Overexpression of Id-1 in prostate cancer cells promotes angiogenesis through the activation of vascular endothelial growth factor (VEGF)

Ming-Tat Ling¹, Tracy C.M.Lau¹, Chun Zhou, Chee Wai Chua, Wai Kei Kwok, Qi Wang, Xianghong Wang² and Yong-Chuan Wong³

Cancer Biology Group, Department of Anatomy, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, SAR, China

¹To whom correspondence should be addressed at: Department of Anatomy, Laboratory Block, Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong, SAR, China. Tel: +852 2819 9226; Fax: +852 2817 0857; Email: ycwong@hkucc.hku.hk or xhwang@hkucc.hku.hk

Androgen-independent metastatic prostate cancer is the main cause of cancer related death in men. One of the reasons for this is the lack of understanding of the molecular mechanisms leading to the metastatic progression of prostate cancer. In this study, we have demonstrated that overexpression of Id-1 (inhibitor of differentiation/DNA synthesis), a member of the helix–loop–helix family proteins, is a key factor in promoting angiogenesis through activation of the vascular endothelial growth factor (VEGF) in prostate cancer cells. Using prostate cancer cells ectopically transfected with the Id-1 gene, we found that upregulation of Id-1 induced VEGF secretion through activation of the VEGF gene transcription. Downregulation of Id-1, however, led to the suppression of VEGF secretion and its gene promoter activity. The association between Id-1 and VEGF was also confirmed on human xenografts by immunohistochemical staining. In addition, the growth medium generated by the Id-1 expressing cells was able to promote morphological changes as well as capillary tube formation in human umbilical vein endothelial cells (HUVECs) at similar degrees to the recombinant human VEGF. Furthermore, inhibition of VEGF function by the treatment with an Flk-1 inhibitor, SU1498, or with the VEGF neutralizing antibody resulted in the reverse of the angiogenic effect on HUVECs. Our results suggest that overexpression of Id-1 in prostate cancer cells may provide an autocrine signal to promote angiogenesis through the activation of VEGF. Since increased Id-1 has been reported in many types of advanced human cancers, our results indicate that down-regulation of Id-1 may be a novel target to inhibit the growth of metastatic cancers through the suppression of angiogenesis.

Introduction

Id-1 (inhibitor of differentiation/DNA synthesis) protein belongs to the Id family of helix–loop–helix proteins. It lacks the basic domain for DNA binding and functions mainly as a dominant inhibitor of the bHLH transcription factor through heterodimerization (1). Id-1 has been shown to play a critical role in the regulation of cell proliferation (2), differentiation (3) and senescence (4–6), and recent studies suggest that Id-1 may function as an oncogene. For example, Id-1 is shown to inhibit replicative senescence and promote life span of primary cells through the inactivation of p16/RB pathway (6). In addition, elevated Id-1 expression either at transcriptional or translational levels has been reported in over 20 types of human cancers including prostate, breast, cervical, colon and liver cancers (7). Furthermore, the ectopic expression of Id-1 promotes cancer cell proliferation and protection against apoptosis under sub-optimal culture conditions (2,8). In addition to its potential oncogenic actions, Id-1 has also been suggested to take part in the malignant progression of human cancer. For example, in breast cancer, Id-1 is found to be constitutively expressed in the highly aggressive but not the non-aggressive cancer cells (9). In endometrial carcinoma, Id-1 expression is also high in high grade and invasive tumours (10). Early stage cervical cancer patients with high Id-1 expression have poor prognosis compared with patients with relatively low Id-1 expression (11). In breast and cervical cancers, increased Id-1 is associated with more aggressive clinical behaviour as well as poor clinical outcome in patients (9,11,12). In our previous studies, in human prostate cancer, Id-1 expression was found to be increased with increased Gleason score of the tumours (13) and ectopic expression of Id-1 in the androgen-sensitive prostate cancer cells led to decreased sensitivity to androgen-induced growth stimulation (14), which is a characteristic of prostate cancer progression. Furthermore, Id-1 is shown to stimulate mitogen-activated protein kinase (MAPK) and nuclear factor-κB pathways (8,15), which are frequently activated in more advanced and aggressive cancers. These lines of evidence strongly suggest that Id-1 may be a key factor not only in promoting human tumourigenesis but it may also play an important part in tumour progression.

One of the striking characteristics of the aggressive cancer cells is their ability to metastasize and develop an ectopic growth. The most convincing evidence associating Id-1 with tumour metastasis are the results generated from knockout mice that Id-1 and Id-3 double knockout results in embryonic lethality in mice due to poor blood vessel formation in the brain. The Id-1⁻⁻ Id-3⁻⁻ mice also fail to support the growth of tumour xenografts owing to the poor vascularization that leads to necrosis of the tumour cells (16). In addition, loss of Id-1 is also associated with downregulation of several pro-angiogenic genes such as integrinα6 and β4 in the tumour endothelial cells (17). Furthermore, downregulation of Id-1 in a breast cancer animal model leads to decreased metastatic
ability of xenografts and reduced invasion ability in cultured cells (18). These results raise a hypothesis that Id-1 may play a positive role in cancer metastasis through the promotion of angiogenesis. However, the molecular basis for its role in cancer metastasis is not clear.

