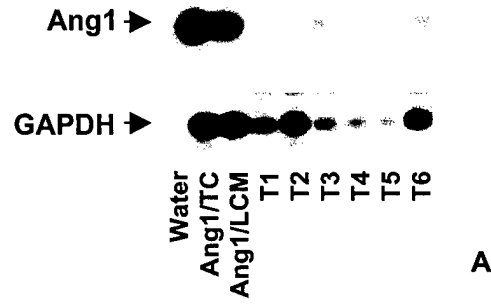


FIGURE 4

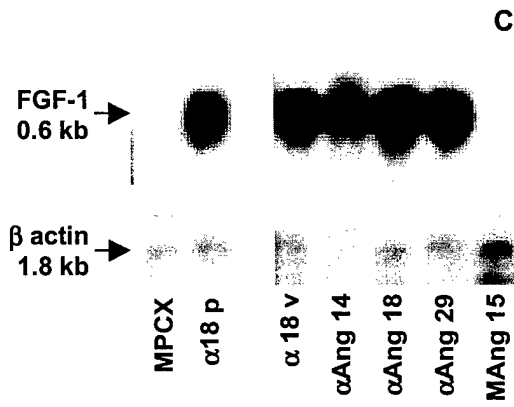
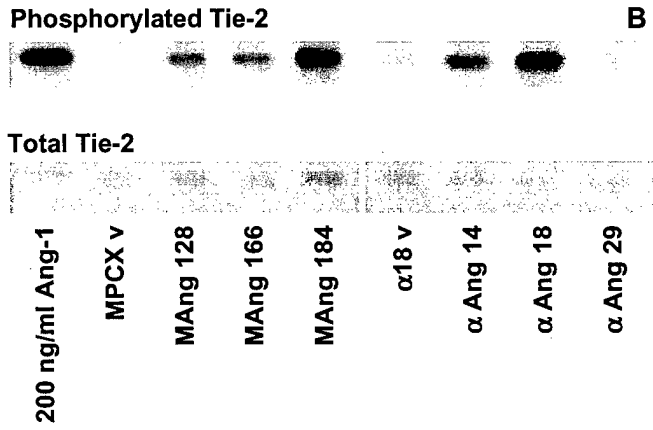
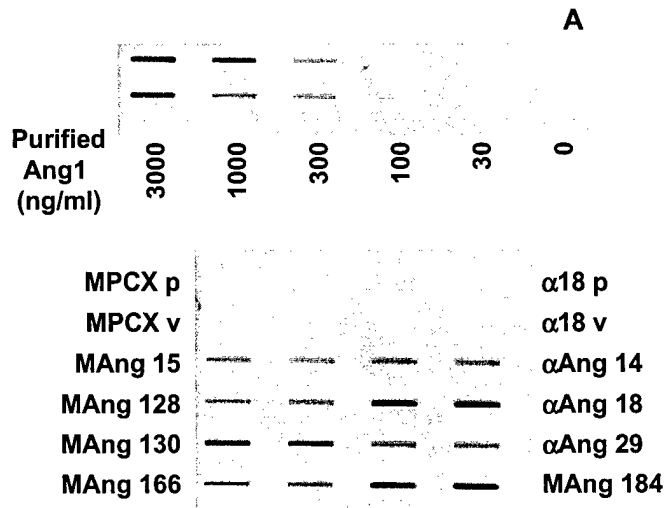


FIGURE 5

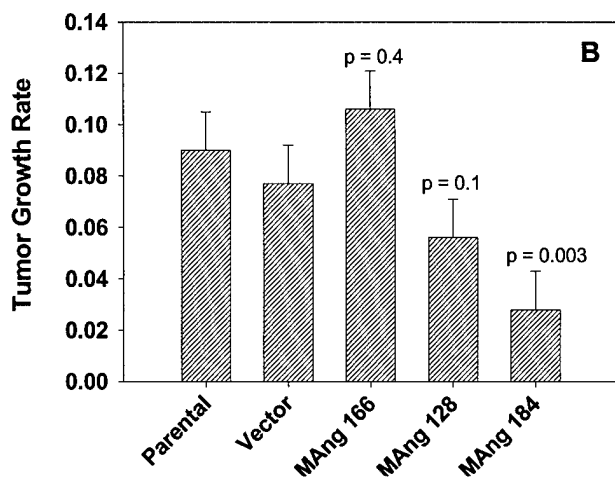
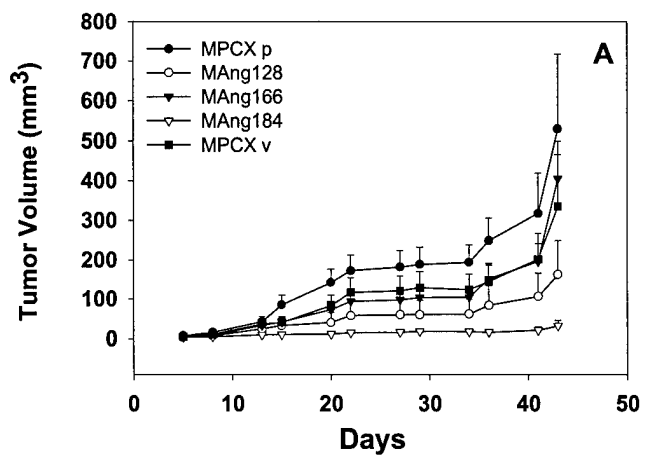


FIGURE 6

