Inhibitory effects of *Yangzheng Xiaoji* on angiogenesis and the role of the focal adhesion kinase pathway

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Abstract. Angiogenesis is an essential event during the excessive growth and metastatic spread of solid tumours. Anti-angiogenic agents have become a new choice of therapy for patients with cancer. In the present study, we investigated the potential effect of *Yangzheng Xiaoji*, a traditional Chinese medicinal formula presently used in the treatment of several solid tumours including liver cancer and gastric cancer, on angiogenesis, *in vitro*. The human vascular endothelial cell line HECV was used. A Matrigel-based sandwich tubule formation assay was employed to assess *in vitro* angiogenesis, a colorimetric method for assessing *in vitro* cell growth. Electric cell-substrate impedance sensing (ECIS) was used to evaluate the adhesion and migration of endothelial cells. The effects on activation of focal adhesion kinase (FAK) were evaluated using western blotting and immunofluorescence methods. *Yangzhen Xiaoji* extract DME25 significantly inhibited tube formation (p=0.046 vs control). This was seen together with a concentration-dependent inhibition on cell-matrix adhesion and cellular migration. It was demonstrated that the focal adhesion kinase (FAK) inhibitor PF557328 had a significant synergistic effect on DME25-induced inhibition of cell adhesion, migration and tube formation. The study showed that DME25 inhibited the phosphorylation of FAK in endothelial cells. In conclusion, *Yangzhen Xiaoji* has a marked effect on angiogenesis, *in vitro* and that this effect is at least partly mediated by the focal adhesion kinase (FAK) pathway.

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Introduction

The growth of solid tumour beyond certain size and the systemic spread of cancer cells are dependent on the presence and degree of angiogenesis in the tumour (1-3). This realisation has led to discovery and development of anti-angiogenesis agents, both naturally occurring (for example endostatin, angiostatin, VEGI), synthetic and biological forms, for example bevacizumab and thalidomide. The last decade has witnessed the translation of anti-angiogenesis agents into clinical practice. For example, some of the anti-angiogenesis drugs are now almost routinely used on eligible patients (4-9). Some of the anti-angiogenic and anti-cancer compounds are either extracts from natural products or derivatives of natural products (7-9).

Yangzheng Xiaoji is a traditional Chinese medical formula that has been redeveloped in recent years and has been shown to have anti-cancer actions in patients with certain solid tumours. In a recent randomised doubled blinded study of patients with primary liver cancer, patients who received conventional chemotherapy combined with *Yangzheng Xiaoji* (n=304) showed significantly increased rate of disease remission (complete and partial remissions) compared with patients who received chemotherapy alone (n=103) (23.3% vs 14%, respectively, p<0.01) (10). In the study, patients who received combinational therapy also had improved quality of life, based on the Karnofsky method. The formula has also been reported to be able to improve atypical dysplasia in the stomach (11).

The mechanisms of the anti-cancer action of *Yangzheng Xiaoji* are not clear. It has been shown that patients who received the *Yangzheng Xiaoji* and chemotherapy combination has less bone marrow suppression compared with those who received chemotherapy (10). It has been suggested therefore that one of mechanisms underlying the clinical observations is that *Yangzheng Xiaoji* may improve the immune function of the body. However, if the formula has an direct effect on cancer cells is not clear.

In addition to the body's defence, cancer progression is also dependent on the biological characteristics of cancer cells, including the rate of cell proliferation, invasiveness, ability to degrade matrix and migration. Angiogenesis is also a key to the distant spread of cancer cells. The latter cell functions are also closely linked to the metastatic potential of cancer cells.

Naturally occurring compounds have been reported to be able influence a number of these cell functions. For example, Taxol, a plant alkaloid, was initially extracted from western yew bark and is a widely used chemotherapeutic agent (12,13). Fumagillin is also a natural product shown to be a strong anti-angiogenic agent (8,14). Artenisinin, an compound extracted from *Qinhao*, a Chinese medical herb used in the treatment of malaria has also been indicated in cancer treatment (15,16).

In the present study, we report the direct effect of *Yangzheng Xiaoji* extract on *in vitro* angiogenesis and the adhesion and migration of vascular endothelial cells. With little effect on the growth of endothelial cells, it has a marked inhibitor effect on the microvessel-like tubules, cell-matrix adhesion and cellular migration, in a concentration dependent manner. Whilst the extract inhibited the phosphorylation of FAK, it synergistically inhibited the angiogenesis with FAK inhibitor.

