Circulating levels of angiogenesis-related growth factors in breast cancer: A study to profile proteins responsible for tubule formation

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Abstract. The present study exploited a versatile *in vitro* endothelial cell/fibroblast co-culture cell system to investigate the association between angiogenesis and breast cancer by comparing the capacity of plasma from women with breast cancer and age-matched controls, to influence tubule formation and modulate angiogenesis *in vitro*, and to identify plasma circulating factors which might be responsible. Plasma from women with breast cancer (n=8) (added on day 7 after co-culture establishment) significantly increased tubule formation by 57% (P<0.01) when compared to cultures grown in culture medium lacking in vascular endothelial growth factor (VEGF) and fetal bovine serum (FBS), whereas plasma from controls (n=8) did not. Higher levels of VEGF, tumour necrosis factor-α (TNFα) and interleukin (IL)-6, but not leptin, were observed in plasma samples of the breast cancer group compared to the control group (n=20 in each group). In independent experiments, the effects of VEGF, TNFα, IL-6 and leptin were assessed and it was found that tubule formation was differentially affected whether these inflammatory cytokines or adipokines were added individually or in combination to the co-culture system. Using Proteome Profiler human angiogenesis array kits, 12 out of 55 angiogenesis-related proteins were differentially expressed in plasma from the breast cancer group compared to the control group. Pro-angiogenic proteins included: amphiregulin, artemin, coagulation factor III, fibroblast growth factor (FGF) acidic, GDNF, IL-8, macrophage inflammatory protein (MIP)-1α, platelet derived growth factor-AB/platelet derived growth factor-BB (PDGF-AB/PDGF-BB) and VEGF, whereas anti-angiogenic proteins were: angiopoietin-2, serpin F1 and serpin B5. In addition, FGF acidic was further identified as differentially expressed, with increased expression, when plasma samples from the normal and cancer groups, which induced an increase in tubule formation, were compared to one another. In conclusion, the present study identified angiogenesis-related proteins circulating in the serum of women with breast cancer that are likely to facilitate the growth and metastasis of breast cancer, in part through their influence on tubule formation, and, therefore, may be potential targets for new cancer therapies.

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Abbreviations: EC, endothelial cells; HUVEC, human umbilical vein endothelial cells; IL-6, interleukin-6; MMP, matrix metalloproteinases; NHDF, normal human dermal fibroblasts; TNFα, tumour necrosis factor-α; VEGF, vascular endothelial growth factor

Key words: angiogenesis, breast cancer, co-culture, cytokines

Introduction

Breast cancer is the most frequently diagnosed cancer, as well as, the leading cause of death in the female population worldwide (1). The aetiology of breast cancer remains largely unknown, probably due to its multifactorial nature, but several risk factors have been identified (2). These include reproductive and hormonal factors, environmental factors (including lifestyle changes with a higher consumption of animal fat, obesity and limited physical activity) and hereditary factors.

Poor prognosis of breast cancer is strongly related to advanced tumour stage and lymph node spread, although the importance of specific molecular markers has also been investigated (3-5). For a tumour to grow and metastasise it needs to develop its own blood supply and this is achieved through angiogenesis. The surrounding endothelial cells are stimulated to form new blood vessels by growth factors secreted from both the tumour and stroma. The process of angiogenesis is not the result of one single growth factor but rather is dependent on the interaction of multiple proteins with pro- or anti-angiogenic properties. In breast cancer, like almost all other solid tumours, growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1β, interleukin (IL)-8 and the regulated on activation, normal T cell expressed and secreted (RANTES), are expressed and, depending on

the subtype of breast cancer, have been identified as negative prognostic factors for patient survival (6-10). These growth factors may be responsible for inducing angiogenesis as they are pro-angiogenic, however, breast cancer cells and tissues can also produce inhibitors of angiogenesis, including thrombospondin, angiostatin and endostatin (11). Thus, it is becoming clear that angiogenesis in cancer progression is the result of a net balance between positive and negative regulators of tubule formation. This evidence directs further investigation into the importance of assessing the roles and levels of angiogenic factors in plasma from women with and without breast cancer. However, despite numerous studies into the detection of circulating biomarkers in breast cancer patients (9,10,12,13), the molecular mechanism(s) by which these circulating factors present in breast cancer may contribute to, and influence angiogenesis, are still poorly understood, suggesting a complex mechanism of interactions. Therefore, the aim of the present study was to assess the ability of circulating factors, present in the plasma from women with breast cancer, to influence tubule formation in an *in vitro* system (14) and to identify key factors responsible for increased angiogenesis.

Materials and methods

Materials. All chemicals and cell culture reagents were purchased from Sigma-Aldrich Ltd. (Dorset, UK), unless otherwise stated.

Cell culture. Primary human umbilical vein endothelial cells (HUVEC) and primary normal human dermal fibroblasts (NHDF) were obtained from Clonetics (Lonza, Slough, UK). HUVEC were maintained in endothelial basal medium (EBM-2) supplemented with EGM-2 SingleQuots (i.e., EGM-2 medium) whilst NHDF were maintained in fibroblast basal medium (FBM) supplemented with FGM-2 SingleQuots; all from Clonetics; Lonza (i.e., FGM-2 medium).

