Vascular endothelial growth inhibitor, expression in human prostate cancer tissue and the impact on adhesion and migration of prostate cancer cells *in vitro*

NING ZHANG[1,2], ANDREW J. SANDERS[1], LIN YE[1], HOWARD G. KYNASTON[1] and WEN G. JIANG[1]

[1]Metastasis & Angiogenesis Research Group, Cardiff University School of Medicine, Cardiff CF14 4XN, UK; [2]Department of Urology, Beijing ChaoYang Hospital, Capital University of Medical Science, Beijing 100020, P.R. China

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Abstract. Vascular endothelial growth inhibitor (VEGI) has been associated with tumour-related vasculature in certain maligancies. However, its implication in prostate cancer remains unknown. We investigated the expression pattern and role of VEGI in prostate cancer and prostate cancer cells. The expression of VEGI was examined in human prostate tissue and prostate cancer cell lines. The biological impact of modifying the expression of VEGI in prostate cancer cells was evaluated using *in vitro* models. VEGI mRNA was expressed in a wide variety of human prostate cancer cell lines and most prostate specimens. VEGI protein was seen in normal prostate epithelium, but was decreased or absent in prostate cancer specimens, particularly in tumours with high Gleason scores. Moreover, forced-expression of VEGI led to a decrease in the motility and adhesion of prostate cancer cells *in vitro*. In contrast, knocking down VEGI in the cells resulted in an increase in motility and adhesion. Interestingly, both forced-expression and knocking down of VEGI had no bearing on growth and invasive capacity of prostate cells. In conclusion, the expression of VEGI is decreased in prostate cancer and is almost absent in tumours with high Gleason scores. Together with its inhibitory effect on cellular motility and adhesion, this suggests that VEGI functions as a negative regulator for aggressiveness during the development and progression of prostate cancer.

Introduction

Prostate cancer is the most commonly diagnosed male cancer in Western countries and the second leading cause of cancer deaths in men (1,2). Despite the improvements in both early detection and treatment, we are still facing significant challenges in managing this disease.

Correspondence to: Professor Wen G. Jiang, Metastasis & Angiogenesis Research Group, Department of Surgery, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, UK
E-mail: jiangw@cf.ac.uk

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Vascular endothelial growth inhibitor, VEGI [also known as tumour necrosis factor superfamily member 15 (TNFSF15) and TNF ligand related molecule 1 (TL1)], is a recently identified anti-angiogenic cytokine that belongs to the TNFSF (3-5). Three isoforms of VEGI have been reported, all sharing a common 151 C-terminal amino acid sequence but differing in their N-terminal regions (6-8). Its transcript was found to be expressed in the placenta, lung, kidney, skeletal muscle, pancreas, spleen, small intestine, prostate and colon. The secreted soluble form of VEGI has been demonstrated as a potent anti-angiogenic factor through inhibiting proliferation of endothelial cells (7-9). Previous studies have demonstrated an intricate relationship between VEGI and carcinoma *in vitro* and *in vivo*. VEGI has been shown to suppress the growth of colon carcinoma cells (murine colon cancer cells, MC-38) both *in vitro* and *in vivo* (4,10). Systemic administration of VEGI markedly inhibited tumour growth and increased survival time in a Lewis lung cancer (LLC) murine tumour model (11). Parr *et al* reported that patients with breast tumours expressing reduced levels of VEGI had a higher local recurrence, shorter survival time and an overall poorer prognosis than those patients expressing high levels of VEGI (12). This suggests that the anti-tumour activity of VEGI may not be due to a direct effect on cancer cells, but rather due to interference with the development of tumour-associated vasculature. VEGI had no inhibitory activity on the growth of cancer cells *in vitro* (4,5,8,10,13).

Despite these observations of VEGI in solid tumours, its role in prostate cancer remains unknown. In the current study, the expression of VEGI was examined in human prostate tissues, prostate tumours, and in prostate cancer cell lines. The biological function of this molecule was investigated in prostate cancer cells, in which the expression of VEGI was manipulated using genetic methods.

Materials and methods

Materials. Prostate tissue samples were snap-frozen in liquid nitrogen immediately after radical prostatectomy, transurethral prostatectomy or prostate biopsy. All protocols were reviewed and approved by the local ethics committee and all patients gave written informed consent. PC-3 (ECACC, Salisbury, UK); DU-145, LNCaP, CA-HPV-10 and PZ-HPV-7 (ATCC);

Table I. PCR primer sequences.

