Effect of YangZheng XiaoJi Extract, DME-25, on Endothelial Cells and their Response to Avastin

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Abstract. *Background: Angiogenesis is a cellular process that has been identified as a key target for therapy in solid cancer. However, over the course of anti-angiogenic therapies, cancer cells acquire resistance to these therapies after an initial period of success. DME-25 is an extract from Yang Zheng Xiao Ji, a traditional Chinese medicine that has been reported to benefit patients with cancer by alleviating chemotherapy-associated symptoms and possibly inhibiting key cancer cell traits. This study aimed to explore if DME-25 on its own and in combination with avastin affected endothelial cell behaviour in vitro in the presence of hypoxic lung cancer-conditioned medium (CM). Materials and Methods: Two lung cancer cell lines, A549 and SK-MES-1, were exposed to hypoxic conditions (O_2 ≤1%) for 4 h, after which CM, and RNA were collected. Transcript expression of several influential angiogenic markers in lung cancer cells were assessed following hypoxic/normoxic conditions. Lung cancer CM was added in combination with avastin and DME-25, before or after vascular endothelial growth factor (VEGF) depletion, to endothelial cells (HECV) and cell migration and microtubule formation were assessed in vitro. Results: HECV cell migration was reduced in the presence of avastin, although less efficiently in the presence of lung cancer CM. A combination of DME-25 and avastin with lung cancer CM significantly reduced HECV cell migration irrespective of culture under hypoxia or normoxia. Depletion of VEGF from the CM reduced the inhibitory capacity of avastin, however, it appeared to have little impact on the anti-angiogenic effects of DME-25. Conclusion: DME-25 inhibits tubule formation irrespectively of the factors secreted by normoxic or hypoxic lung cancer cell CM depleted of VEGF.*

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Lung cancer is the most common cause of cancer-related mortality in the UK accounting for more than one in five deaths, a survival rate that has not improved since the 1970s (1). One of the main reasons for this remains the late diagnosis of the disease, which often leaves treatment options limited, potentially even palliative, as illustrated by the poor 5-year survival rate associated with lung cancer. Anti-angiogenic therapies have demonstrated great promise, particularly in lung cancer; however, due to acquired resistance which develops, new treatments and targets continue to be sought.

Due to the advanced understanding of tumour biology, the focus on identifying novel diagnostic, prognostic and therapeutic molecular targets, especially with high specificity for targeted processes, including angiogenesis, has gained momentum. An essential protein that has been identified in tumour angiogenesis is vascular endothelial growth factor (VEGF), as well as its receptor signaling. With the development and licensing of avastin (bevacizumab), a monoclonal antibody against VEGF, the promise of preventing tumour angiogenesis became tangible (2). Avastin as a stand-alone treatment initially yielded good results, however, it ultimately results in tumour cells developing resistance. This resistance was achieved by both cancer and normal cells re-balancing and compensating with other pro-angiogenic mechanisms. Therefore, avastin continues to be used in combination with chemotherapy for advanced non-small cell lung cancer but other ways of inhibiting tumour angiogenesis continue to be sought.

It has long been thought that hypoxia occurs within some parts of malignant tissues, allowing a small subset of highly aggressive tumour cells to adapt and survive in both oxygen- and nutrient-deprived microenvironments. It has been also demonstrated that an increase in genetic instability and decrease in cell proliferation potentially contribute to an increased capacity to metastasise in the presence of hypoxia (3, 4). Often the use of VEGF-targeted therapies, such as avastin, results in tumour hypoxia. Kim *et al*. also demonstrated that the regulator of oxygen homeostasis hypoxia-inducible factor 1α (HIF1α) contributes to the development of resistance to avastin, whilst Rapisarda *et al*. showed increased antitumour activity achieved by combining avastin with HIF1α inhibition (5, 6). Hypoxia-induced reactive oxygen species have also been shown to alter endothelial cell genetic profiles, potentially further enhancing the metastatic potential of cancer cells (5, 7).

Complementary and alternative medicines and treatments are gaining greater recognition throughout the Western world as alternatives to standard therapies. In recent years, several publications focusing on traditional Asian medicines for cancer management have placed focus on these for further scientific interest, resulting in several studies attempting to elucidate their mechanisms of action. One such combination of traditional Chinese herbs, Yangzheng Xiao Ji (YZXJ), demonstrated an improvement in the quality of life of patients with cancer through alleviation of chemotherapeutic adverse effects (8). Subsequent controlled clinical trials and meta-analyses have shown benefits for patients with a broad range of cancer types, including both lung and gastric cancer (9-12). Recent exploration into the anticancer properties of YZXJ *in vitro* by our group has shown inhibition of the adhesion and migration of a range of cancer cells (13-15). YZXJ has also been reported to benefit patients with lung cancer through its ability to disrupt angiogenesis by interfering with several common pathways utilised by endothelial cells. These include inhibition of the focal adhesion kinase (FAK) and RAC-alpha serine/threonine protein kinase (AKT) pathways, as well as mitogen-induced cellular migration, all of which are beyond the traditional angiogenic factor activated pathways (*e.g*. the VEGF pathway), thus potentially providing a method of overcoming acquired resistance to current anti-angiogenic therapies (14, 16).

Materials and Methods

Cell lines. Lung cancer cell lines A549 and SK-MES-1 were purchased from the American Type Culture Collection (Teddington, Middlesex, UK) and the human vascular endothelial cell line HECV was a kind gift from the National Institute for Cancer Research (Genova, Italy). Lung cancer and endothelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotic antimycotic solution (containing penicillin, streptomycin and amphotericin B) (Sigma Aldrich, Poole, Dorset, UK) and incubated at 37˚C, with 5% CO_2 and 95% humidity. For experiments, cells were washed twice and subsequently incubated in DMEM supplemented with Serum Replacement factor 1 (Sigma-Aldrich). Hypoxic conditions were induced using an EVOS chamber (Life Technologies, Paisley, UK) with oxygen levels set at 1%.

Table I. *Primer sequences used in the quantitative gene transcript analyses*.

Target sequence	Sequence (5'-3')	Predicted product size (bp)
GAPDH F	CTGAGTACGTCGTGGAGTC	93
GAPDH ZR	CAGAGATGATGATGACCCTTTTG	
PDPN F	GAATCATCGTTGTGGTTATG	
PDPN ZR	CTTTCATTTGCCTATCACAT	
IL8 F	TCTCTTGGCAGCCTTCCT	94
IL8 ZR	TGTCTTTATGCACTGACATCT	
VEGFA F	GAGCCGGAGAGGGAG	127
VEGFA ZR	CTGGGACCACTTGGCAT	
VEGFC F	CTACAGATGTGGGGGTTGCT	64
VEGFC ZR	GTAGCTCGTGCTGGTGTTCA	
VEGFD F	CAGGGCTGCTTCTAGTTTGG	67
VEGFD ZR	TCTCCACAGCTTCCAGTCCT	
HIF1α F	TTGGAGATGTTAGCTCCCTA	87
HIF1α ZR	GGAACTGCTTTCTAATGGTG	
HGF F	TACGCTACGAAGTCTGTGAC	105
HGF ZR	TCTTGCCTGATTCTGTATGA	

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; *PDPN*: podoplanin; *IL8*: Interleukin-8; *VEGFA/B/C/D*: Vascular endothelial growth factor A/B/C/D; HIF1α: Hypoxic inducible factor 1 alpha; *HGF*: Hepatocyte growth factor. ACTGAACCTGACCGTACA – ZR probe sequence, attached to the 5' end of each of the reverse primers.