The co-culture system was set up using HUVEC (between passages 1-8) and NHDF (between passages 1-12) following the protocol established by Bishop *et al* (15) and adapted by Barron *et al* (14). HUVEC and NHDF were mixed and seeded in 24-well plates (Thermo Fisher Scientific Nunc, Loughborough, UK) in EGM-2 medium. Co-cultured cells were incubated for up to 14 days at 37˚C in a 5% CO_2 in air humidified incubator.

Study subjects. Participants in the study were recruited through the Breast Clinic in Aberdeen Royal Infirmary, Scotland. The study was approved by the Grampian Ethics Committee. Signed informed consent was obtained from each study participant. Venous blood from 40 volunteers was used in this study: 20 women with breast cancer and 20 age-matched control individuals. The women with breast cancer had a life-time risk estimated to be double that of the normal female population, based on their history, and screen of no known genetic basis for the development of their breast cancer (e.g., *BRCA1* or *BRCA2* mutations) (16). The control group included individuals with the normal population risk of breast cancer who did not have breast cancer but were undergoing other forms of breast surgery (16).

Plasma isolation. Plasma was isolated from venous blood from all of the participants using a Histopaque gradient (16). Briefly, 20 ml of blood was layered on top of 20 ml Histopaque-1077 and centrifuged at 800 x g for 30 min at room temperature. The top layer containing the plasma was collected and stored at -80˚C until required.

Plasma samples (n=40) were assayed in duplicate to measure the concentration of VEGF, TNFα, IL-6 and leptin (DuoSet ELISA cat. no. DY293B, DY210, DY206 and DY398-05, respectively; R&D Systems, Abingdon, UK) according to the manufacturer's instructions. These assays have a sensitivity of ~30, 15, 9 and 20 pg/ml, respectively, and quantify both the natural and recombinant forms of each protein.

Co-culture treatment with human plasma and combinations of cytokines. The co-culture system was incubated with EGM-2 medium to initiate tubule formation and, 7 days after co-culture establishment, the medium was changed to EGM-2ϴFBS [i.e., EGM-2 medium minus VEGF SingleQuot and 2% (v/v) fetal bovine serum (FBS) SingleQuot] or EGM-2ϴFBS supplemented with 2% plasma from a random subset of women with breast cancer (n=8) or control individuals (n=8). The medium was collected every 4 days and replaced with respective treatments.

In order to examine the effects of inflammatory cytokines, the co-culture system was incubated with EGM-2 medium to initiate tubule formation and, 7 days after the co-culture establishment, medium was changed to EGM-2+VEGF, EGM-2ϴ, EGM-2ϴ+Leptin, or cytokine combinations: EGM-2ϴ+TNFα+IL-6, EGM-2ϴ+TNFα+Leptin or EGM-2ϴ+IL-6+Leptin. The medium codes are summarised in Table I. The medium was collected every 4 days and was replaced with respective treatments.

Immunostaining. After 14 days, co-cultures were fixed at room temperature with ice-cold 70% (v/v) ethanol and stained with myeloma cell adhesion molecule [MCAM (CD146); specific to HUVEC] to assess the extent of tubule formation as previously described by Barron *et al* (14). Co-cultures were viewed using an Olympus 1XS1 microscope with Olympus TL4 Light Box Infinity Capture Application version 5.0.0 (Lumenera Corp., Nepean, ON, Canada) with camera model Infinity 2-2c was used for image capture. Three images per well were captured and saved as BMP images of 1616 x 1216 pixels. Illumination was set to give a best contrast between stained tubules and unstained fibroblasts. Tubule formation was defined by total tubule length and quantified using AngioSys® software version 1.0 (TCS Cellworks Ltd., Buckingham, UK).

Proteome Profiler™ Human Angiogenesis Antibody array. The relative expression of 55 angiogenesis-related proteins was determined in human plasma (4 women per group) using a Proteome Profiler™ Human Angiogenesis Antibody Array according to the manufacturer's instructions (cat. no. ARY007; R&D Systems). Briefly, after a 1-h membrane blocking step, human plasma (200 μl) was pre-incubated with a cocktail of biotinylated detection antibodies (15 μl) and added to the membrane before incubating overnight at 4˚C. After a series of washes, the membrane was incubated with streptavidin-horseradish peroxidase (HRP; 2 ml) for

Table I. Details of medium employed for co-culture treatments.

Code	Details
EBM-2	Endothelial basal medium
EGM-2	EBM-2 supplemented with EGM-2 SingleQuots
EGM-2Θ	EGM-2 medium minus VEGF SingleQuot
EBM-2ΘFBS	EGM-2 medium minus VEGF and FBS SingleQuots
EGM-2Θ+Leptin	EGM-2Θ supplemented with leptin
EGM-2+VEGF	EGM-2 supplemented with VEGF
EGM-2Θ+TNFα+IL-6	EGM-2Θ supplemented with TNFα and IL-6
EGM-2Θ+TNFα+Leptin	EGM-2Θ supplemented with TNFα and leptin
EGM-2Θ+IL-6+Leptin	EGM-2Θ supplemented with IL-6 and leptin

30 min, before chemiluminescence detection reagents were added in equal volumes for 1 min. The signal was detected by exposing the membrane to CL-XPosure X-ray film (Thermo Fisher Scientific) over a variety of exposure times (1, 3, 5 and 10 min). The light produced at each spot is proportional to the amount of analyte bound and the mean pixel density of the duplicate spots produced on the film was determined using a FUSION FX7™ imaging instrument with Fusion 1 and BIO-1D™ imaging software (PeqLab; VWR International Ltd., Lutterworth, UK).