Primer	Forward	Reverse
hGAPDH	5'-AGCTTGTCATCAATGGAAAT	5'-CTTCACCACCTTCTTGATGT
hGAPDH(Q-PCR)	5'-CTGAGTACGTCGTGGAGTC	5'-ACTGAACCTGACCGTACACAGAGATG ATGACCCTTTTG Z-sequence
VEGI	5'-ATGAGACGCTTTTTAAGCAA	5'-CTATAGTAAGAAGGCTCCAAAG
VEGI (Q-PCR)	5'-CAAAGTCTACAGTTTCCCAAT	5'-ACTGAACCTGACCGTACATGATTTTTA AAGTGCTGTGTG Z-sequence
VEGI (ribozyme1)	5'-CTGCAGTCATTGGGAAACTGT ACTGATGAGTCCGTGAGGA	5'-ACTAGTGAGACGCTTTTTAAGCAAAGT TTCGTCCTCACGGACT
VEGI(ribozyme2)	5'-CTGCAGTCTCACAACTGGAAA CTGATGAGTCCGTGAGGA	5'-ACTAGTTAATCCTCTTTCTTGTTTCGTC ACGGACT
VEGI (expression)	5'-ATGAGACGCTTTTTAAGCAA	5'-CTATAGTAAGAAGGCTCCAAAGA
Primers for detecting plasmid	T7F: TAATACGACTCACTATAGGG	BGHR: TAGAAGGCACAGTCGAGG
Primers to detect ribozymes	RBTPF: CTGATGAGTCCGTGAGG ACGAA	RBBMR: TTCGTCCTCACGGACTCATCAG

PNT-1A and PNT-2C2 (kindly provided by Professor N. Maitland, University of York) were used. Monoclonal mouse anti-human-VEGI was purchased from Santa Cruz Biotechnology, Inc. (SC-53975, CA, USA).

RNA isolation and reverse transcription PCR. RNA was isolated using total RNA isolation reagent (ABgene, Epsom, UK). Reverse transcription and PCR was performed using standard methods.

Immunohistochemical and immunocytochemical staining of prostate specimens and cells. Prostate tumour (n=20) and normal prostate (n=11) tissue was sectioned using a cryostat, and verified by two pathologists. For immunocytochemical staining of VEGI expression, cells were fixed and then permeabilized with 0.1% Triton for 5 min in TBS. Following blocking with horse serum, the samples were probed with anti-VEGI antibody, and labelled with a biotinylated secondary antibody, and subsequently visualised using Vectastain Universal Elite ABC kit (Vector Laboratories, Peterborough, UK).

Construction of VEGI expressing and ribozyme transgenes and transfection (14,15). The full-length human VEGI coding sequence and hammerhead ribozyme targeting human VEGI were cloned into a mammalian expression plasmid vector (pEF/His TOPO TA, Invitrogen, Inc., Paisley, UK). Full primer sequences are provided in Table I. Empty control plasmids or plasmids containing VEGI expression sequence or ribozyme transgenes were then transfected into cells using electroporation. After selection with blasticidin, the transfectants were used in the studies.

Real-time quantitative polymerase chain reaction (Q-PCR) (12,16). Real-time quantitative PCR was carried out using the iCycler^{iQ5} system (Bio-Rad, Hemel Hemstead, UK) to determine the level of expression of the VEGI transcripts in the cell lines. GAPDH was used as a quantitative control.

Cell growth assay (17). Cells were seeded into a 96-well plate (2,500 cells/well). Cell growth was assessed after 1, 3 and 5 days. Crystal violet was used to stain the cells, and the absorbance was determined at a wavelength of 540 nm using a spectrophotometer (BIO-TEK, Elx800, UK).

Wounding assay (14,15). The migration of the cells was determined using wounding assay. A monolayer of cells was scraped with a fine gauge needle. The movement of cells close to the wound was recorded on a digitised time lapse video recorder and analyzed using an imaging analysis tool (OPTIMAS).

Motility assay using Cytodex-2 beads (14,15). Cells ($1x10^6$) were incubated with 100 μl of cytocarrier beads overnight. The beads were washed twice to remove any dead cells, and then re-suspended. One hundred microliters of beads/cells were transferred into a 24-well plate. After incubation for 4 h, the cells were fixed in 4% formalin and stained with 0.5% crystal violet, before counting.