Materials and methods

Human endothelial HECV cells were purchased from Interlab (Milan, Italy). The cells were maintained in Dubecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Poole, Dorset, UK) supplemented with penicillin, streptomycin and 10% foetal calf serum (Sigma-Aldrich). The cells were incubated at 37˚C, 5% CO_2 and 95% humidity. Matrigel (reconstituted basement membrane) was purchased from Collaborative Research Products (Bedford, MA, USA). A selective small inhibitor to FAK (FP573228) was from Tocris (Bristol, UK). Antibody to Paxillin were from Transduction Laboratories and phospho-specific antibodies (anti-FAK, anti-pFAK and anti-pPaxillin) were from Santa-Cruz Biotechnologies (Santa Cruz, CA, USA).

Preparation of extract DME25 from Yangzheng Xiaoji for experimental use. Medicinal preparation of *Yangzheng Xiaoji* (Yiling Pharmaceutical, Hebei) was subject to extraction using DMSO, balanced salt solution and ethanol, on rotating wheel for 24 h at 4˚C as we recently reported (17). Insolubles were removed after centrifugation at 15,000 x g. DMSO preparation was found to be more consistent, reproducible and with better yield compared with the other two solvents. DMSO extract was hence used in the subsequent experiments. The extract was standardised by quantifying the optical density of the preparation using a spectrophotometer at a wavelength of 405 nM. A master preparation of the extract which gave 0.25 OD was stocked as the master stock and so named as DME25 for the experiments.

In vitro cell growth assay. This was based on a previous published method (18). HECV cells were seeded into 96 well plates at a density of 3,000 cells/well. Triplicate plates were set up for incubation periods of overnight, 3 days and 5 days. Following sufficient incubation, the plates were removed from the incubator, fixed in 4% formaldehyde (v/v) and stained with 0.5% (w/v) crystal violet. The crystal violet stain was subsequently extracted using a 10% acetic acid (v/v) allowing the detection of cell density through spectrophotmeric analysis of the resulting solutions absorbance using a Bio-Tek ELx800 multi-plate reader (Bio-Tek Instruments Inc., VT, USA).

Electric cell-substrate impedance sensing (ECIS) based cellular adhesion and migration assays. ECIS-Zθ instrument (Applied Biophysics Inc., NJ, USA) were used for cell adhesion and motility (wounding assay) assays in the study (19,20). Cell modelling was carried out using the ECIS RbA modelling software, supplied by the manufacturer. The 96W1E ECIS arrays were used in the present study. ECIS measures the interaction between cells and the substrate to which they were attached via gold-film electrodes placed on the surface of culture dishes. Following treating the array surface with a cysteine solution, the arrays were incubated with complete medium for 1 h. The same number of the respective cells was added to each well. In the cell adhesion assay, the adhesion was tracked immediately after adding the cells into the arrays. For cell migration assay, the arrays with cells were allowed to reach confluence after 3 h. The monolayer of the cells was electrically wounded at 2,000 mA for 20 sec. Impedance and resistance of the cell layer were immediately recorded for a period of up to 20 h. For signalling transduction inhibitor assays, the respective inhibitors was included in the wells. Adhesion and migration were modelled using the ECIS RbA cell modelling software as we recently reported (21,22).

In vitro tubule formation assay. *In vitro* microvessel tubule formation was assessed using a Matrigel endothelial cell tubule formation assay. This was modified from a previously reported method (23,24). Briefly, 250 μg of Matrigel was seeded, in serum-free medium, into a 96-well plate and placed in an incubator for a minimum of 40 min to set. Following this, 35,000 HECV were seeded onto the Matrigel layer and incubated for 4-5 h, in the presence or absence of DME25, FAK inhibitor or their combination. Tubule formation that occurred over the incubation period was visualised under low magnification and images captured. Total tubule perimeter per field was quantified using ImageJ software.

Cell-matrix adhesion. Cell culture plate (96-well) was first coated with 2 μg Matrigel and allowed to air dry. After rehydration for 1 h, the wells were gently washed with DMEM medium, HECV cells (20,000 per well) were added together with the respective treatments. Wells were gently washed with BSS 5 times, before cells were fixed with 4% formalin and stained with crystal violet. Adherent cells were counted and shown as number of adherent cell per high power field of an upright microscope.

Immunofluorescent staining (IFC). HECV cells were seeded at a density of 20,000 cells per well in a 16-well chamber slide (LAB-TEK Fisher Scientific UK, Longhborough, UK), together with the respective treatment for 2 h (25,26). Medium was carefully aspirated from the wells and the cells were fixed in 4% formalin for 20 min. Following fixation, the cells were permeabilised for 5 min in a 0.1% Triton X-100 BSS solution. A blocking solution of (Tris buffer 25 mM Tris, pH 7.4) with 10% semi-skimmed milk was used to block non-specific binding for 40 min. Cells were subsequently washed twice with wash buffer before probing for specific antibodies to FAK and phopho-FAK (SC-1688 and SC-11766, respectively, from Santa-Cruz Biotechnologies, Inc., Santa Cruz, CA, USA). Primary antibodies were made up in the Tris buffer with 3%