In addition to the 55 angiogenesis-related proteins, each membrane contained three pairs of positive reference spots and one pair of negative control spots. Following subtraction of the mean pixel density of the negative control spots from all values, the level of each protein was expressed as a ratio relative to the mean of the positive reference spots which were assigned a value of 1. Relative expression ratios for all 55 angiogenesis-related proteins were compared between plasma from women with breast cancer (n=3) and plasma from control individuals (n=4).

Statistical analysis. Unless otherwise stated, all data are expressed as mean ± SEM of three independent experiments from different cell passage numbers. Statistical differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison t-test (GraphPad Prism). $P<0.05$ was considered to be statistically significant.

Results

Effect of human plasma on tubule formation. The effect of human plasma on tubule formation was tested with EGM-2ΘFBS: human plasma replaced FBS (2% v/v) present in EGM-2 medium minus VEGF. Co-cultures were initially incubated in EGM-2 medium (day 0) and, after co-culture establishment (day 7): EGM-2ΘFBS or EGM-2ΘFBS supplemented with either human plasma from women with breast cancer (cancer group, n=8) or control (control group, n=8) individuals was added. These conditions were used to assess the ability of angiogenesis-related growth factors present in human plasma to modulate tubule formation once the angiogenesis process had already started in our *in vitro* co-culture model.

Table II. Baseline characteristics of control and cancer groups.

Characteristics	Control group (n=20)	Cancer group (n=20)
Age (years)	41.9±1.3	41.1±1.2
VEGF (pg/ml)	23.7±12.2	142.3±58.2
TNFα (pg/ml)	159.9±90.6	491.4±225.8
IL-6 (pg/ml)	43.1±31.2	153.1±79.5
Leptin (ng/ml)	15.9±1.5	17.8±1.7

Results are expressed as mean ± SEM.

Tubules were clearly formed and visualised after 14 days (Fig. 1A) and similar results were obtained in each experiment. The extent of tubule formation was quantified by measuring total tubule length and the percentage change was normalised to EGM-2ΘFBS (Fig. 1B). Tubule formation was significantly increased by 57% ($P<0.01$) when plasma from women with breast cancer was used in the co-culture system compared to EGM-2ΘFBS. Although tubule formation was increased (by 21%) with plasma from control individuals; no significant difference was found compared to EGM-2ΘFBS.

Levels of VEGF, TNFα, IL-6 and leptin in human plasma. Higher levels of VEGF (~6-fold increase, $P=0.053$), TNFα (~3-fold increase) and IL-6 (~3.5-fold increase) were present in plasma samples of the breast cancer group compared to the control group (n=20 in each group), but differences were not statistically significant (Table II) ($P>0.05$). No changes in leptin levels were observed between groups (Table II).

Effect of the adipokine leptin and of a combination of inflammatory cytokines/adipokine on tubule formation. We have previously shown that angiogenic factors and inflammatory markers such as VEGF, TNFα and IL-6 play an important role in modulating angiogenesis (14); however, their effects on modulating angiogenesis when used in combination with the adipokine leptin or other inflammatory cytokines have not been previously reported.

The effect of leptin on tubule formation was first examined in the absence of VEGF (i.e., EGM-2Θ medium) in a similar