Treatment of cancer cells under hypoxia and normoxia. Avastin was bought from University Hospital of Wales Pharmacy, reconstituted in a 0.1% solution of bovine serum albumin/balanced saline solution (BSA/BSS) and used for all experiments at a final concentration of 10 μg/ml.

DME-25, an extract of YZXJ capsules (Yiling Pharmaceutical, Hebei, China) containing a combination of 16 herbs was prepared as previously described (17). In brief, the herbal combination of YZXJ was incubated with DMSO for 24 h at 4˚C. Insoluble constituents were subsequently removed by centrifugation and the remaining extract called DME-25. The DME-25 extract was used at a final concentration of 1:1,000 for all experiments.

Lung cancer cells were treated under normoxia in a routinely maintained incubator supplied with 5% CO_2 and air. For hypoxia, lung cells were placed in a special chamber linked to nitrogen, CO_2 and oxygen, and was programmed to supply 1% oxygen for 4 h. Cells were processed for RNA extraction at the end of the incubation and prepared for quantitative gene-transcript analysis.

Preparation of conditioned medium. Lung cancer cells were seeded into duplicate individual 35×10 mm with vent cell culture dishes

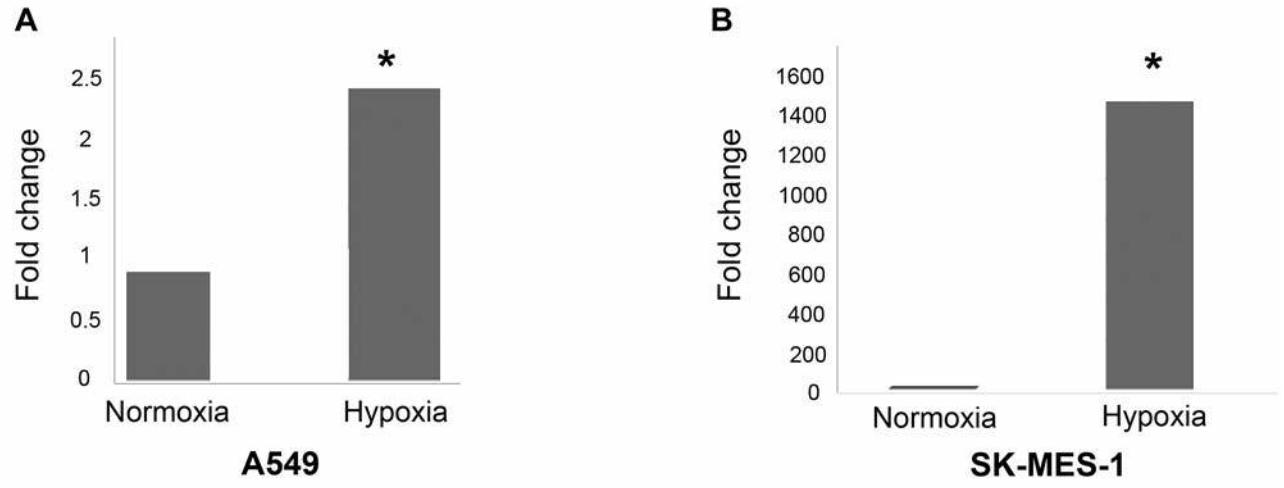

Figure 1. *Transcript expression of HIF-1α in lung cancer cells. A: In A549 lung cancer cells exposed to hypoxia for 4 hours HIF1α transcript expression significantly increased compared to the normoxic control (*p=0.03). B: In SK-MES-1 lung cancer cells exposed to hypoxia for 4 hours HIF1α transcript expression significantly increased compared to the normoxic control (*p=0.03).*

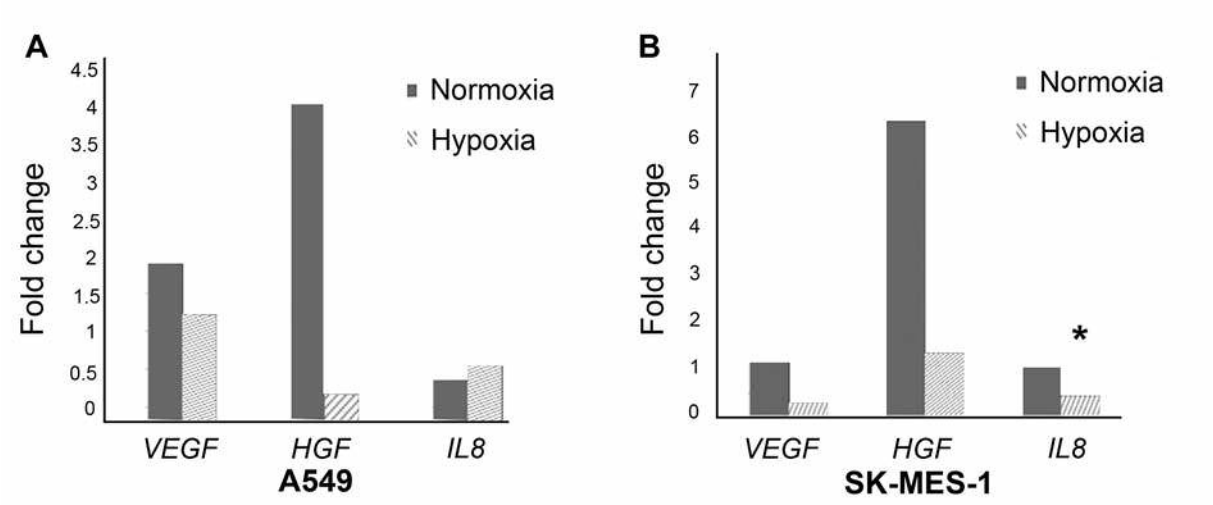

Figure 2. *Transcript expression of vascular endothelial growth factor (VEGFA), hepatocyte growth factor (HGF) and interleukin 8 (IL8) in lung cancer cells. A: In A549 lung cancer cells exposed to hypoxia for 4 h, VEGFA and HGF transcript expression decreased, although this did not reach significance. IL8 transcript expression increased, although again not reaching significance. B: In SK-MES-1 lung cancer cells exposed to hypoxia for 4 h, VEGF and HGF transcript expression was reduced, although not reaching significance. IL8 transcript expression was significantly reduced (p=0.03) compared to the normoxic control.*

(Greiner Bio-one, Stonehouse, Gloucestershire, UK) at a density of 7.5×10^5/ml and left to settle overnight. Cell monolayers were washed twice in BSS and replaced with DMEM supplemented with 1.5ml of serum replacement factor 1. Dishes were placed either into an incubator set at 37˚C, with 5% CO_2 and 95% humidity (considered to be normoxia) or in the EVOS chamber with oxygen levels set at 1% for 4 h (hypoxia). Media was subsequently collected and separated into two Eppendorfs for subsequent migration and tubule formation assays.