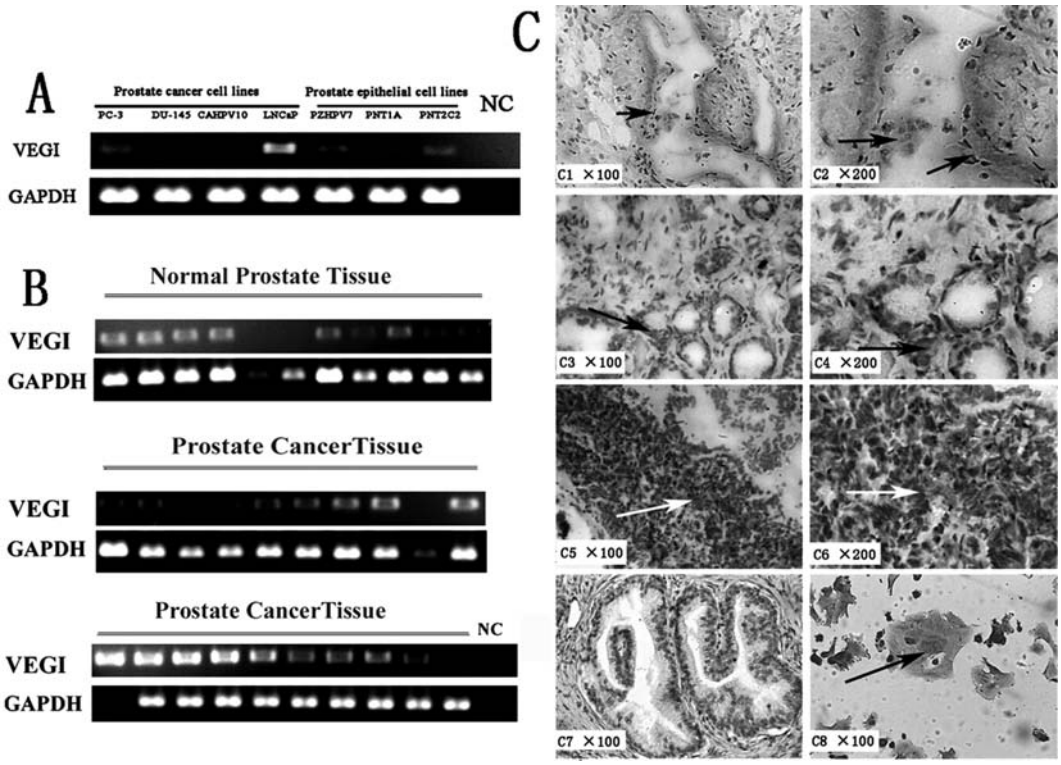

Figure 1. VEGI expression in prostate cancer tissues and cell lines. (A) Detection of the VEGI transcript using RT-PCR in a panel of human prostate cell lines. (B) Detection of the VEGI transcript in a panel of normal and tumour tissues. GAPDH was used as the housekeeping control. (C) Immunohistochemical staining of human prostate specimens. C1 and C2 are normal prostate tissues. The VEGI protein was found to be intensively stained in the cytoplasmic area of normal prostate epithelial cells (indicated by black arrows). C3 and C4 are prostate cancer tissues with a Gleason score of 7. VEGI was only weakly stained in these cells (black arrows). C5 and C6 are prostate cancer tissues with a Gleason score of 9. The VEGI protein staining was nearly absent (white arrows). That is, VEGI expression was seen to be negative or weakly positive in the cancer cells of prostate cancer tissue. C7 is a negative control with normal prostate tissue. C8 is a positive control with DU-145^{VEGIexp4}. The VEGI was forcibly-expressed in the cytoplasmic area.

Invasion assay (14,15). Transwell inserts with 8 μm pore size were coated with 50 μg Matrigel (BD Matrigel™ Basement Membrane Matrix) and air dried. After rehydration, 20,000 cells were added to each well. After 96 h cells that had migrated through the matrix to the other side of the insert were fixed, stained and then counted under a microscope.

Cell-matrix adhesion assay (15,18). Cells were seeded into a 96-well plate which had been pre-coated with Matrigel (5 μg/well). After 40 min of incubation, non adherent cells were washed off using BSS. The adhered cells were fixed, stained and then counted.

Electric cell-substrate impedance sensing (ECIS) based motility assay (19). The ECIS-1600R model instrument and 8W10 arrays (Applied Biophysics, Inc., NJ) were used in this study. Cells (300,000) were added to each well of the ECIS arrays. After 3 h when confluency was reached, the monolayer was electrically wounded at 6 V for 30 sec for each well. Impedance and resistance of the cell layer were immediately recorded for a period of up to 20 h.

Statistical analysis. All statistical analysis was performed using the SPSS 16.0 software. The two sample t-test was used for normally distributed data. Fisher's exact test was used for analysing immunohistochemical staining in prostate tissues. Differences were considered to be statistically significant at $P<0.05$.

Results

The expression of VEGI in prostate tissues and cell lines. The expression of VEGI was examined in seven prostate cell lines and human prostate tissues using conventional RT-PCR. VEGI transcript was detectable in most cell lines except CA-HPV-10 (Fig. 1A). It was also detected in 9 of 11 normal prostate tissues, and 17 of 20 prostate cancer tissues (Fig. 1B). In immunohistochemical staining, VEGI was seen in normal prostate epithelia cells, but the staining was decreased or absent in prostate cancer cells, particularly in specimens with higher Gleason scores (Fig. 1C). The positive staining of normal tissue (81.8%, 9/11) was significantly higher than that of prostate cancer tissues (25%, 5/20), P=0.007.