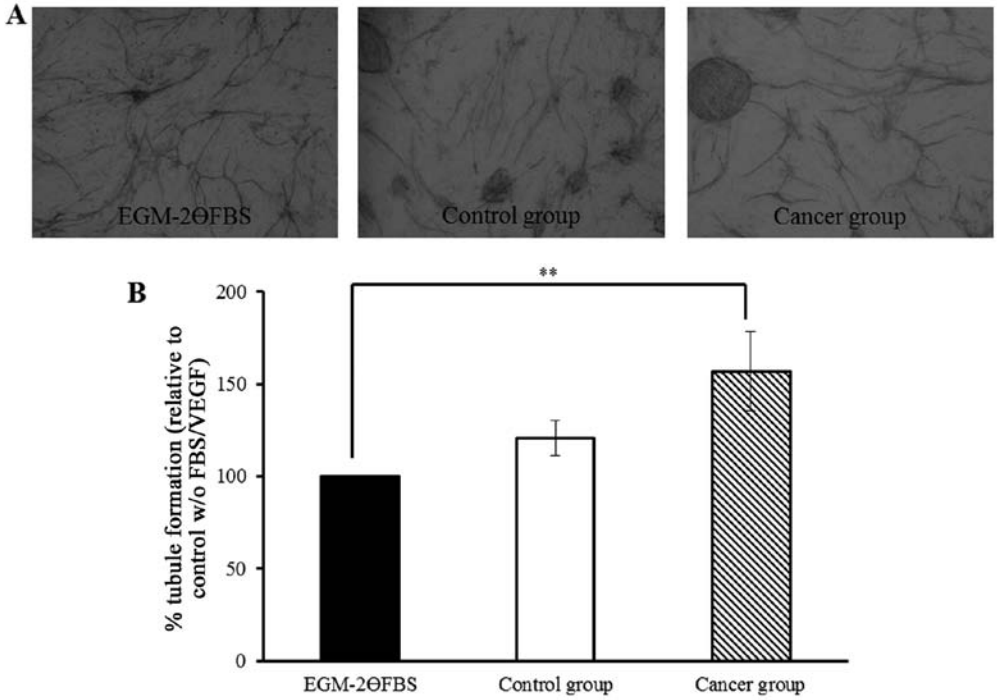

Figure 1. (A) Tubule formation assessed by MCAM staining on day 14 in co-cultures grown in EGM-2ΘFBS, EGM-2ΘFBS supplemented with 2% plasma from control individuals (control group) or EGM-2ΘFBS supplemented with 2% plasma from women with breast cancer (cancer group) added on day 7 after co-culture establishment. Medium was changed every 4 days. Images presented are representative of at least 3 independent experiments/plasma samples; within an experiment, each treatment was carried out in duplicate and observed in three randomly chosen fields. Magnification, x40. (B) Quantification of total tubule length determined using AngioSys software. Plasma was added on day 7. Results are presented as a percentage of EGM-2ΘFBS as mean ± SEM of at least 3 independent experiments/plasma sample; within each experiment, each treatment was carried out in duplicate; $^{**}P<0.01$.

manner to Barron *et al* (14). Tubule formation was quantified by measuring total tubule length and the percentage change was normalised to EGM-2Θ (Fig. 2A). Incubating co-cultures with leptin (10 nM, added on day 7; EGM-2Θ+leptin) did not alter tubule formation compared to EGM-2Θ. Whereas, tubule formation was significantly increased (43%) when complete EGM-2 medium was added at day 7 compared to EGM-2Θ, confirming the ability of the co-culture system to further produce tubules with addition of VEGF. However, addition of VEGF (2 ng/ml; EGM-2+VEGF) to complete EGM-2 medium after 7 days did not induce further tubule formation (Fig. 2B), suggesting an inability of the *in vitro* system to create more new tubules than were normally formed with complete EGM-2 medium as clearly visualised after staining with MCAM on day 14 (Fig. 2C).

Further studies were undertaken to determine the effect of cytokine/adipokine combinations on tubule formation. Co-cultures were initially incubated with EGM-2 medium (day 0) and, after co-culture establishment (day 7), EGM-2Θ, EGM-2Θ+TNFα+IL-6, EGM-2Θ+TNFα+Leptin or EGM-2Θ+IL-6+Leptin were added. As combination studies in which cytokines and adipokines are tested together in equal or different concentrations have not been previously carried out, these studies, which use an *in vitro* system reflecting an *in vivo* situation where cytokines and adipokines do not exist alone, are therefore very important to identify factors responsible for angiogenesis.

Tubules were clearly formed and visualised after 14 days (Fig. 3A) and similar results were obtained in each experiment. The extent of tubule formation was quantified by measuring total tubule length and the percentage change was normalised to EGM-2Θ (Fig. 3B). TNFα (10 ng/ml) combined with IL-6 (10 ng/ml) or TNFα (10 ng/ml) combined with leptin (10 nM) ($P<0.05$), reduced tubule formation by 53 and 58%, respectively. IL-6 (10 ng/ml) combined with leptin (10 nM) did not alter tubule formation compared to EGM-2Θ but, tubule formation was increased when a higher concentration of IL-6 (15 ng/ml, increased by 26%) or leptin (15 nM, increased by 16%) were combined with TNFα (5 ng/ml) but, these changes were not statistically significant when compared to EGM-2Θ. A 114% increase in tubule formation was observed with IL-6 (15 ng/ml) combined with leptin (5 nM) but again, this was not statistically significant when compared to EGM-2Θ. No statistically significant differences were observed between the equivalent (white bars) and different (black bars) concentrations of each combination (Fig. 3B). These results indicate that TNFα, IL-6 and leptin may differentially modulate tubule formation either added individually or in combination, but the limitation of the co-culture system does not allow this or, alternatively, additional key factors are required.

Comparison of angiogenesis-related protein expression between plasma from women with breast cancer and control group. Although higher levels of VEGF, TNFα, IL-6 or leptin were observed in the breast cancer group (Table II), the addition of exogenous VEGF, TNFα, IL-6 or leptin alone (Fig. 2) and Barron *et al* (14) or in combination (Fig. 3) showed that these molecules are not solely responsible for the increase in tubule formation observed in the breast cancer group (Fig. 1). Proteome profiler human angiogenesis antibody arrays were,