RNA isolation, cDNA generation and quantitative PCR (qPCR). RNA was isolated from cultured cells using the Trizol protocol as stated by Sigma Aldrich. In brief, 1 ml of Tri reagent was added to the monolayer of cells to induce lysis. Subsequently the supernatant was transferred to a 1.5 ml Eppendorf containing 200 µl of chloroform (Sigma-Aldrich). Samples were shaken and then centrifuged at 12,000 × *g* for 15 min at 4˚C. The upper aqueous phase was carefully removed and transferred to a new Eppendorf tube containing 500 µl of isopropanol (Sigma-Aldrich). The samples

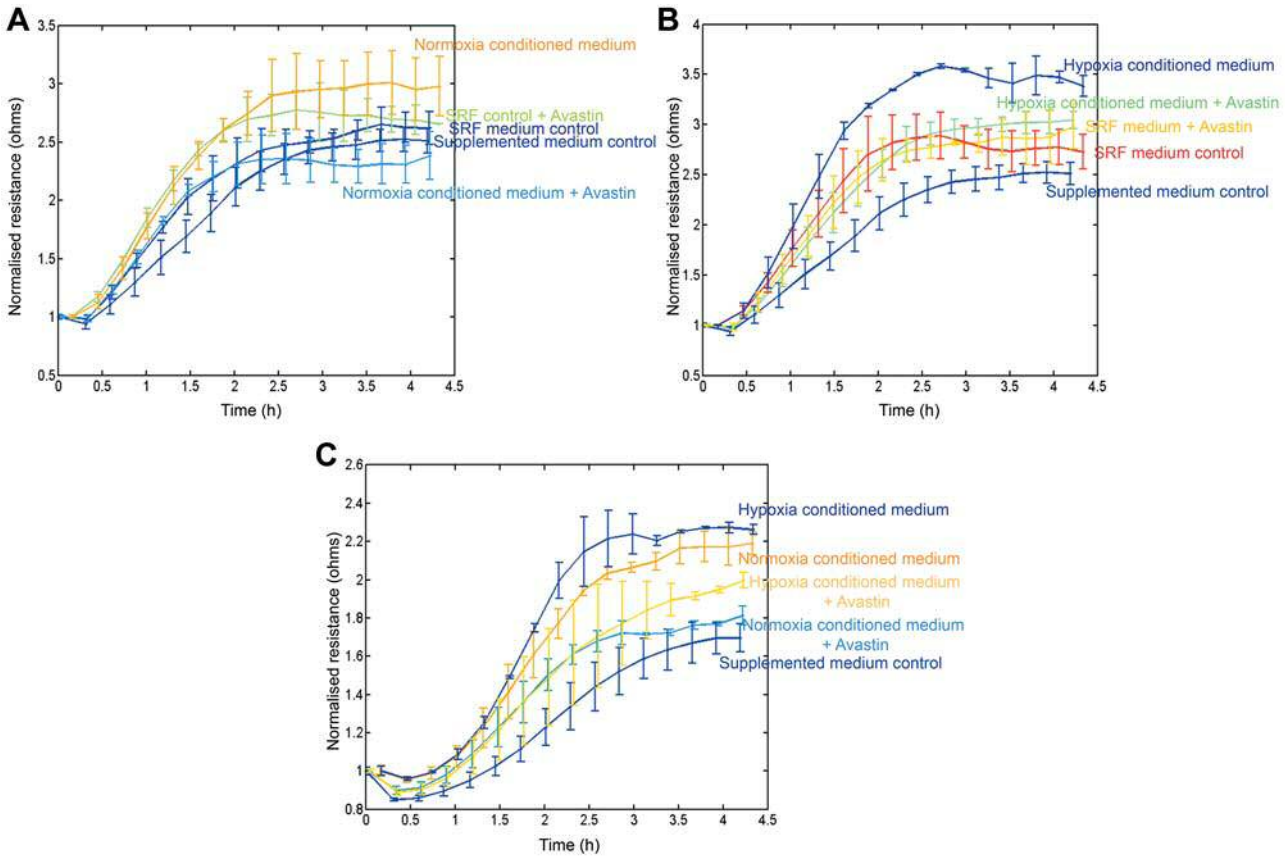

Figure 3. *Impact of avastin in the presence of medium from hypoxic lung cancer cells. A: Conditioned medium from normoxic A549 lung cancer cells increased human vascular endothelial (HECV) cell migration, that was negated by the addition of avastin. B: Conditioned medium from hypoxic A549 lung cancer cells increased HECV cell migration, that was negated by the addition of avastin. C: The combination of hypoxic conditioned media and avastin reduced HECV cell migration less than that observed under normoxic conditions. SRF: Serum replacement factor.*

were shaken and then centrifuged at 12,000 × *g* for 10 min at 4˚C. The supernatant was removed from the Eppendorf tube and replaced with 1 ml 75% ethanol: diethyl pyrocarbonate (DEPC) water after which it was centrifuged at 7,500 × *g* for 5 min. Ethanol:DEPC was removed and the pellet left to air dry at room temperature for a few minutes. The pellet was subsequently resuspended in DEPC water prior to quantification.

RNA was quantified using an IMPLEN Nanophotometer (Munchen, Germany) which was programmed to detect single-stranded RNA (μg/μl) at 260:280 nm ratio using DEPC water as a control. All RNA was standardised to 500 ng.

cDNA to screen for the genes listed in Table I was generated using Precision nanoScript 2 Reverse Transcription kit (Primer Design Southampton, UK). The quantitative analyses were carried out using quantitative transcript analysis with Ampliflor™ technology, which uses a universal probe and a pair of gene-specific primers (one of which carries an additional Z-sequence). qPCR was carried out on a StepOne-Plus unit (ABI, Loughborough, Leicestershire, UK) with a custom master mix from Primer Design. An internal standard was included in each of the assays and used to calculate the sample transcript levels. All PCR reactions, following cDNA synthesis, were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Supernatant VEGF depletion. The supernatant from lung cancer cells was used to treat HECV cells with and without VEGF depletion. VEGF depletion was achieved using immunoprecipitation with neutralizing antibody to VEGF (ab9570; ABCAM, Cambridge, Cambridgeshire, UK) as described below. Supernatant was divided into two equal volumes (750 μl). One aliquot was incubated with agarose beads coated in BSS, while the other aliquot was incubated with agarose beads and anti-VEGF (Neutralisation density (ND_{50}) 10 ng/ml) for 2 h at 4˚C rotating on a blood wheel. Agarose beads were subsequently removed from the samples by centrifugation at 5000 × *g* for 5 min, after which the supernatants were transferred to fresh Eppendorf tubes and labelled as VEGF-containing (control) or VEGF-depleted samples.

Electric cell-substrate impedance sensing (ECIS) for analysing cell adhesion and migration. Cell adhesion and migration were assessed using the high-throughput ECIS method as previously reported (18). ECIS was conducted using an ECIS-Theta model equipped with a