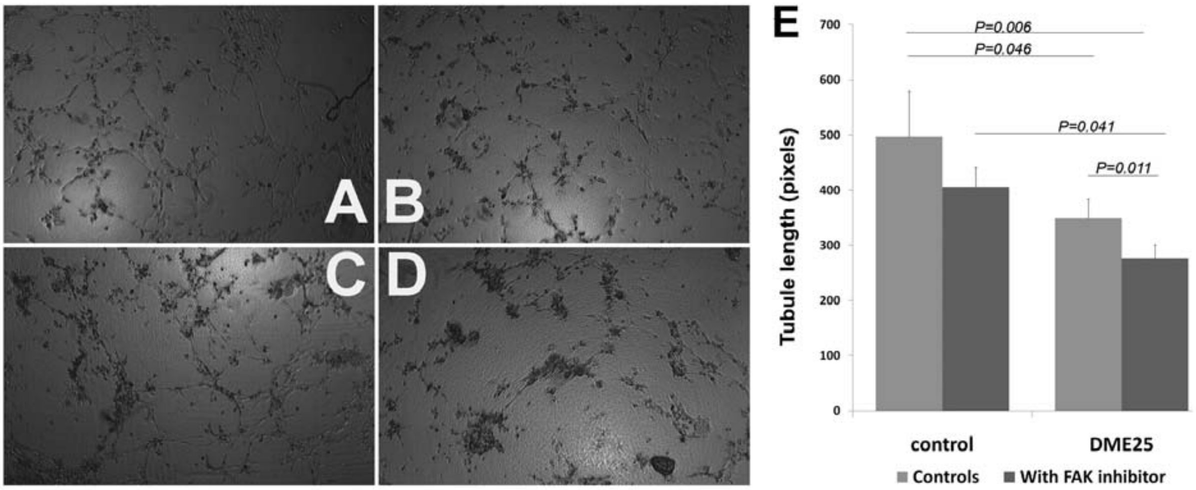

Figure 1. Effects of DME25 on *in vitro* microvessel tubule formation from HECV cells. HECV cells were sandwiched between Matrigel layers and treated with either medium as control (A), with FAK inhibitor (25 nM) (B), with DME25 (1:2,000) (C) or the combination FAK inhibitor and DME25 (D). Tubules are shown as images from inverted microscope (A-D) and as quantified length (E) using digital image analysis. DME25 significantly inhibited tubule forming and had a synergistic effect with FAK inhibitor.

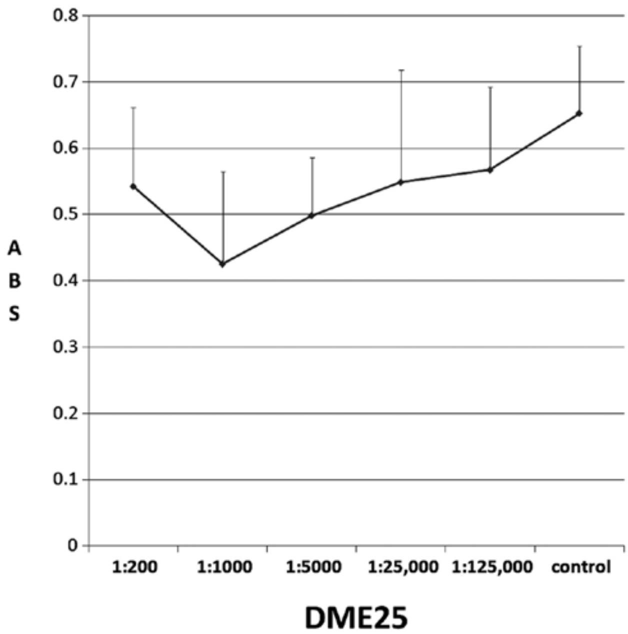

Figure 2. DME25 had little impact of the *in vitro* growth of endothelial cells. Cells were treated with DME25 at difference dilution for 72 h. Cells numbers were evaluated using crystal violet. There was no significant effect on the growth of HECV cells.

milk at a 1:100 concentration for 1 h. The primary antibody was then completely removed by washing the cells 5 times in the same buffer. FITC conjugated anti-mouse and anti-rabbit secondary antibodies (Sigma-Aldrich) was subsequently added to the cells and the slides were incubated on a shaker platform in the dark for 1 h. The slides were finally washed 3 times to remove unbound secondary antibody, mounted with Fluor-save (Calbiochem-Novabiochem Ltd., Nottingham, UK) and visualised under an Olympus BX51 fluorescent microscope at x100 objective magnification.