Genetic manipulation of VEGI levels in prostate cancer cell lines. PC-3 cells, which expressed modest levels of VEGI transcript, were transfected with a VEGI expression construct and anti-VEGI ribozyme transgenes, to respectively create sublines showing enhanced or suppressed levels of VEGI expression. DU-145 cells, which expressed lower levels of VEGI transcript, were only transfected with the VEGI expression construct. As shown by RT-PCR analysis, VEGI mRNA expression was significantly increased in DU-145^{VEGIexp4} cells compared with wild-type (DU-145^{wt}) and plasmid control (DU-145$^{pEF/His}$) cells (Fig. 2A). Q-PCR also demonstrates that VEGI mRNA expression was significantly increased in DU-145^{VEGIexp4} and PC-3^{VEGIexp4} cells, and

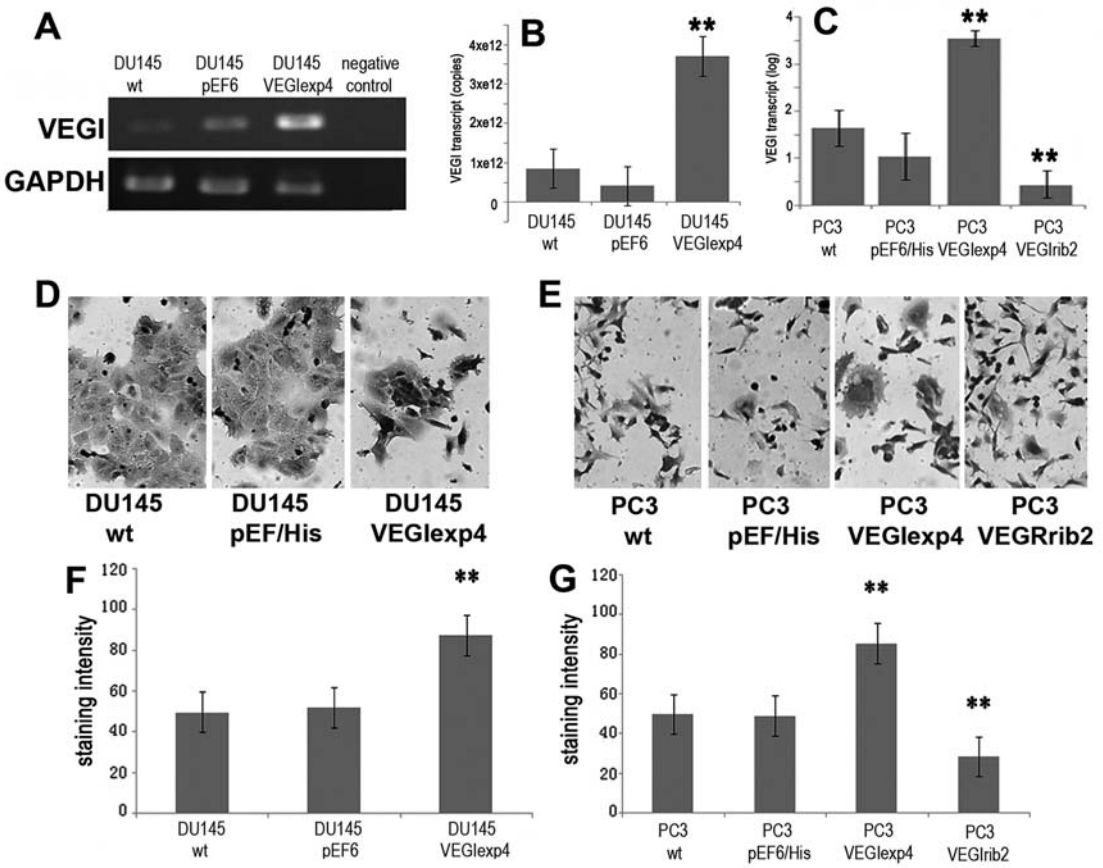

Figure 2. Confirmation of manipulation of VEGI expression in prostate cancer cells. (A and B) Verification of forced expression of the VEGI transcript in DU-145 cells. Representative image from 4 experiments showing results of the RT-PCR (A), where VEGI mRNA was overexpressed in DU-145^{VEGIexp4} cells compared with the wild-type and empty plasmid control cells. Q-PCR confirmed the overexpression of VEGI mRNA in DU-145^{VEGIexp4} cells compared with DU-145^{wt} and DU-145$^{pEF/His}$ ($p<0.01$) cells. Asterisk indicates $p<0.01$ vs. DU-145^{wt} and DU-145$^{pEF/His}$ cells. (C) Verification of forced expression and knockdown of VEGI transcript in PC-3 cells, using Q-PCR. Asterisk indicates $p<0.01$ vs. PC-3^{wt} and PC-3$^{pEF/His}$ cells. Q-PCR was performed in triplicates for each cell line and repeated independently three times. (D and E) Forced expression and knockdown of VEGI at protein level using immunocytochemistry staining for DU-145 and PC-3 cells. VEGI protein was stained stronger in the cytoplasmic areas of DU-145^{VEGIexp4} and PC-3^{VEGIexp4} cells than that of wild-type and empty plasmid control cells. VEGI protein level was almost eliminated in PC-3^{VEGIrib2} cells compared with the wild-type and empty plasmid control cells. (F and G) The staining density of VEGI was quantified and normalised for PC-3 and DU-145 cells. Asterisk indicates $p<0.01$ vs. wild-type and empty plasmid control cells. Error bars represent the SD.

decreased in PC-3^{VEGIrib2} cells, compared to corresponding controls (Fig. 2B and C). Forced expression and knockdown of VEGI was also reflected at the protein level in immunocytochemical staining: a decrease in the VEGI protein level was seen in PC-3^{VEGIrib2} cells, and increased staining was seen in DU-145^{VEGIexp4} and PC-3^{VEGIexp4}, compared to controls (Fig. 2D-G).