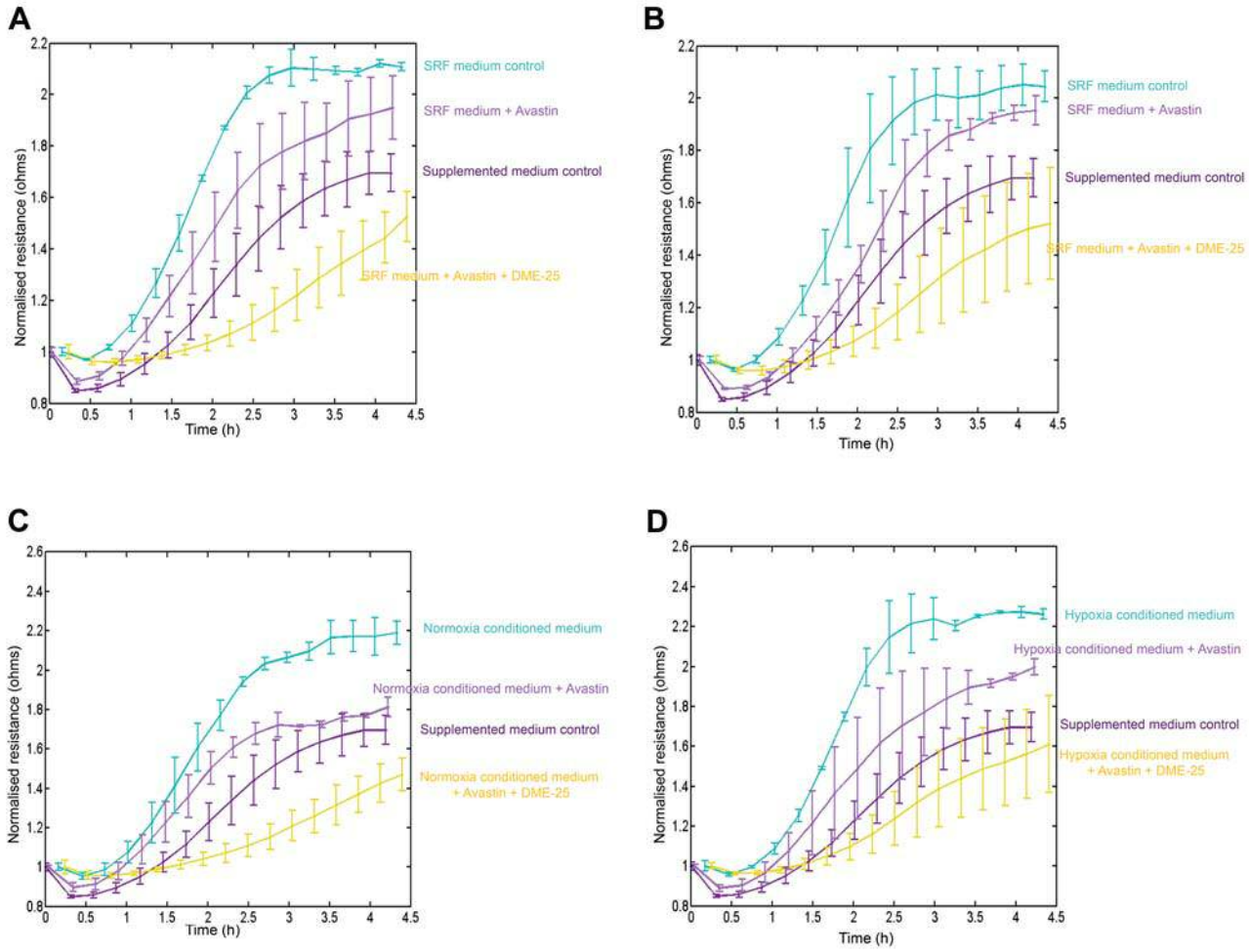

Figure 4. *Impact of avastin and DME-25 in the presence of hypoxic lung cancer media. A) The combination of avastin and DME-25 with A549 normoxic conditioned media significantly reduced HECV cell migration. B) The combination of avastin and DME-25 with A549 hypoxic conditioned media significantly reduced HECV cell migration. C) The combination of avastin and DME-25 with A549 4h normoxic conditioned media significantly reduced HECV cell migration, compared to control and avastin alone. D) The combination of avastin and DME-25 with SK-MES-1 4h normoxic conditioned media significantly reduced HECV cell migration, compared to control and avastin alone. SRF: Serum replacement factor.*

wounding module and in the 96W1E cell arrays, both supplied by Applied Biophysics (Troy, NJ, USA). In brief, 1×10^5 HECV cells were seeded per well and treated with combinations of the supernatant, both control and VEGF-depleted, avastin with/without DME-25. Cells were monitored and the change in resistance over time measured until levels plateaued. Subsequently, in order to assess cell migration, a wound was created by passing an electrical current through the gold electrodes, and cell migration was determined using changes in resistance as cells migrated back over the edges of the artificial wound.

Microtubule-formation assay. A 96-well plate was pre-coated with 10 mg/ml of Matrigel (Corning, St. Davids Park, Flintshire, UK) (~50 μl/well) and left to set at room temperature for approximately 30 min. Each well was seeded with 4×10^4 HECV cells resuspended in DMEM supplemented with serum replacement factor and appropriate treatment combinations of avastin and DME-25.

Results

Changes of angiogenic and other factors associated with angiogenesis under normoxia and hypoxia in human lung cancer cells. In both A549 and SK-MES-1 lung cancer cells exposed to hypoxic conditions for 4 h, there was a significant increase in HIF-1α transcript expression compared to the respective normoxic control (p=0.01) (Figure 1A and B, respectively). The other angiogenic markers screened, *VEGFA*, hepatocyte growth factor (*HGF*) and interleukin 8 (IL8) appeared to differ in their response between cell lines. In A459 cells, *VEGFA* and *HGF* transcript expression seemed to be reduced after 4 h of hypoxia, whereas IL8 transcript expression increased, however, none of these changes crossed the statistical