SDS-PAGE and western blotting. Cells were grow to confluence in a 25 cm^3 tissue culture flask, detached and lysed in HCMF buffer containing 1% Triton X-100, 2 mM $CaCl_2$, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin and, 10 mM sodium orthovanadate on a rotor wheel for 1 h before being spun at 13,000 x g to remove insolubles. The protein levels in the samples were subsequently quantified using the Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, CA, USA).

Once sufficient separation had occurred the proteins were blotted onto a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences UK Ltd., Bucks, UK), blocked in 10% milk and probed for the expression of specific proteins. Anti-pFAK and anti-pPaxillin were used to probe the phophorylated FAK and paxillin, respectively (27). In addition to this, GAPDH expression was also assessed using an antibody specific to this molecule (Santa Cruz Biotechnology Inc.) to assess total protein levels and uniformity throughout the test samples. Protein bands were then visualised through the Supersignal West Dura Extended Duration substrate chemiluminescent system (Perbio Science UK Ltd., Cramlington, UK) and detected using a UVIProChem camera system (UVItec Ltd., Cambridge, UK).

Results

DME25 inhibited formation of microvessel-like tubules without affecting the growth of endothelial cells. Using an *in vitro* tubule formation assay, it was shown that DME25 (shown in Fig. 1 are 1:1000 dilution) significantly reduced tubule length compared with control (p=0.046). This was seen at concentrations at which no growth inhibition was achieved at the concentrations without cytotoxicity on HECV cells (Fig. 2). DME25 over a