The influence of VEGI expression on cell matrix adhesion and motility of prostate cancer cells. We first examined the effect of VEGI on cell-matrix adhesion of prostate cancer cell lines. Overexpression of VEGI exhibited a significant inhibitory effect on cell-matrix adhesion of the cells (Fig. 3A). Compared with DU-145^{wt} (32.4±4.27) and DU-145$^{pEF/His}$ (31.6±4.95), the number of adherent cells for DU-145^{VEGIexp4} (16.1±5.28) was significantly reduced ($p<0.001$ vs. both controls). In line with the observation from DU-145 cells, the number of adherent cells for PC-3^{VEGIexp4} and PC-3^{VEGIrib2} was 35.2±13.40 and 89.4±22.06, respectively (Fig. 3B), $p<0.001$ compared with both PC-3^{wt} (61.8±6.62) and PC-3$^{pEF/His}$ cells (61.8±12.52).

In the cytocarrier based cell motility assay, cell motility was significantly reduced in DU-145^{VEGIexp4} and PC-3^{VEGIexp4} cells. The number of migrating DU-145^{VEGIexp4} cells was 11.0±3.77 compared with 26.3±6.46 for DU-145^{wt} cells and 28.8±6.94 for DU-145$^{pEF/His}$ cells ($p<0.001$). The number of migrating PC-3^{VEGIexp4} cells was 28.5±8.89, compared with 46.0±10.49 for PC-3^{wt} cells and 38.8±11.97 for PC-3$^{pEF/His}$ cells ($p<0.001$). The number of migrating PC-3^{VEGIrib2} cells was 90.8±29.02. It was also significantly higher than that of PC-3^{wt} cells and PC-3$^{pEF/His}$ cells, $p<0.001$ (Fig. 4A and B).

To further investigate the effect of forcibly expressing VEGI on cell motility, the ECIS system was utilised to determine the migration of DU-145 cells. The migrating capacity was remarkably reduced in DU-145^{VEGIexp4} cells compared with DU-145^{wt} and DU-145$^{pEF/His}$ cells (Fig. 4C).

In the wounding assay, we also found that the motility was reduced significantly in cells containing the VEGI expression construct, and increased in cells showing VEGI knockdown. The average migrating distance of DU-145^{VEGIexp4} and PC-3^{VEGIexp4} was 12.1±11.97 μm and 56.7±9.24 μm, respectively; $p<0.001$ compared to both DU-145^{wt} (74.7±7.76 μm)

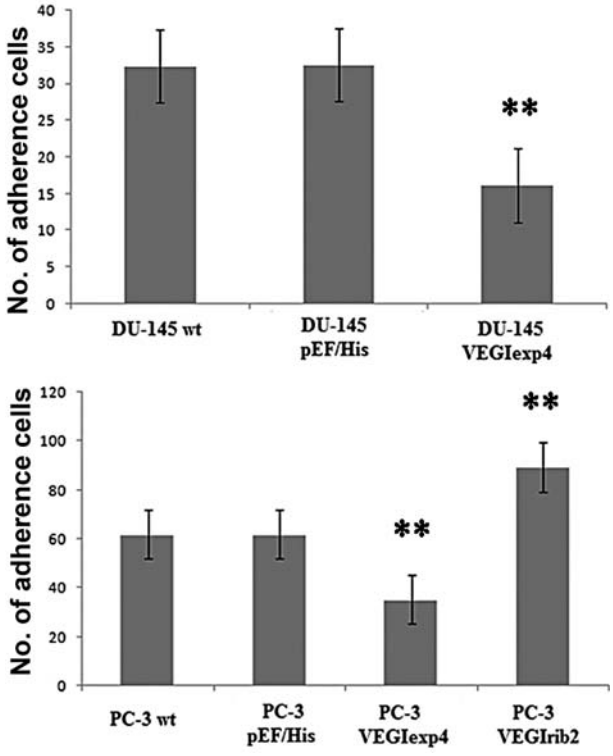

Figure 3. VEGI expression and *in vitro* cell-matrix adhesion. All experiments were repeated 5 times. (A) Forced expression of VEGI reduced the number of adherent cells in DU-145^{VEGIexp4} cells. Asterisk indicates p<0.01 vs. DU-145^{wt} and DU-145$^{pEF/His}$ cells. (B) Reduced adhesion was also seen in PC-3^{VEGIexp4} cells. Whereas, knockdown of VEGI inversely increased adhesion in PC-3^{VEGIrib2} cells. Asterisk indicates p<0.01 vs. PC-3^{wt} and PC-3$^{pEF/His}$ cells. Cell matrix adhesion assay carried out using 5 wells per cell line per experiment. Error bars represent the SD.