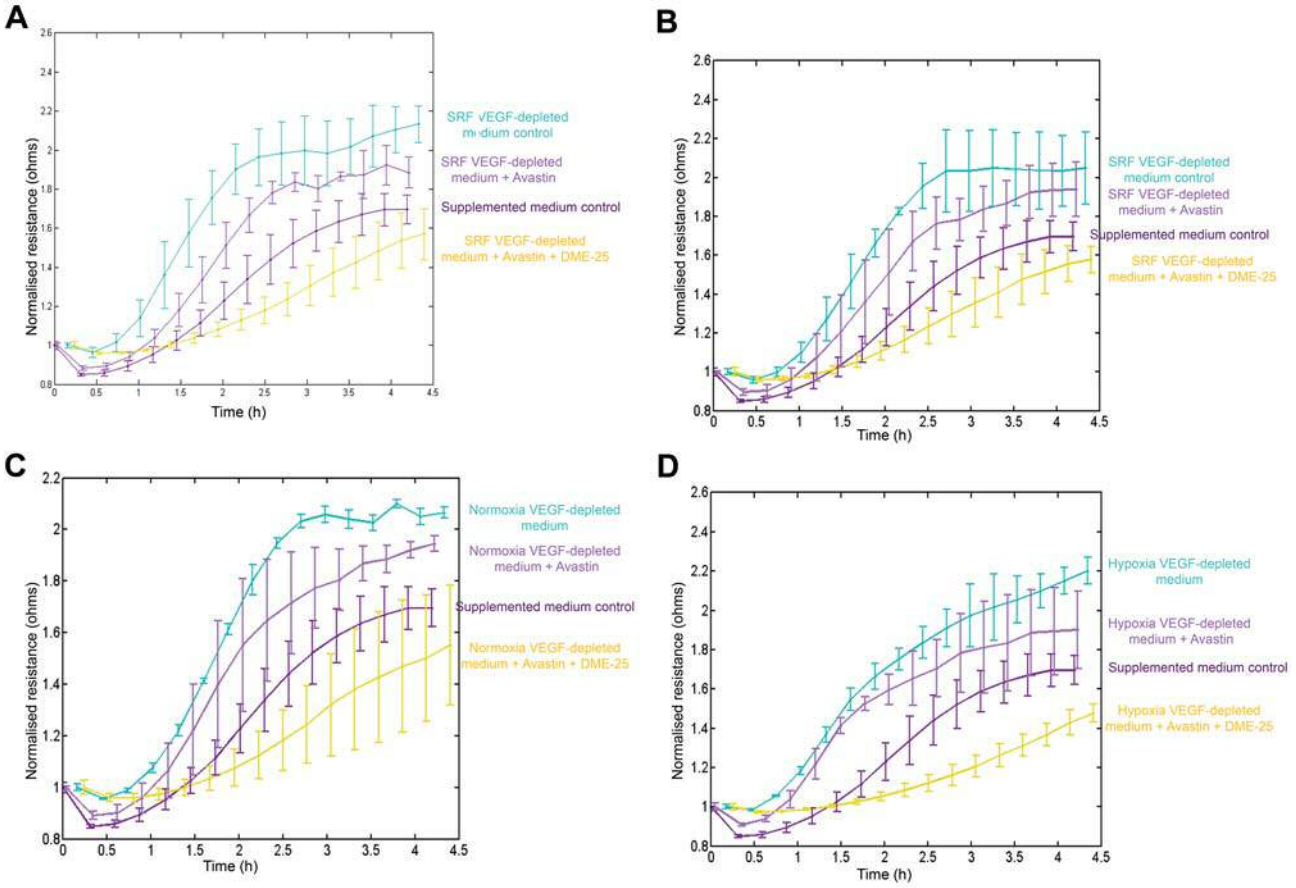

Figure 5. *Impact of avastin and DME-25 in the presence of hypoxic lung cancer media after VEGF-depletion. A) The combination of avastin and DME-25 with A549 normoxic conditioned media significantly reduced HECV cell migration compared to the VEGF-depleted. B) The combination of avastin and DME-25 with A549 hypoxic conditioned media significantly reduced HECV cell migration. C) The combination of avastin and DME-25 with A549 4hrs normoxic conditioned media significantly reduced HECV cell migration, compared to control and avastin alone. D) The combination of avastin and DME-25 with SK-MES-1 4h hypoxic conditioned media significantly reduced HECV cell migration, compared to control and avastin alone. SRF: Serum replacement factor.*

threshold ($p \leq 0.05$) when compared to the normoxic control (Figure 2A). However, in the SK-MES-1 lung cancer cells exposed to hypoxia for 4 h *VEGFA*, *HGF* and *IL8* transcript expression was reduced (Figure 2B). Of these reductions, *IL8* was the only one to cross the significance threshold ($p=0.03$). However, the reduction in *VEGFA* was close to significance ($p=0.06$) when compared to the normoxic control. Multiple other members of the VEGF family transcript expressions (*VEGF-C* and *-D*) were screened. After 4 h hypoxia when compared to the normoxic control; no difference in expression patterns was observed between these family members and VEGFA (Data not shown).

Evaluation of the response of HECV cells to conditioned medium from lung cancer cells. When HECV cells were treated with the serum replacement factor (SRF) medium and compared to a supplemented medium control there was no difference (Figure 3A). There was also no difference in cell migration when the SRF medium was added in addition to avastin (Figure 3A). When A549 lung cancer-conditioned medium was added to HECV cells, there was an increase in HECV cell migration, although this did not reach statistical difference compared to the serum replacement control (Figure 3A). As expected, the conditioned medium from A549 lung cancer cells when combined with avastin resulted in a significant decrease in HECV cell migration (Figure 3A, $p=0.01$ *vs*. SRF control). The addition of avastin to the A549 conditioned medium appeared to restore the level of HECV cell migration to that seen in the SRF control. A similar trend in HECV cell migration was observed when cells were exposed to conditioned medium from hypoxic A549 cells (Figure 3B), although the effect of avastin appeared to be reduced, and did not reach significance compared to the SRF control

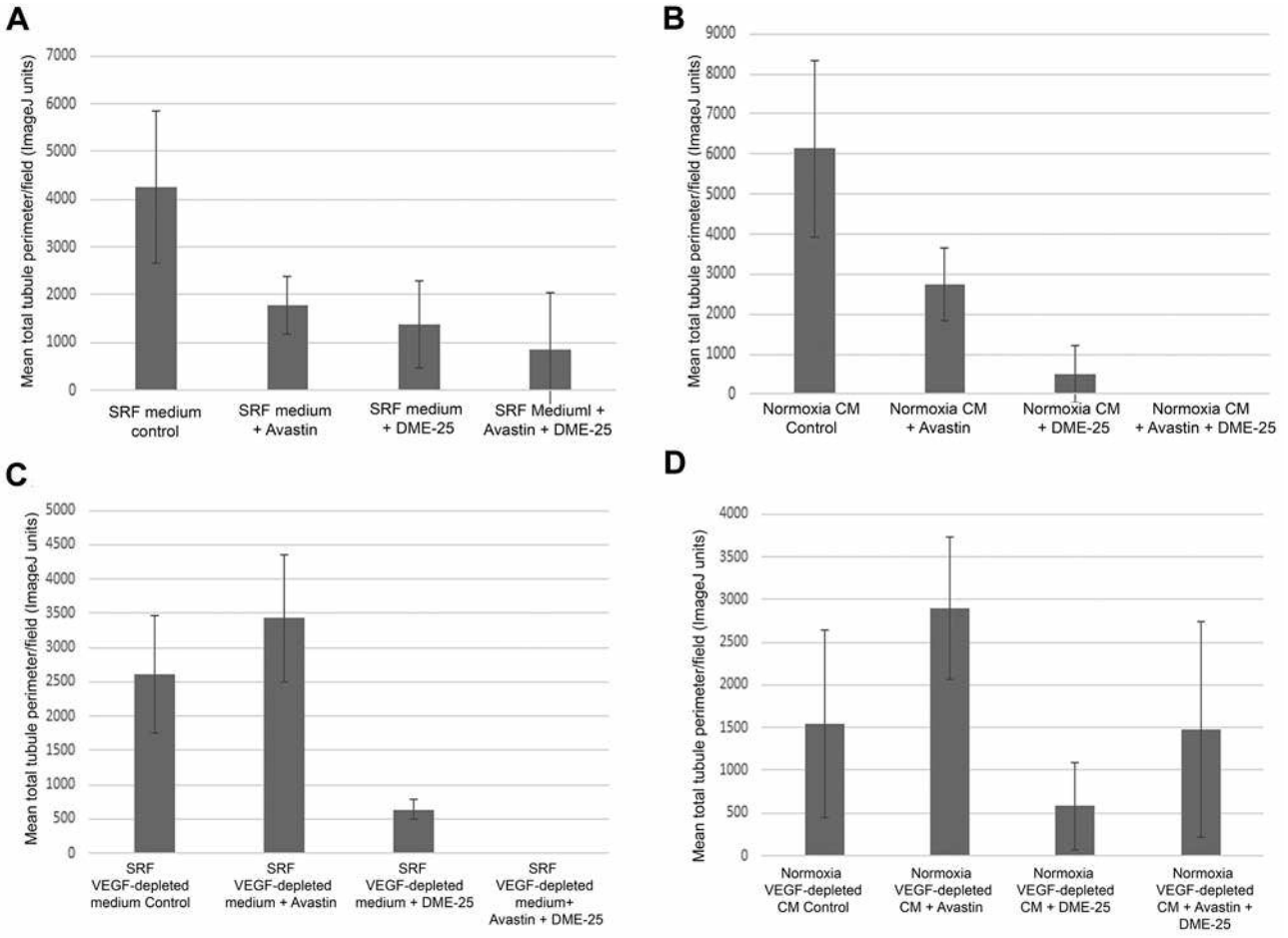

Figure 6. *Impact of avastin and DME-25 on HECV tubule formation in the presence of A549 lung cancer media. A) Micro-tubule formation was reduced in HECV cells treated with A549 normoxic media in combination with avastin, DME-25 or a combination of DME-25 and avastin. B) Micro-tubule formation was reduced in HECV cells treated with A549 4h normoxic media in combination with avastin, DME-25 or a combination of DME-25 and avastin. C) VEGF-depletion of A549 normoxia media removed the inhibitory influence of avastin, however DME-25 and a combination of DME-25 and avastin still inhibited micro-tubule formation. D) VEGF-depletion of A549 4h normoxia media removed the inhibitory influence of avastin, however DME-25 and a combination of DME-25 and avastin still inhibited micro-tubule formation. E) Media control with avastin, DME-25 or avastin and DME-25 all inhibited microtubule formation in HECV cells. SRF: Serum replacement factor, CM: conditioned medium, VEGF: vascular endothelial growth factor.*

(p=0.198). When conditioned medium from cells under hypoxia and under normoxia were directly compared, there was no difference in HECV cell migration (Figure 3C). There was a reduction in HECV cell migration response when avastin was added in combination with the respective conditioned medium. The addition of avastin resulted in a greater reduction in HECV migration when added to conditioned medium from normoxic cells rather than hypoxic ones (Figure 3C).