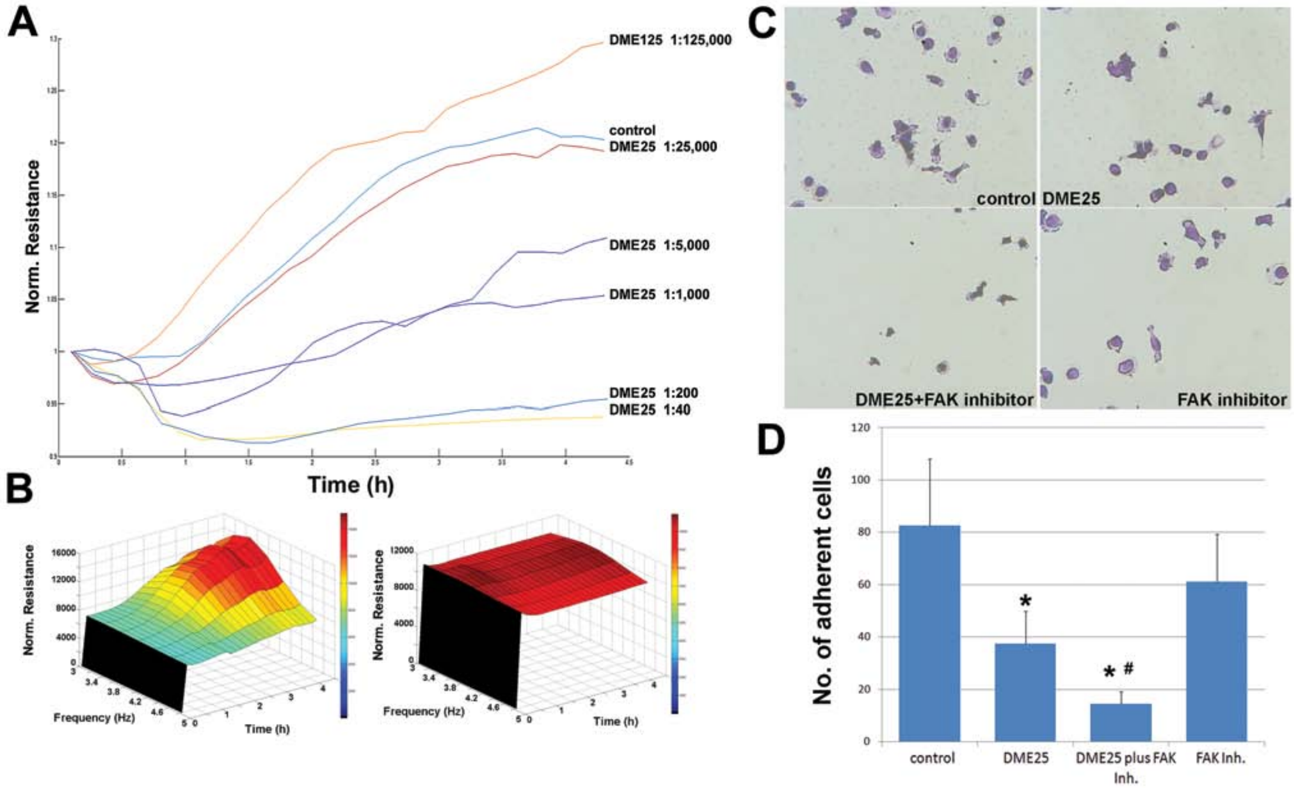

Figure 3. (A and B) Concentration-dependent inhibition of the matrix adhesion of HECV cells by DME25. DME25 was diluted from 1:40 to 1:125,000 (A). Dilutions below 1:25,000 showed inhibitory effect. (B) 3D imaging of the adhesion. Left, control; right, cells with DME25 at 1:1,000. X-axis, frequencies; Y-axis, resistance; Z-axis, time. (C and D) Cell-matrix adhesion investigated by conventional method. (C) Images from crystal violet stained adherence cells; (D) number of adherent cell per high power field. *p<0.01 vs control; #p<0.01 vs DME alone and FAK inhibitor alone.

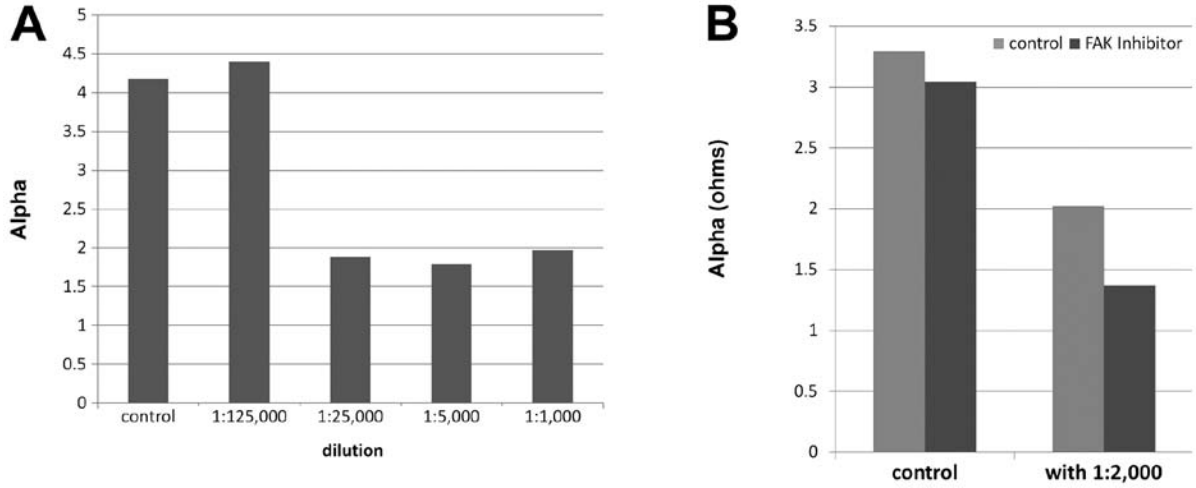

Figure 4. DME25 and cell adhesion at a concentration-dependent matter (A) and is dependent on FAK pathway (B). Shown are data obtained from cell Rb modelling.

wide concentration did not have a significant influence on the growth of endothelial cells.

DME25 exerted an inhibitory effect on cell-matrix adhesion. DME25 demonstrated a concentration-dependent inhibitory effect on the adhesion of HECV cells, with marked inhibitory effects seen at dilutions of 1:5,000 or lower (Figs. 3A and 4). Using 3D modelling, it was seen that the inhibitory effects by DME25 were seen across the frequencies tested (Figs. 3B and 5). Using conventional cell-matrix adhesion method, DME25 had a significant inhibitory effect on the adhesion (Fig. 3C and D).