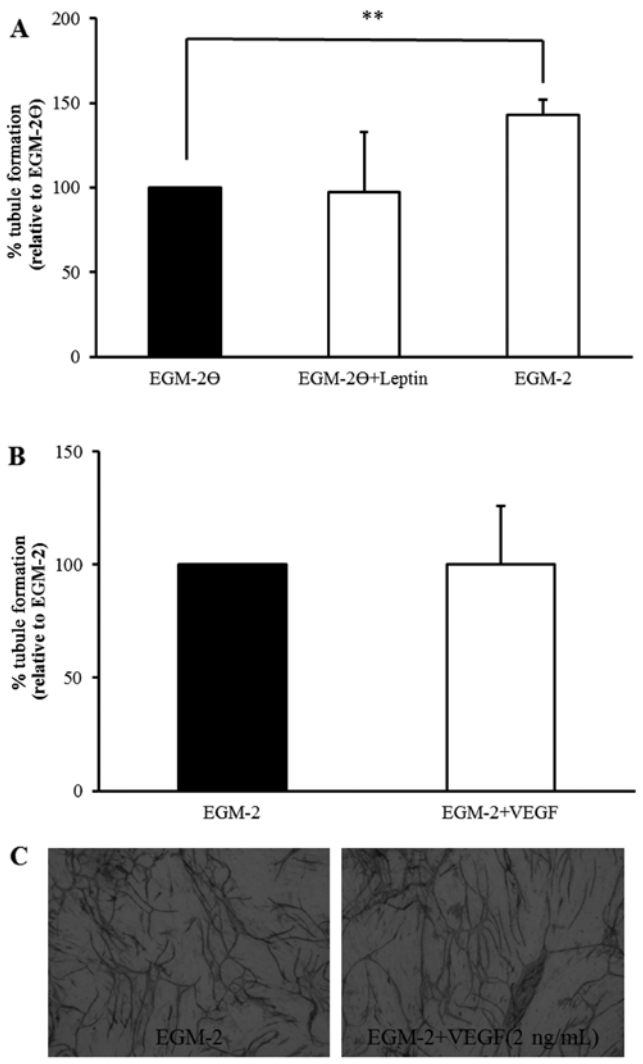

Figure 2. (A) Quantification of total tubule length determined using AngioSys software. Medium changed from EGM-2 on day 7 to EGM-2Θ, EGM-2Θ+Leptin and EGM-2. Medium was changed every 4 days. Results presented as a percentage of EGM-2Θ as mean ± SEM of 3 independent experiments, within each experiment, each treatment was carried out in duplicate; $^{**}P<0.01$. (B) Quantification of total tubule length determined using AngioSys software. VEGF (2 ng/ml) added on day 7 and medium was changed every 4 days. Results presented as a percentage of EGM-2 as mean ± SEM of 3 independent experiments, within each experiment, each treatment was carried out in duplicate. (C) Tubule formation assessed by MCAM staining on day 14 in co-cultures grown in EGM-2 and EGM-2+VEGF (2 ng/ml) added on day 7 after co-culture establishment. Medium was changed every 4 days. Images presented are representative of treatments from 3 independent experiments; within an experiment, each treatment was carried out in duplicate and tubule formation was observed in three randomly chosen fields. Magnification, x40.

Table III. Relative expression of angiogenic factors which demonstrated no change in protein expression.

Angiogenic factor	Control group (n=4)	Cancer group (n=3)
Activin A	0.07	0.07
ADAMTS-1	0.08	0.10
Angiogenin	1.14	1.23
Angiopoietin-1	0.50	0.65
Angiostatin/plasminogen	0.14	0.13
CXCL16	1.01	1.08
DPPIV	1.07	1.04
EGF	0.14	0.12
EG-VEGF	0.19	0.15
Endoglin	0.97	0.89
Endostatin/collagen XVIII	1.05	1.15
Endothelin-1	0.54	0.67
FGF-4	0.10	0.09
FGF-7	0.06	0.07
GM-CSF	0.12	0.09
HB-EGF	0.19	0.16
HGF	0.14	0.12
IGFBP-1	1.01	1.15
IGFBP-2	1.03	1.15
IGFBP-3	1.02	1.12
IL-1β	0.09	0.07
LAP (TGF-β1)	0.10	0.08
Leptin	1.00	1.07
MCP-1	0.06	0.06
MMP-8	0.97	1.06
MMP-9	1.03	1.04
NRG1-β1	0.15	0.15
Pentraxin 3 (PTX3)	0.45	0.59
PD-ECGF	0.21	0.22
PDGF-AA	0.60	0.78
Platelet factor 4 (PF4)	1.02	1.09
PIGF	0.11	0.11
Prolactin	0.86	0.99
TIMP-4	1.07	0.73
Thrombospondin-1	1.05	0.73
Thrombospondin-2	0.05	0.05
VEGF-C	0.04	0.03

Values (to 2 decimal places) shown are relative expression of angiogenic factor over positive control.

therefore, used to identify differences in protein expression that could be responsible for the increase in tubule formation. Angiogenesis protein profiles in plasma from women with breast cancer (n=3) were compared to profiles from control individuals (n=4) (plasma from one breast cancer individual was not acceptable for protein array analysis and was excluded).

Levels of proteins expressed in plasma from the control group were compared to those in plasma from women with breast cancer, irrespective of whether the plasma sample increased, or not, tubule formation in the *in vitro* co-culture system. A cut-off of 1.5-fold increase difference and a cut-off of 0.5-fold decrease difference was used as considered threshold for meaningful physiological changes in protein arrays studies. Thirty-seven proteins in the array showed no change in expression relative to the positive reference spots (value of 1) and were not further examined (Table III). Eighteen angiogenic-related factors demonstrated a level of expression that was 1.5-fold different in either plasma obtained from control or women with cancer. For these