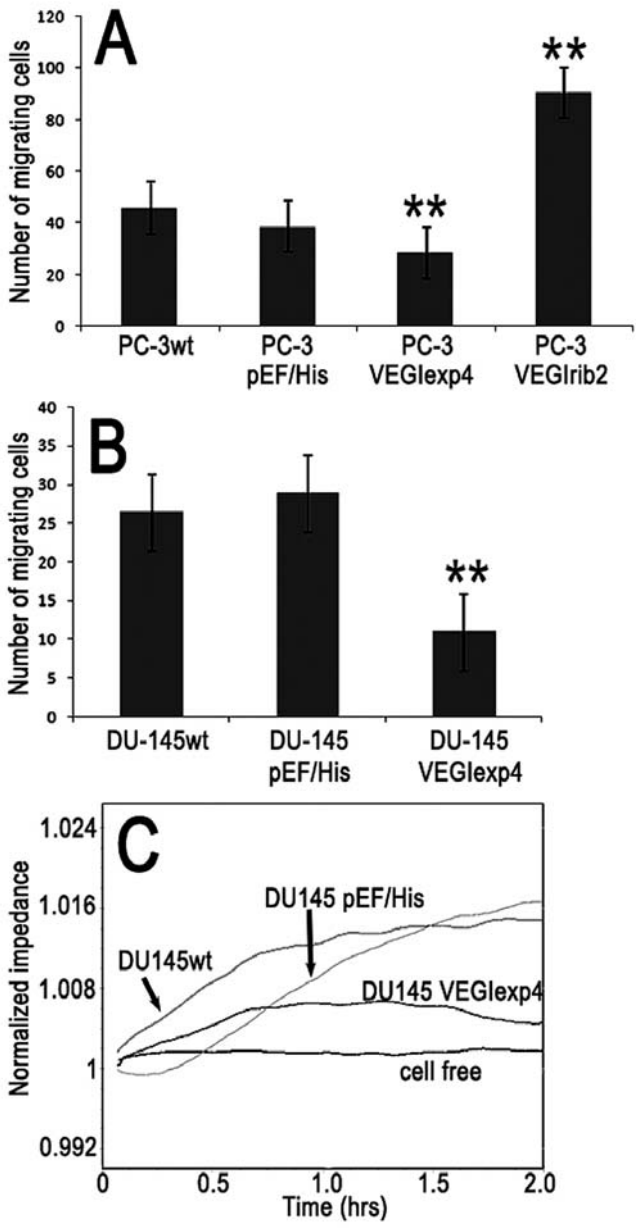

Figure 4. The effect of forced expression VEGI on migration of PC3 and DU145 cells. All experiments were repeated 4 times. (A) The motility of PC-3^{VEGIexp4} cells was markedly reduced, and that of PC-3^{VEGIrib2} cells was increased inversely, compared with wild-type and empty plasmid control cells. Experiments were done in 6 wells per cell. Asterisk indicates p<0.01 versus PC-3^{wt} and PC-3$^{pEF/His}$ cells. (B) A decrease in motility was seen in DU-145^{VEGIexp4} cells. Asterisk indicates p<0.001 vs. DU-145^{wt} and DU-145$^{pEF/His}$ cells. (C) Effect of VEGI expression on cell migration as analysed by ECIS (wounding assays). Cells were first wounded at 6 V for 30 sec. The impedance change is shown. The DU-145^{VEGIexp4} cells which overexpress VEGI showed a marked reduction in migration. Error bars represent the SD.

and DU-145$^{pEF/His}$ (61.2±22.08 μm) cells, PC-3^{wt} (97.7± 12.93 μm) and PC-3$^{pEF/His}$ (69.4±15.17 μm) cells. Knockdown of VEGI increased the motility of PC-3^{VEGIrib2} cells, the distance of cell movement was 117.7±19.96 μm, p=0.067 and 0.016 compared to both PC-3^{wt} and PC-3$^{pEF/His}$ cells, respectively (Fig. 5A and B).

Manipulation of VEGI expression had no impact on growth and invasiveness of prostate cells. The influence on cell growth and invasion by VEGI was also examined using *in vitro* growth and invasion assay, respectively. There was no difference seen in the growth of prostate cancer cells by modified VEGI expression. The invasive capacity of PC-3^{VEGIexp4}, PC-3^{VEGIrib2} and DU-145^{VEGIexp4} cells did not show any change compared with that of wild-type and empty plasmid control cells (p>0.05) (Fig. 6).