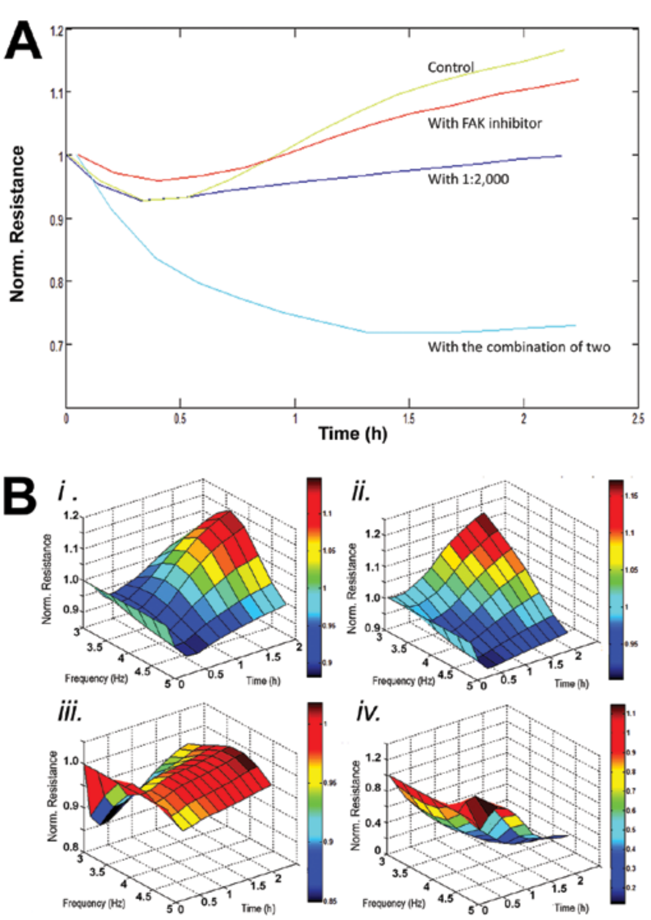

Figure 5. The inhibitory effect of DME25 and the role of FAK. DME25 marked reduced the adhesion, an effect was further strengthened by FAK inhibitor as shown by both the migration trace (A) and 3D modelling (B). (i) Control; (ii) cells treated with FAK inhibitor (25 nM); (iii) cells treated with DME25 (1:1000) and (iv) cells treated with a combination of (ii) and (iii). X-axis, frequencies; Y-axis, resistance; Z-axis, time.

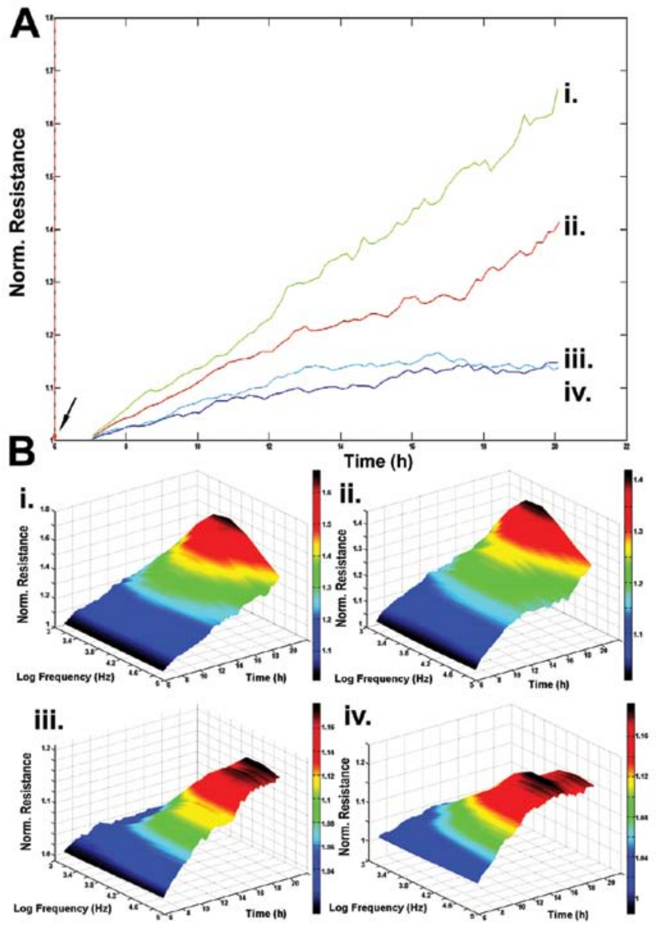

Figure 6. The inhibitory effect on cell migration by DME25 and the role of FAK. DME25 marked reduced the migration, an effect was further strengthened by FAK inhibitor as shown by both the migration trace (A) and 3D modelling (B). (i) Control; (ii) cells treated with FAK inhibitor (25 nM); (iii) cells treated with DME25 (1:1,000) and (iv) cells treated with a combination of (ii) and (iii). X-axis, frequencies; Y-axis, resistance; Z-axis, time. Arrow in A indicates the time point at which electric wounding was conducted.

Endothelial cell migration was reduced by DME25. In a similar fashion to cell-matrix adhesion, cellular migration was similarly inhibited by the presence of DME25 and was further inhibited when FAK inhibitor was used together with DME25 (Fig. 6).

DME25 and FAK inhibitor had a synergistic effect on the adhesion, migration and tubule formation of endothelial cells. FAK inhibitor has a marked effect on the adhesion of HECV cells (Figs. 3C and D, 4B, 5). When administered together with DME25, the inhibitory effect appears to be synergistically strengthened as seen in these figures.

FAK inhibitor appears to have an inhibitory effect on tubule formation although this is not statistically significant (p=0.14) (Fig. 1E). However, the combination between DME25 and FAK inhibitor had a marked inhibition on tubule formation, compared with control, with FAK inhibitor along and DME25 along (p=0.006, p=0.041, p=0.011, respectively).