When SRF medium was added to HECV cells in combination with avastin and DME-25, HECV cell migration was significantly reduced compared to avastin and the serum replacement factor medium control (Figure 4A and B respectively, p<0.001 *vs*. control). A similar reduction in HECV cell migration was also seen when A549 normoxic (Figure 4C, p<0.001 *vs*. control) and hypoxia (Figure 4D, p<0.001 *vs*. control) conditioned medium was added. Under both conditions, the combined addition of avastin and DME-25 resulted in significant reductions in HECV cell migration (p<0.001). With normoxic conditioned medium, this reduction reached significance compared both to the normoxic control and avastin standalone treatment (p=0.01). HECV cell migration was also significantly reduced when compared to the hypoxic control (p<0.001), however, this was not noted when compared to the control with avastin alone.

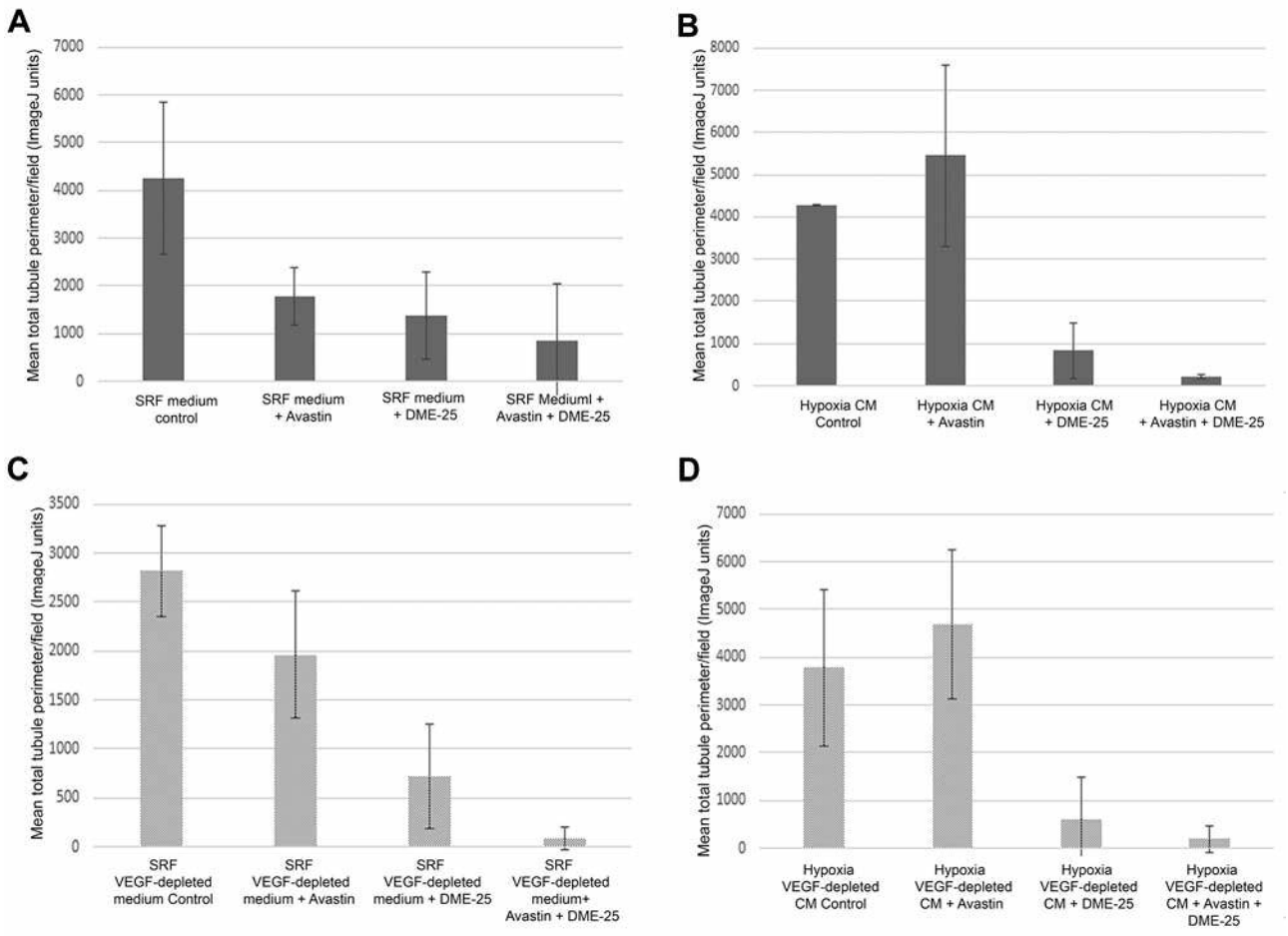

Figure 7. *Impact of avastin and DME-25 on HECV tubule formation in the presence of hypoxic A549 lung cancer media. A) Micro-tubule formation was reduced in HECV cells treated with A549 hypoxic media in combination with avastin, DME-25 or a combination of DME-25 and avastin. B) Micro-tubule formation was unaffected in HECV cells treated with A549 4h hypoxic media and avastin. However, DME-25 or a combination of avastin and DME-25 inhibited HECV tubule formation. C) VEGF-depletion of A549 hypoxic media removed the inhibitory influence of avastin, however DME-25 and a combination of DME-25 and avastin still inhibited micro-tubule formation. D) VEGF-depletion of A549 4h hypoxic media removed the inhibitory influence of avastin, however DME-25 and a combination of DME-25 and avastin still inhibited micro-tubule formation. E) Media control with avastin, DME-25 or avastin and DME-25 all inhibited microtubule formation in HECV cells. SRF: Serum replacement factor, CM: conditioned medium, VEGF: vascular endothelial growth factor.*

The conditioned medium from A549 lung cancer cells was also added to HECV cells after VEGF depletion (Figure 5). As expected, when avastin was added to these cells, less of an effect was noticed on HECV cell migration, with both the hypoxic and normoxic samples with avastin failing to cross the significance threshold (p=0.121 and 0.771, respectively). This was particularly noticeable under treatment with normoxic-conditioned medium (Figure 5C). The addition of both avastin and DME-25 in combination did significantly reduce HECV cell migration compared to the VEGF-depleted controls in both the normoxic and hypoxic conditioned medium (both p<0.001) (Figure 5C and D).