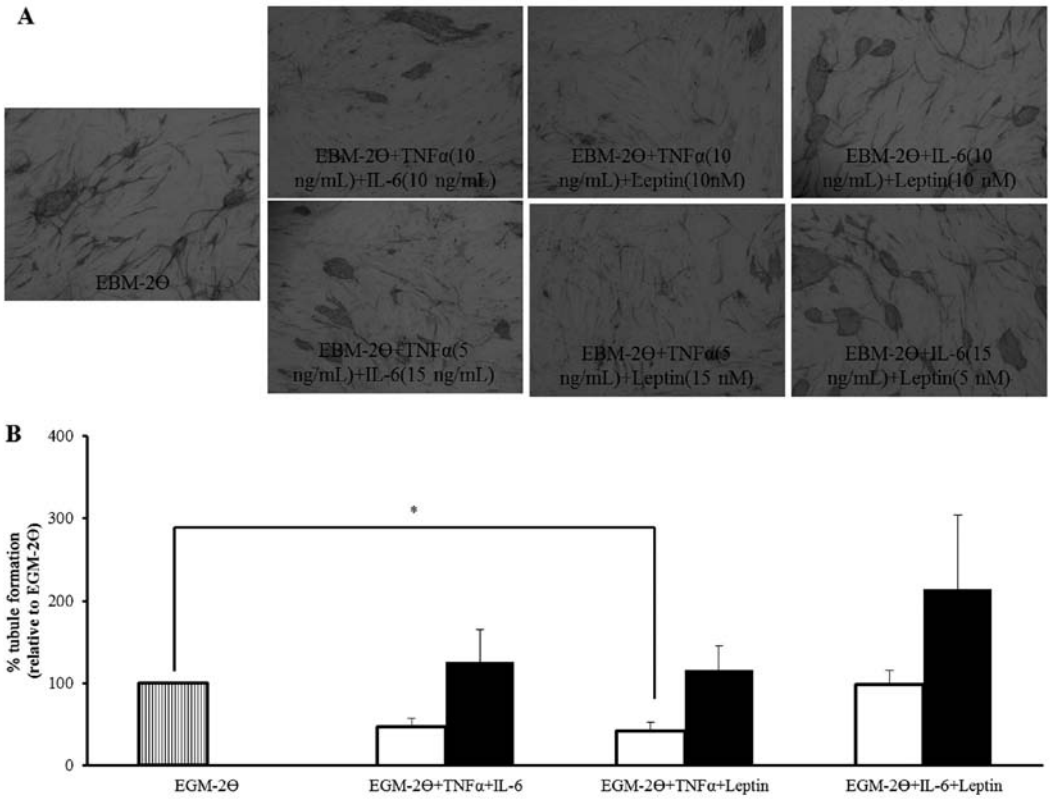

Figure 3. (A) Tubule formation assessed by MCAM staining on day 14 in co-cultures grown in EBM-2Θ, EBM-2Θ+TNFα+IL-6, EBM-2Θ+TNFα+Leptin or EBM-2Θ+IL-6+Leptin added on day 7 after co-culture establishment. Medium was changed every 4 days. Images presented are representative of at least 3 independent experiments; within an experiment, each treatment was carried out in duplicate and observed in three randomly chosen fields. Magnification, x40. (B) Quantification of total tubule length determined using AngioSys software. TNFα, IL-6 and leptin added at equivalent concentrations (white bars) (related to top row of images in A) and different concentrations (black bars) (related to bottom row of images in A). Results are presented as a percentage of EGM-2Θ (striped bar) as mean ± SEM of at least 3 independent experiments; within each experiment, each treatment was carried out in duplicate; *P<0.05.

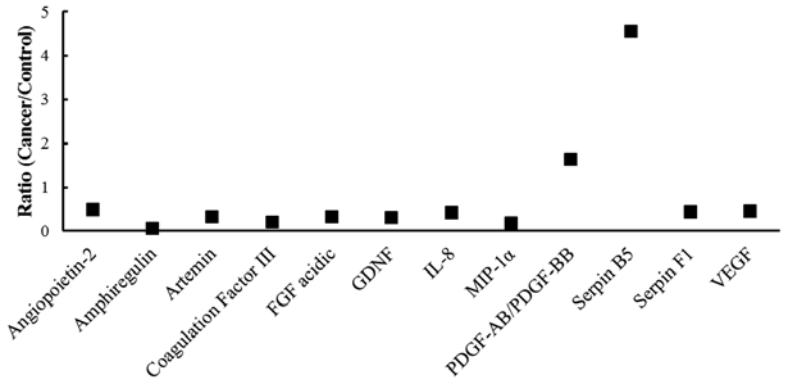

Figure 4. Relative expression of angiogenic factors in breast cancer/control samples.

18 proteins, cancer-to-control ratios were calculated. Overall, 6 proteins were excluded that had ratios between 0.5-1.5 (i.e. pro-angiogenic: FGF basic, persephin, uPA and vasohibin; anti-angiogenic: serpin E1 and TIMP-1), 10 proteins showed reduced expression (ratio <0.5) (pro-angiogenic: amphiregulin, artemin, coagulation factor III, FGF acidic, GDNF, IL-8, MIP-1α and VEGF; anti-angiogenic: angiopoietin-2 and serpin F1) (Fig. 4); whilst, 2 proteins showed increased expression (ratio >1.5): PDGF-AB/PDGF-BB (ratio of 1.64, pro-angiogenic) and serpin B5 (ratio of 4.56, anti-angiogenic) (Fig. 4).

Furthermore, plasma samples, from both the normal and cancer groups, which induced an increase in tubule formation in the *in vitro* co-culture model, were compared to identify which angiogenesis-related proteins could be responsible for tubule formation. For array analysis, a cut-off of 1.5-fold difference was set as before. In the normal group, 42 out of the 55 angiogenesis-related proteins showed no change in protein expression and were not further examined. Thirteen of the 55 angiogenic-related factors had levels of expression that were 1.5-fold different. In relation to the expression of these 13 proteins, further analysis was carried out by comparing their

Table IV. Relative expression of angiogenic factors which demonstrated an increase in tubule formation when plasma from both the control and breast cancer groups was used in the *in vitro* co-culture system.