DME25 inhibited phosphorylation of FAK in endothelial cells. We further evaluated the effect of DME25 on the activation of FAK and paxillin in HECV cells, namely tyrosine phosphorylation in these proteins, using phospho-tyrosine specific antibodies. As shown in Fig. 7, DME25 suppressed phosphorylation of FAK and produced more profound inhibition together with FAK inhibitor. Neither DME25 nor FAK inhibitor or their combinations had marked effect on the phosphorylation of paxillin.

Using immunofluorescence method, FAK was seen to be stained strongly in control cells at the focal adhesion sites (Fig. 8, left panel, indicated by arrows). Addition of DME25, FAK inhibitor and the combination of DMA25 and FAK inhibitor render the cells with less focal adhesion complex although the degree of staining was unchanged compared with control (Fig. 8, left panel). It is very interesting to observe the marked changes of phosphorylated FAK which was stained with phosphorylation specific anti-pFAK antibody. As shown in Fig. 8 (right panel, with extended exposure time), control cells had visible stainings of pFAK at the focal adhesion sites in control cells. Both DME25 and FAK inhibitor resulted in reduction of staining of pFAK. However, cells treated with the combination of DME25 and FAK inhibitor almost completely lost staining of pFAK (Fig. 8 right panel).

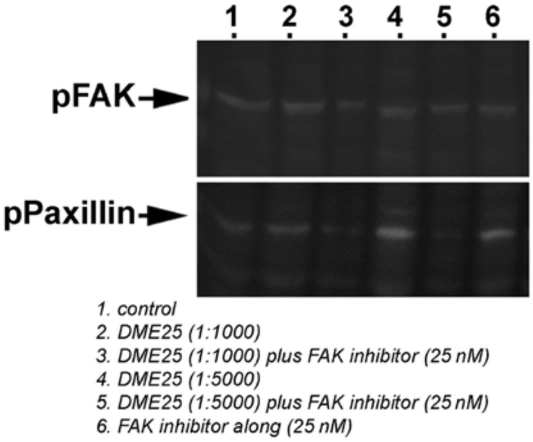

Figure 7. DME25 suppressed phosphorylation of FAK and paxillin in HECV endothelial cells. After serum starvation for 2 h, HECV cells were treated with DME25 (at two different concentrations, namely 1:1,000 and 1:5,000 dilutions), FAK inhibitor (25 nM) or their combination for 60 min. After separation of the equal amount of proteins on SDS PAGE gels, phosphorylated FAK and paxillin were probed using phospho-specific antibodies to FAK and paxillin.

Discussion

Anti-angiogenesis therapies, for example Avastin, have now been used as new line of therapies in solid tumours and have been shown to have their clinical worthiness in some tumour types. A few of the traditional anti-cancer compounds have also been found to have their role in anti-angiogenesis. *Yangzheng Xiaoji* is a new formula developed from traditional Chinese medicine and has been shown to have clinical benefit in patients with cancers, namely liver cancer and gastric cancer, two of the leading cancer types in China (10,11). The precise mechanism(s) of the formula is not clear, although there has been indication that it may have some immune protective effects when administered during chemotherapy. However, in a recent preliminary study (17), *Yangzheng Xiaoji* has been shown to have an inhibitory effect on the adhesion and migration of cancer cells. These two cell functions are also critical during the angiogenic process of endothelial cells.

The present study attempted to examine the potential effect of *Yangzheng Xiaoji* on angiogenesis. Our initial screening met with a surprising finding that extract from *Yangzheng Xiaoji*, DME25 markedly inhibited *in vitro* tubule formation from vascular endothelial cells. We further demonstrated that the