Discussion

VEGI was originally thought to be exclusively expressed in endothelial cells (20). Subsequently, it has been reported that VEGI is also expressed in a wide variety of human cancer cell lines, including breast, prostate, bladder, colorectal and liver (12). The role of VEGI in human cancer cells has been investigated recently (21). Chew *et al* reported that overexpression of VEGI in endothelial cells caused dose-dependent cell death (8). VEGI is also able to inhibit the growth of xenograft tumours and reduce microvessel density. In 2005, Hou *et al*, using a Lewis lung cancer (LLC) murine tumour model, demonstrated that systemic administration of VEGI gave rise to a marked inhibition of tumour growth and to an increase in survival time of the treated animals (11). Zhai *et al* also reported that VEGI markedly inhibited the growth of breast and colon xenograft tumours, and suggested that this may be indirectly through the ability of VEGI to inhibit

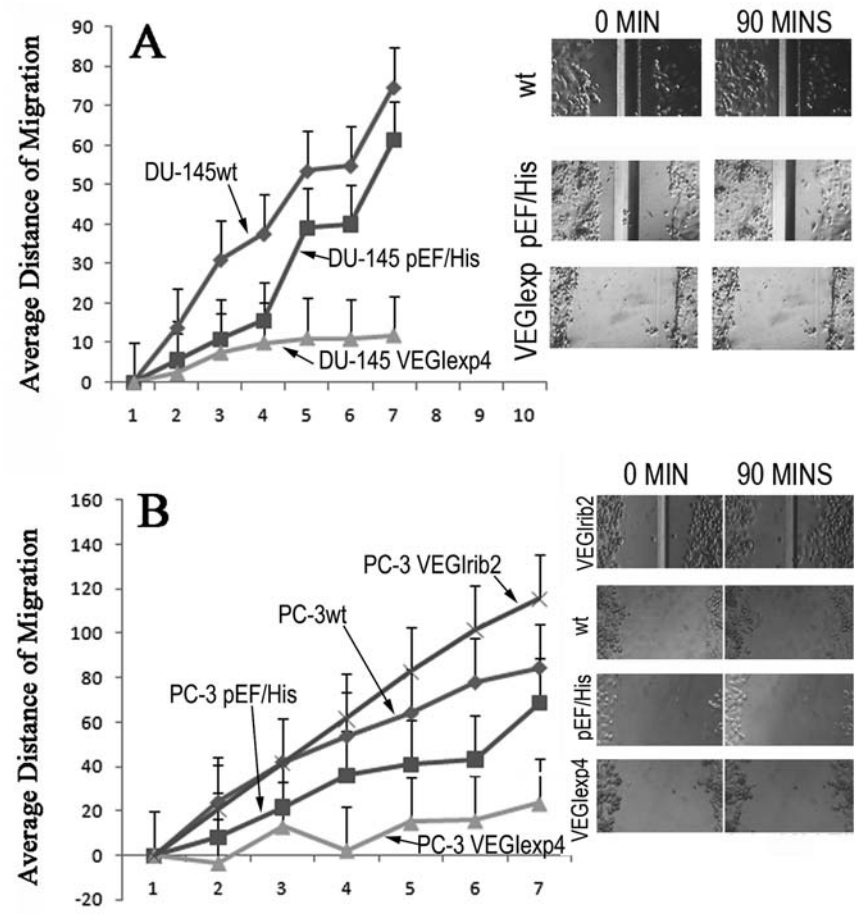

Figure 5. The effect of forced expression of VEGI on migration assay (scratch wounding assay). All experiments were repeated 3 times. (A) The movement power was reduced significantly in DU-145^{VEGIexp4} cells compared with wild-type and empty plasmid control cells ($p<0.01$). (B) The movement power was reduced significantly in PC-3^{VEGIexp4} cells (both $p<0.01$) and increased in PC-3^{VEGIrib2} cells ($p=0.067$ and 0.016, respectively) compared with wild-type and empty plasmid control cells. Error bars represent the SD.