Angiogenic factor	Increase in tubule formation Control group (n=2)	Increase in tubule formation Cancer group (n=2)
Activin A	2.28[c]	0.36[b]
ADAMTS-1	-	0.60[a]
Angiopoietin-1	0.64[a]	3.54[c]
Angiostatin/ plasminogen	-	3.10[c]
Artemin	1.45[a]	3.23[c]
EGF	-	0.68[a]
EG-VEGF	-	0.64[a]
Endoglin	-	0.61[a]
FGF-4	-	2.02[c]
FGF acidic	1.78[c]	49.39[c]
GDNF	2.07[c]	-
GM-CSF	1.56[c]	0.48[b]
HGF	-	0.62[a]
IL-1β	-	2.22[c]
IL-8	0.61[a]	1.55[c]
LAP (TGF-β1)	-	1.49[a]
MIP-1α	1.54[c]	-
Pentraxin 3 (PTX3)	-	0.43[b]
PD-ECGF	-	0.61[a]
PDGF-AA	0.61[a]	2.54[c]
PDGF-AB/PDGF-BB	0.37[b]	4.31[c]
Persephin	-	2.02[c]
PIGF	-	1.63[c]
Serpin B5	-	30.86[c]
TIMP-1	-	0.01[b]
TIMP-4	-	0.04[b]
Thrombospondin-2	8.12[c]	-
uPA	0.61[a]	0.11[b]
VEGF	-	0.24[b]
VEGF-C	0.57[a]	1.54[c]

Values (to 2 decimal places) shown are the ratios of protein expression in plasma samples which induced an increase compared to no change in tubule formation when compared to EBM-2ΘFBS, for control group and cancer group independently. [a]Angiogenic-related proteins that had ratios between 0.5-1.5; [b]angiogenic-related proteins that had ratios <0.5; and [c]angiogenic-related proteins that had ratios >1.5.

expression and calculating a ratio between the same proteins in plasma samples which induced an increase-to-no change in tubule formation when compared to EGM-2ΘFBS (Fig. 1). After exclusion of 6 proteins that had ratios between 0.5-1.5 (i.e., pro-angiogenic: angiopoietin-1, artemin, IL-8, PDGF-AA, uPA and VEGF-C), 1 angiogenesis-related protein (i.e. PDGF-AB/PDGF-BB, pro-angiogenic) showed reduced expression (ratio <0.5) whereas 6 angiogenesis-related proteins (i.e. pro-angiogenic: FGF acidic, GDNF, GM-CSF and MIP-1α; anti-angiogenic: activin A and thrombospondin-2), had increased expression (ratio >1.5) (Table IV).

Conversely, in the cancer group, 28 of the 55 angiogenesis-related proteins showed no change in protein expression and were not examined further. Twenty-seven of the 55 angiogenesis-related factors identified had levels of expression that were 1.5-fold different (Table IV) and, of these 27 remaining angiogenesis-related proteins, the increase in tubule formation-to-no change in tubule formation ratios was calculated. Overall, after exclusion of 7 angiogenesis-related proteins that had ratios between 0.5-1.5 [i.e. pro-angiogenic: EGF, EG-VEGF, endoglin, HGF, LAP (TGF-β1), and PD-ECGF; anti-angiogenic: ADAMTS-1], 20 showed differences in levels: the level of expression was reduced (ratio <0.5) for 7 proteins (i.e. pro-angiogenic: GM-CSF, pentraxin 3, uPA and VEGF; anti-angiogenic: activin A, TIMP-1 and TIMP-4) whereas 13 protein levels were increased (ratio >1.5) (i.e. pro-angiogenic: angiopoietin-1, artemin, FGF acidic, FGF-4, IL-1β, IL-8, PDGF-AA, PDGF-AB/PGDF-BB, persephin, PIGF and VEGF-C; anti-angiogenic: angiostatin/plasminogen, and serpin B5) in the cancer group (Table IV).

Discussion

Angiogenesis is one of the major causes for cancer metastasis. The formation of new blood vessels is essential for cancer growth and metastasis; therefore, the development of angiogenic characteristics is vital in a number of tumour types including breast cancer (17,18). Many studies have measured the production of angiogenesis-related factors in serum from women with breast cancer *via* ELISA analysis; however, the majority of these have only examined one or two angiogenesis-related factors, including VEGF (6,8,19,20), basic FGF (6), IL-6 (21), leptin and prolactin (8) and thrombospondin-1 (19). To date, there have only been three studies which have looked at the expression of multiple angiogenic factors in serum from women with breast cancer (10,12,22).

The present study evaluated the effect of factors present in plasma obtained from women with breast cancer compared to age-matched control women in relation to tubule formation, using a versatile *in vitro* co-culture model established in our laboratory (14) in addition to testing the effect of cytokines/adipokines added singularly or in combination to the system. Finally it examined the relative expression of 55 angiogenesis-related proteins in a subset of plasma samples from cancer and control individuals in relation to tubule formation. This study therefore provides additional information on proteins differentially expressed and on the importance of a balance between pro-angiogenic/anti-angiogenic factors in tubule formation.