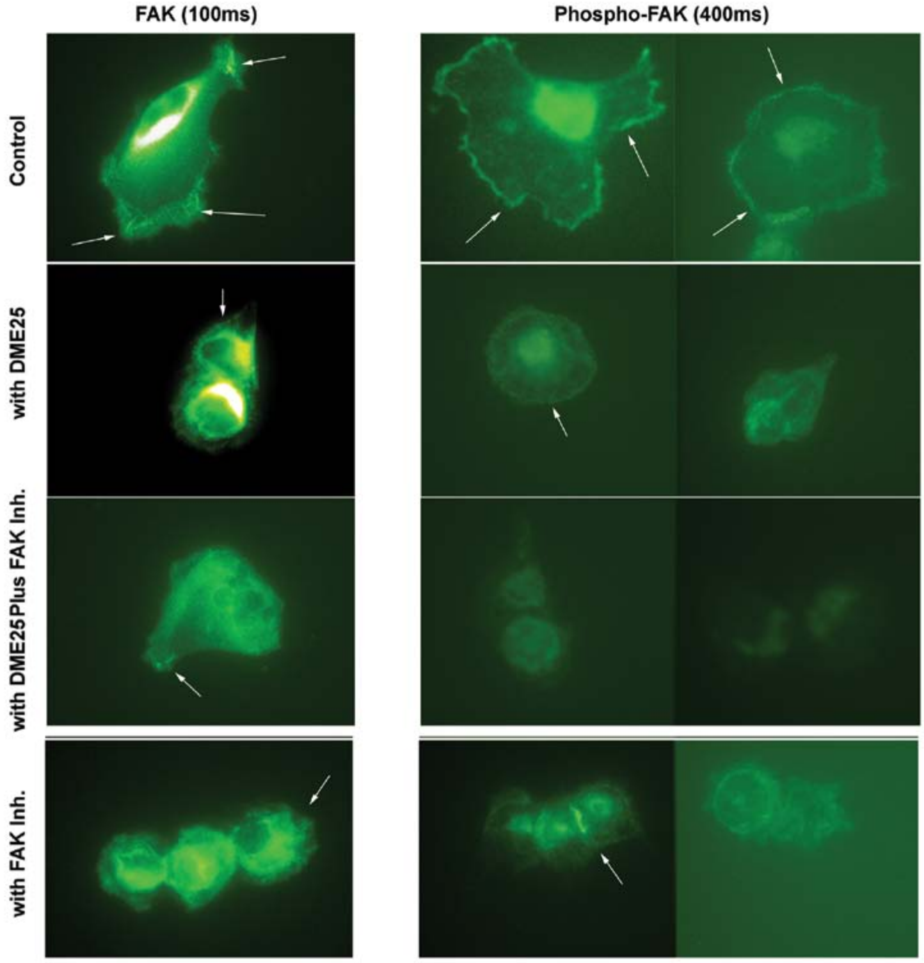

Figure 8. Immunofluorescence staining of focal adhesion kinase (FAK) and phospho-FAK in HECV cells. 20,000 HECV cells were added together with medium as control, DME25 (1:1,000), FAK inhibitor or the combination of DME25 and FAK inhibitor. Cells were washed and fixed after 2 h. Total FAK (left panel) and phosphorylated FAK (right) were stained, respectively, using anti-FAK and anti-pFAK antibodies. Images for FAK was obtained with 100 msec exposure and that of pFAK 400 msec.

extract has a concentration-dependent inhibitory effect on cell-matrix adhesion and cellular migration. These results are interesting and appear to be connected. Cell-matrix adhesion is an important part during cellular migration and both the adhesion and migration are essential during angiogenesis and in particular during tubule formation in the model of the present study, namely sandwich based tubule formation assay. Orchestrated adhesion to matrix and migration over matrix are necessary for the endothelial cells to join and form vessel-like tubules. Thus, it is plausible to suggest that the effect on adhesion and migration is likely to be the key contributing factor to the inhibition on tubule formation.

The other interesting finding of the present study is that blocking FAK using a small FAK inhibitor markedly strengthened the effect of *Yangzheng Xiaoji* extract and that the extract itself has an inhibitory effect on the activation of FAK, namely tyrosine phosphorylation of FAK, which was seen by both western blotting and immunofluorescence methods. FAK pathway is essential during cell-matrix adhesion and cellular adhesion over extracellular matrix (27-30). Upon interacting with matrix, cells utilise the membrane integrins to bind to the matrix and trigger the activation of series intracellular events, one of the key pathway is the activation of focal adhesion kinase, which in turn leads to activating the integrin interaction with the cytoskeletal system (31). This forms an essential component during matrix adhesion and subsequent cell migration. FAK has been shown to be amongst key signalling pathways during angiogenesis (31-35). FAK inhibitors, such as the one used in the present study, has been shown in early clinical trials to have anti-cancer effects in patients with lung cancer and breast cancer (36-40). Extract from herbs has been previously shown to affect the activities of FAK in endothelial cells (41,42). Together, it can be argued that one of the key pathways that *Yangzheng Xiaoji* targets is FAK pathway during the angiogenic process. However, these results should be interpreted with caution for the following reasons. First, *Yangzheng Xiaoji* is a mixture of herbal medicine. The active ingredient(s) in the formula is yet to be found. The effect seen in the present study may well be a mixed effect of the extract. Second, angiogenesis requires a great deal more coordination of endothelial cells, than cell adhesion and cellular migration. Effects on other cellular events should also be examined.

In conclusion, the anti-cancer traditional formula, *Yangzheng Xiaoji*, has a profound effect on angiogenesis, *in vitro*. This is seen together with the reduction of cell-matrix adhesion and cellular migration and is likely to be mediated by the focal adhesion kinase (FAK) pathway.

Acknowledgements

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