Tubule formation by HECV cells in response to conditioned media from lung cancer cells. As has been previously reported, the addition of avastin inhibits HECV microtubule formation *in vitro* compared to a serum replacement factor medium control. DME-25 extract also inhibited HECV microtubule formation, both on its own and in combination with avastin (Figure 6A). This pattern was mirrored in the presence of A549 lung cancer cell-conditioned media (Figure 6B). After VEGF depletion of the A549 cell-conditioned media, the addition of avastin appeared to have little impact on microtubule formation *in vitro* compared to the controls (Figure 6C and D). Irrespective of VEGF depletion, the addition of DME-25 in combination with

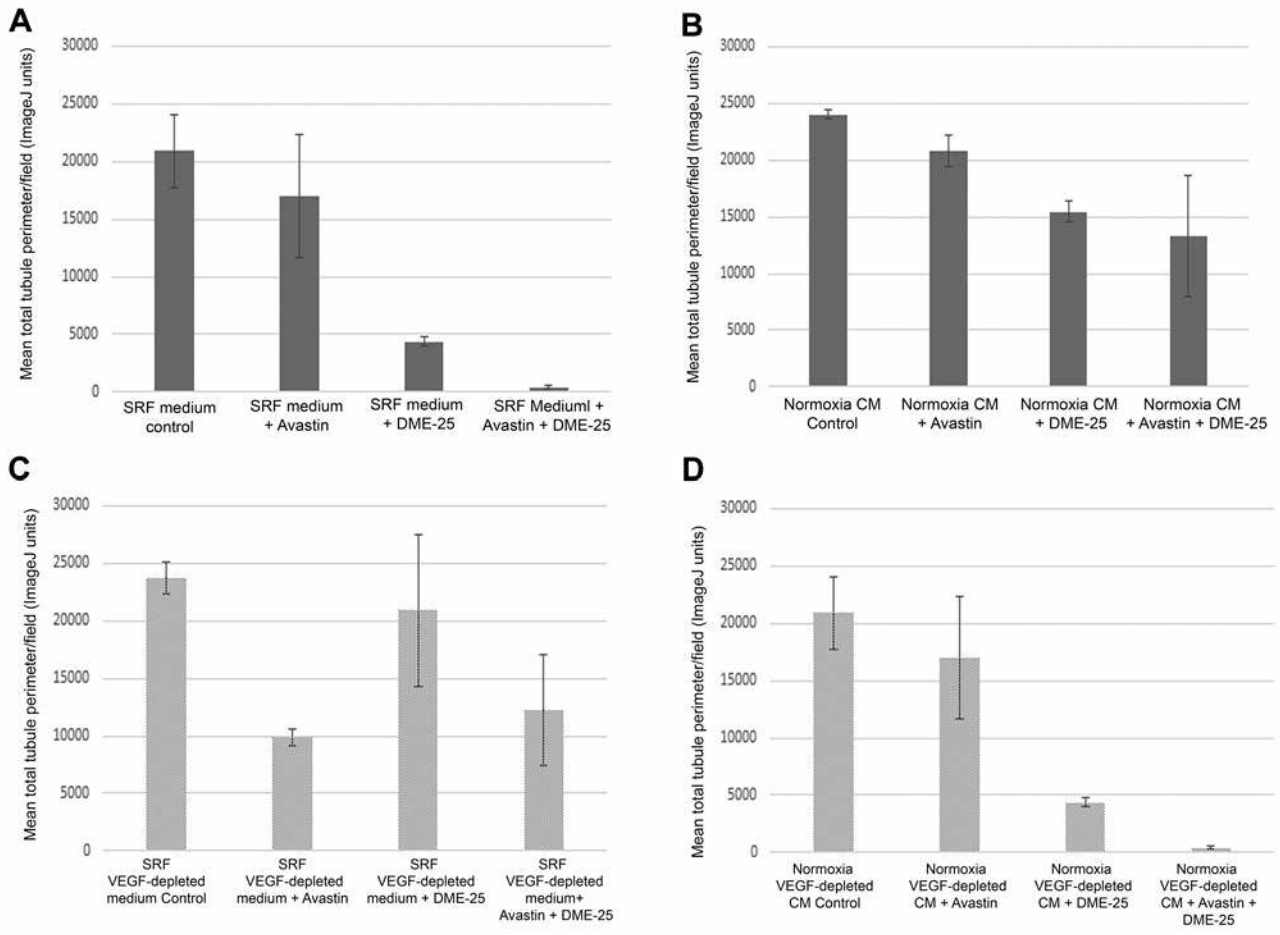

Figure 8. *Impact of avastin and DME-25 on HECV tubule formation in the presence of normoxic SK-MES-1 lung cancer media. A) Micro-tubule formation was reduced in HECV cells treated with SK-MES-1 noroxic media in combination with avastin, DME-25 or a combination of DME-25 and avastin. B) Micro-tubule formation was reduced in HECV cells treated with SK-MES-1 4h noroxic media in combination with avastin, DME-25 or a combination of DME-25 and avastin. C) VEGF-depletion of SK-MES-1 normoxia media did not remove the inhibitory influence of avastin, however DME-25 appeared to have little impact on HECV micro-tubule formation. A combination of DME-25 and avastin inhibited micro-tubule formation. D) VEGF-depletion of SK-MES-1 4h normoxia media removed the inhibitory influence of avastin, however DME-25 and a combination of DME-25 and avastin still inhibited micro-tubule formation. E) Media control with DME-25 or avastin and DME-25 all inhibited microtubule formation in HECV cells. SRF: Serum replacement factor, CM: conditioned medium, VEGF: vascular endothelial growth factor.*

A549 cell-conditioned media continued to inhibit HECV cell tubule formation. However, the addition of a combination of A549 VEGF-depleted conditioned media, avastin and DME-25 did not lead to the same level of inhibition as DME-25 on its own, microtubule formation appeared to have returned to levels similar to those seen in the control (Figure 6D).

When HECV cells were exposed to avastin and SRF medium tubule formation appeared to be inhibited (Figure 7A). Conditioned medium from A549 cells under hypoxic conditions and avastin did not appear to inhibit tubule formation (Figure 7B). The addition of DME-25 or avastin and DME-25 in combination with conditioned medium from A549 cells under hypoxic conditions did reduce HECV cell microtubule formation (Figures 7A and B respectively). As noted, under the normoxic conditioned medium (Figure 6D) VEGF-depletion from hypoxic conditioned medium prevented avastin from inhibiting microtubule formation (Figure 7C and D respectively). However, unlike in normoxic conditions the addition of DME-25 or a combination of avastin and DME-25 appeared to continue inhibiting microtubule formation under the influence of A549 hypoxic conditioned medium.