The *in vitro* co-culture system used to study tubule formation has been optimised so that molecules of interest could be added 7 days after initiation of tubule formation to make it relevant when considering breast tumour progression and metastasis. By using plasma samples, it was found that tubule formation *in vitro* was significantly increased ($P<0.01$) after incubation with plasma from women with breast cancer (n=8). Indeed, a higher level of VEGF was present in the breast cancer

group compared to the control group. However, not all individual plasma samples increased tubule formation, which could be due to low/absent levels of VEGF but also, to higher levels of the anti-angiogenic growth factor, TNFα. As inflammatory cytokines such as TNFα (23) and IL-6 (unpublished data) and, the adipokine leptin have been shown, *in vitro*, to play a putative role in breast cancer progression (24), it is important to investigate the effect that these exogenous cytokines/adipokines might have on angiogenesis in isolation, but also to study their effect when present in combination as these growth factors do not exist in isolation in human plasma. Using our *in vitro* co-culture cell model, it was found that leptin did not alter tubule formation when VEGF was lacking but, addition of complete EGM-2 medium supplemented with VEGF did further increase tubule formation. These results are in contrast with the pro-angiogenic effect of leptin (10, 100 and 1000 ng/ml) on endothelial tube formation previously described in single-cell conditions using HUVECs and a matrigel matrix (25-27) or, HUVECs and a collagen I matrix (28); suggesting that the system used for studying angiogenesis *in vitro* might be critical even if the use of *in vitro* co-culture systems are more closely related to *in vivo* conditions.

This study is also the first to use an *in vitro* endothelial cell/fibroblast co-culture cell system to investigate the effect of combining exogenous cytokines/adipokines. Results from this study revealed that when TNFα was combined with either IL-6 or leptin, tubule formation was reduced but, not to the same extent as TNFα alone as it stopped tubule formation (14); therefore, pro-angiogenic IL-6 and leptin may counteract the anti-angiogenic effect of TNFα, but not completely. An increase in tubule formation was observed when IL-6 was combined with leptin. These observations further indicate that angiogenesis is modulated by specific pro- and anti-angiogenic inflammatory markers. More specifically, TNFα, IL-6 and leptin differentially affect tubule formation either individually or in combination. Thus, other angiogenesis-related proteins may be involved. Since angiogenesis is a complex process that involves many proteins; angiogenesis protein arrays were used to assess the expression of a large group of proteins. Several angiogenesis-related proteins (both pro- and anti-angiogenic) were observed to have altered expression in the plasma from women with breast cancer which may correlate with the increase in tubule formation and therefore, may be involved in breast tumour progression and metastasis. Accordingly, angiogenesis in breast cancer is regulated by a net balance between pro- and anti-angiogenic proteins. In the present study, 12 out of 55 angiogenesis-related proteins analysed had altered expression in the breast cancer group compared to the control group; with 10 proteins observed to have reduced expression (pro-angiogenic: amphiregulin, artemin, coagulation factor III, FGF acidic, GDNF, IL-8, MIP-1α and VEGF; anti-angiogenic: angiopoietin-2 and serpin F1); whilst, 2 proteins showed increased expression: PDGF-AB/PDGF-BB (ratio of 1.64, pro-angiogenic) and serpin B5 (ratio of 4.56, anti-angiogenic). To the authors' knowledge, this is the first study to directly examine angiogenesis-related protein expression and tubule formation *via* a co-culture cell system *in vitro* using human plasma samples. However, a study by Morelli *et al* (19), investigated the *in vitro* effect of sera from patients with breast or gastrointestinal cancers on HUVEC proliferation using a colourimetric assay. Morelli *et al* (19) observed that sera from 19% (15 of 78) of breast cancer patients induced growth of HUVECs whilst, sera from 5% (4 of 78) of breast cancer patients inhibited endothelial cell proliferation. Furthermore, when sera stimulated endothelial growth, it was correlated with high levels of VEGF detected by ELISA whilst, when sera inhibited endothelial cell growth, it was correlated with high levels of soluble thrombospondin revealed by western blot analysis (19). These results show that individual variations of angiogenesis-related proteins are evident in specific cohorts of study volunteers and could depend on disease stage, subtype and treatment. In relation to this, we recognise a limitation of this study to analyse the effects on tubule formation in the co-culture stystem in function of disease stage and subtype: no treatment differences were present as samples were collected just after diagnosis of breast cancer and before treatment commenced.

The present study focused on the identification of factors which drive angiogenesis, however, it also highlighted the importance of the fine balance between pro- and anti-angiogenic factors present in cancer patients' plasma and driving angiogenesis. To date, anti-angiogenic drugs for cancer treatment have been of relative success and have predominantly targeted the expression/binding of pro-angiogenic factors such as VEGF (29). Our data emphasise the need for development of drug therapies which target, at the same time, not only the expression of pro-angiogenic factors but also affect the expression of some anti-angiogenic factors as it is the result of their differential expression which affects angiogenesis and cell proliferation.

In conclusion, many angiogenesis-related proteins may be involved in the growth, spread and progression of breast tumours. This study identified potential key factors which might be responsible to drive angiogenesis in breast cancer and, once their role has been defined further, they might provide targets for future clinical therapies. The identified factors have been shown to be relevant in other malignancies including gastrointestinal (19) and laryngeal cancer (30), confirming their potential importance in breast cancer.

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