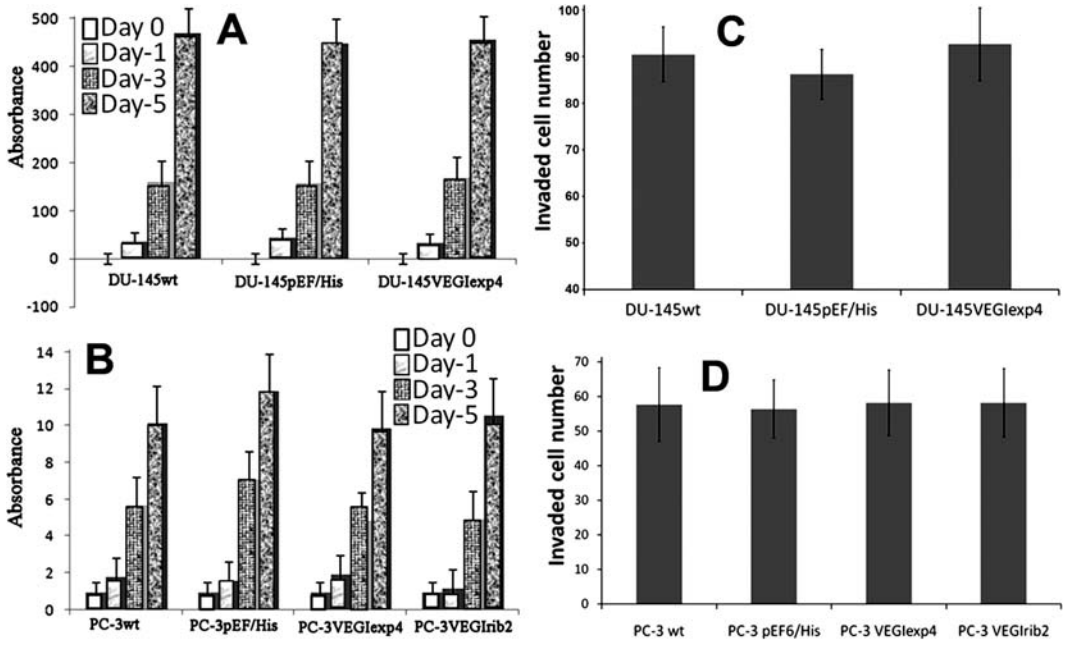

Figure 6. The effect of modified expression of VEGI on growth and invasiveness of prostate cells. (A and B) The growth capacity of PC-3^{VEGIexp4}, PC-3^{VEGIrib2} and DU-145^{VEGIexp4} cells did not show any difference from that of wild-type and empty plasmid control cells ($p>0.05$). (C and D) The invasiveness of PC-3^{VEGIexp4}, PC-3^{VEGIrib2} and DU-145^{VEGIexp4} cells did not show any difference from that of wild-type and empty plasmid control cells ($p>0.05$).

capillary-like structure growth (4,5). These studies suggest that VEGI is able to induce apoptosis in endothelial cells via an autocrine pathway but the same was not seen in other cell types examined (5,9). Overall, early evidence has indicated that the anti-tumour activity of VEGI is more likely to be the result of an interference with the development of tumour-associated vasculature rather than that of a direct effect on tumour cells (10,11,13,22). However, it has also been suggested that VEGI may inhibit the growth of human tumour cell lines, including human histiocytic lymphomas (U-937), human breast carcinomas (MCF-7), human epithelial carcinoma (HeLa) and human myeloid lymphomas ML-1a (23).

So far, there is little work on the significance of VEGI in prostate cancer. The present study revealed that VEGI mRNA was expressed in a wide variety of human prostate cancer cell lines, and most prostate specimens. Due to the fact that mRNA samples used in the present study were a result of homogenisation of biopsied tissues, it is difficult to decipher the source (normal epithelial, cancer, or stromal cells) of the VEGI mRNA transcript, rather than showing the presence of the transcript in the test tissues. However, the immunohistochemical analysis clearly indicates the origin of VEGI protein in human prostate cancer tissue. VEGI protein was found to be expressed at low levels within the prostate cancer cells, and was almost absent in tumours with high Gleason scores. The absence or reduction of tumour VEGI expression suggests that there may be a shift in the balance between pro- and anti-angiogenic stimuli. This loss of balance may subsequently produce a microenvironment that is conducive to tumour growth and survival (12,22,24). Moreover, our results confirm that forced-expression of VEGI can directly affect the motility and adhesion of prostate cancer cells. The number of adherent cells in VEGI forced-expression cells exhibited a near 50% decrease compared with the wild-type cells. The average distance of VEGI force expression cells was also decreased dramatically compared with controls. This was also supported by the observations from VEGI knockdown cells, which exhibited increased motility and adhesion. VEGI can directly reduce aggressiveness of prostate cancer cells, which is in line with the decreased expression of VEGI in prostate cancer specimens. Together with the observations that VEGI has little bearing on growth of prostate cancer cells, these data argue for a pivotal role of VEGI as a cell adhesion and migration regulator. This presents an exciting direction to pursue in future scientific research.

In addition to the effect on endothelial cells and cancer cells, VEGI may be involved in other aspects of cancer. VEGI was highly expressed in activated dendritic cells (DCs), in activated lymphocytes, and in plasma cells, and monocytes (20,25-27). It is also a T cell co-stimulator. It can directly stimulate DC maturation, and induce nuclear factor-κB activation, and apoptosis in death receptor-3-expressing cell lines. However, it is unclear whether activation of tumour-specific or non-specific B or T lymphocytes, or induction of cytokines may also operate in VEGI-mediated tumour suppression. Collectively, it is suggested that modulating VEGI may eventually be exploited as a therapeutic strategy in prostate cancer.

In conclusion, the present study shows that VEGI expression is decreased in prostate cancer, particularly in tumours with higher Gleason Scores. VEGI, a potential cell migration and adhesion regulating protein of TNFSF, is inversely associated with the aggressiveness of human prostate cancer cells. This is likely via its inhibitory role on cell migration and adhesion. Our results suggest that VEGI may be a putative tumour suppressor and a potential therapeutic target.

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