The SK-MES-1 normoxia conditioned medium appeared to have little impact on microtubule formation *in vitro* irrespective of VEGF-depletion or in the presence of avastin

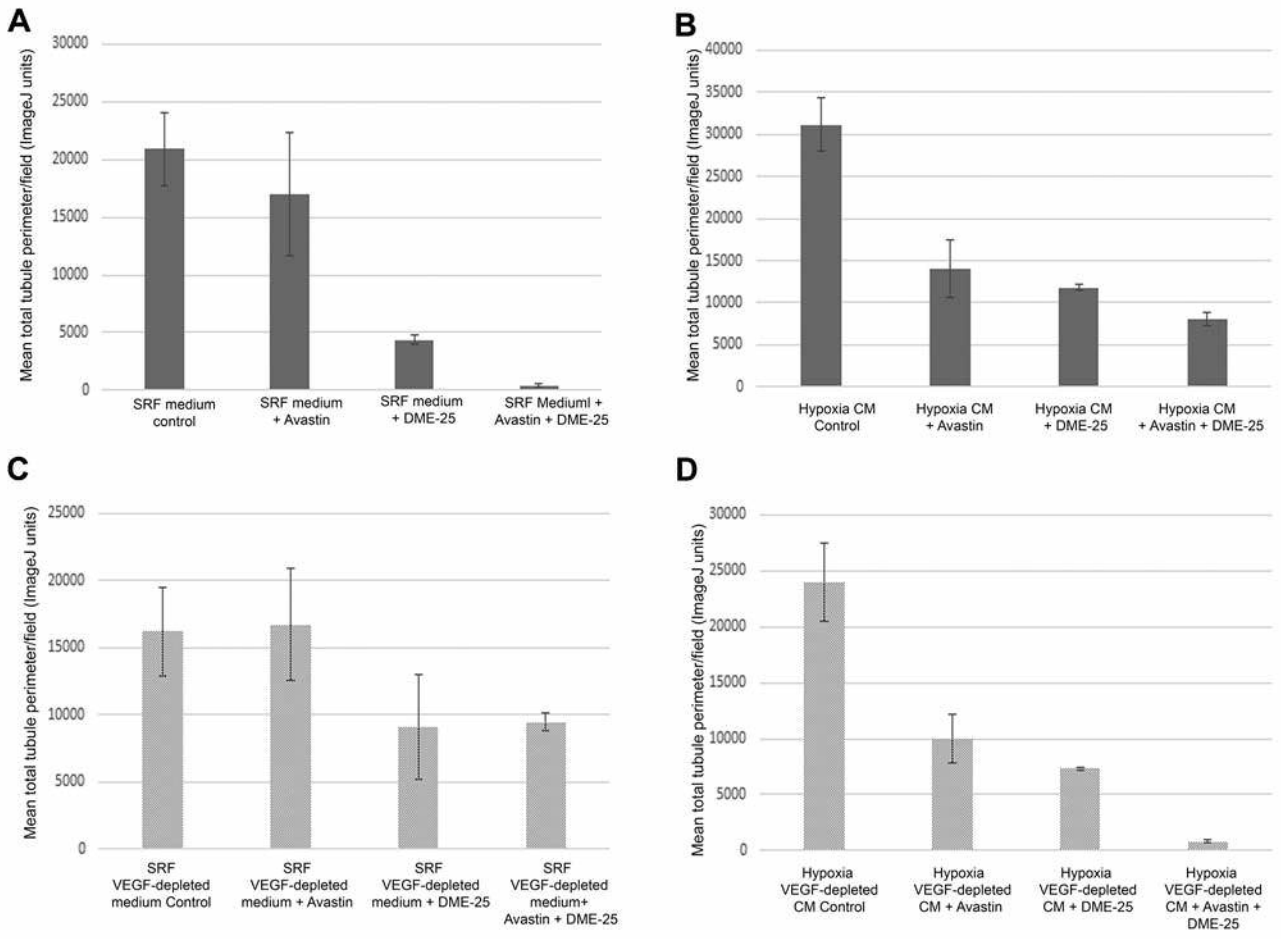

Figure 9. *Impact of avastin and DME-25 on HECV tubule formation in the presence of hypoxic SK-MES-1 lung cancer media. A) Micro-tubule formation was reduced in HECV cells treated with SK-MES-1 hypoxic media in combination with avastin, DME-25 or a combination of DME-25 and avastin. B) Micro-tubule formation was reduced in HECV cells treated with SK-MES-1 4h hypoxic media in combination with avastin, DME-25 or a combination of DME-25 and avastin. C) VEGF-depletion of SK-MES-1 hypoxic media removed the inhibitory influence of avastin, however DME-25 or a combination of avastin and DME-25 still inhibited HECV micro-tubule formation. D) VEGF-depletion of SK-MES-1 4h hypoxia media however did not remove the inhibitory influence of avastin, whilst DME-25 and a combination of DME-25 and avastin continued to inhibit micro-tubule formation. E) Media control with DME-25 or avastin and DME-25 all inhibited microtubule formation in HECV cells. SRF: Serum replacement factor, CM: conditioned medium, VEGF: vascular endothelial growth factor.*

compared to the SRF medium control (Figure 8A –D). The addition of DME-25 to the SK-MES-1 normoxia conditioned medium reduced HECV microtubule formation despite VEGF depletion (Figures 8B and D). This trend was also mirrored when SK-MES-1 conditioned medium was added in combination with avastin and DME-25.

In the conditioned medium from hypoxic SK-MES-1 cells, avastin inhibited microtubule formation compared to the SRF controls (Figure 9A and B respectively). As was noted in the normoxic conditions the addition of DME-25 also inhibited microtubule formation. In the VEGF-depleted control SRF HECV microtubule formation was unaffected by the presence of avastin (Figure 9C). However, this trend was not maintained in the SK-MES-1 hypoxic conditioned medium group. When VEGF-depleted SK-MES-1 conditioned medium was added in combination with avastin, HECV microtubule formation was inhibited. VEGF-depletion did not appear to alter the inhibitory effects that DME-25 or the combination of DME-25 and avastin had on HECV microtubule formation in the presence of SK-MES-1 conditioned media (Figure 9D).

Discussion

With the limitations of avastin as a standalone anti-angiogenic treatment now known, research is continuing in order to identify new angiogenic factors that can be targeted in

combination with VEGF. This approach has resulted in modern medicine embracing some of the ancient traditional Asian medicines, as demonstrated by the active ongoing research investigating the use and mechanisms of action of YZXJ and its extract DME-25. This study suggests that the combination of DME-25 and avastin can affect vascular endothelial cell behaviour (migration and microtubule formation) when exposed to pro-angiogenic factors secreted by A549 and SK-MES-1 cells *in vitro*. This study also demonstrated that factors secreted under hypoxic conditions, particularly by SK-MES-1 cells, result in HECV cells responding less effectively to avastin, although the combination of the YZXJ extract, DME-25, with avastin negated this.

Our group has previously the shown that YZXJ and its extract DME-25 can influence migratory behaviour of HECV cells and a variety of cancer cells *in vitro* through inhibition of the tyrosine phosphorylation of FAK (17). FAK has since long been linked with cell migration and angiogenesis (19). Although the active component(s) of YZXJ remain unknown, this small study demonstrates that it inhibits key pro-tubule formation behaviour of endothelial cells on its own and in the presence of avastin, in the presence of normoxic and hypoxic conditioned media from lung cancer cells. Thus, targeting of the FAK pathway may offer greater benefit in targeting cells that are exposed to hypoxic conditions, not only by inhibition of angiogenesis but also through reducing the migratory potential of endothelial cells. FAK inhibitors are already undergoing testing and have yielded some promising results. The combination of FAK and proto-oncogene tyrosine-protein kinase src inhibitors have demonstrated increased antitumour activity against non-small cell lung cancer (20), while in ovarian cancer, a combination of FAK inhibitors and chemotherapy agents have demonstrated promise in overcoming resistance (21, 22). Herein, and in other previous studies, we showed that this combination of herbs affects a diverse range of cancer cell types and endothelial cell adhesive and migratory properties, this may also provide additional benefits in cancer therapy by limiting the metastatic potential of cells. Therefore, given the potential of hypoxic conditions to drive genetic changes in cancer cells that result in a more metastatic phenotype, discovering and embracing therapeutics that could combat this behaviour may provide novel outlooks in cancer treatment.

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