Prostate cancer is one of the most commonly diagnosed cancers in American men representing one-third of all new cancer cases each year. This means one out of six American men being diagnosed with prostate cancer over the course of their lifetimes. As a result, over 30,000 men die each year from prostate cancer in the US (19). Advanced androgen-sensitive prostate cancer is manageable with androgen depletion therapy. However, once prostate cancer recurs and becomes androgen-independent, especially at metastatic stage, the median survival of this group of patients is short due to the lack of effective treatment strategies (20). Therefore, androgen-independent metastatic prostate cancer remains the main cause of cancer related death in men. However, the molecular mechanisms leading to metastatic progression of prostate cancer are not known. In this study, we have demonstrated that overexpression of Id-1 may be a key factor in promoting metastatic growth of prostate cancer through the activation of vascular endothelial growth factor (VEGF). Our results suggest that overexpression of Id-1 may provide an autocrine signal to facilitate ectopic growth of prostate cancer cells through the promotion of angiogenesis. Downregulation of Id-1 may provide a novel target to inhibit the growth of metastatic prostate cancer.

Materials and methods

Cell culture conditions

Human prostate adenocarcinoma cell lines (LNCaP and DU145) and human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (Rockville, MD). LNCaP and DU145 cells were maintained in RPMI1640 medium (Sigma, St. Louis, MO) supplemented with 5% fetal calf serum (FCS) and penicillin/streptomycin. HUVEC was grown in Endothelial Cell Medium-2 (EGM2: Clonetics, Camber Bio Science Walkersville Inc., Walkersville, MD) supplemented with 2% fetal bovine serum, hydrocortisone, ascorbic acid, hFGF, VEGF, hEGF, r-rIGF-1, GA-1000 and Heparin as recommended by the manufacturer. HUVECs were used between passages 2 and 8. All cells were maintained at 37°C in 5% CO₂.

Generation of Id-1 and Si-Id-1 transfectants

LNCaP-pBabe (vector control) and LNCaP-Id-1 transfectants (C1–4) were established as described previously (2). The vector control and the stable transfectants were maintained under the same conditions as the parental LNCaP cell line. Before use for the treatment of HUVECs, the culture medium was changed to RPMI containing 1% FCS for 24 h.

A stable Id-1-Si-RNA vector was generated using the GeneSuppressor system kit according to the manufacturer’s instructions. Briefly, the primers containing the short hairpin RNA sequence targeting the Id-1 coding region were annealed and cloned into pSuppressor-Retro vector to generate the Si-RNA expression vector. The sequences of the Si-Id-1 primers were: si-Id-1-F, TCG AGG CGT TTA CTC ACC GTA CAA GGA AGG TTG CAG GGC TGA TGA ACA GCC TCT TTT; si-Id-1-R, CTA GAA AAA GGC TGC TAC GTA CAA GGT TCC TGG AGG CTT GAG TAA CAG CC. The vector control was generated using the same procedures as the Si-RNA vector except that the short hairpin RNA sequence was replaced with non-sense sequences that are not homologous to the human genome. The sequences of the control primers were: si-Con-F, TCG AGC GTA TTG CTT AGC ATT AGG TGA TGG TAC ACG TAA CAG TAG GCA ATG CCG TTT TT; si-Con-R, CTA GAA AAA GGC TAT TGC CTA GCA TTS CGT CAA GGT CTA CGT AAT GCT AGG CCA TAC GC. The resulting vectors were then transfected into the packaging cell line 293 using Fugene 6 reagent. Retroviruses were collected 48 h later, mixed with polybrene (8 μg/ml), and then incubated with DU145 cells. Positive Si-Id-1 clones were then selected in Neomycin (400 μg/ml) and stable transfectants were isolated after ~14 days drug selection. A vector control was generated from a pool of > 20 individual clones transfected with the control vector.

Vascular endothelial growth factor protein quantification by enzyme-linked immunosorbent assay (ELISA)

Same number of LNCaP, LNCaP-Id-1 transfectants (C1–4), LNCaP-pBabe, DU145-Si-Id-1 transfectants and the vector control cells (2 × 10⁵ cells/well) were plated in six-well plates in RPMI containing 5% FCS. The cells were allowed to grow for 48 h until they were ~60–70% confluent. The growth medium was then removed and replaced with fresh RPMI containing 1% FCS. The cells were incubated for a further 24 h until ~80% confluency was attained. The medium was then harvested and filtered for the measurement of secreted VEGF. The remaining cells were collected and the viable cells were counted. VEGF present in the growth medium was measured using a Quantakine Human VEGF ELISA kit according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). The concentration of VEGF was measured as picograms per millilitre (pg/ml) in the growth medium and the results were then calculated as picograms of secreted VEGF per cell. Each experiment was performed at least three times and the mean concentration of VEGF secretion was presented as the final result. Standard deviation (SD) of the means was used as error bars.

Luciferase assay

LNCaP cells (1 × 10⁵ cells/well) were plated into 12-well culture plates and allowed to grow for 24 h. pGL-V109 (luciferase reporter containing the VEGF promoter, kindly donated by Dr K. Xie, The University of Texas, Houston, TX) and pRL-CMV-Luc were co-transfected with either the pCDNA-Id-1 (a gift from Dr Eiji Hara, Kyoto Prefectural University of Medicine, Kyoto, Japan) or pcDNA into the cells using Fugene 6 reagent (Roche Diagnostics, Indianapolis, IN). Cells were lysed 48 h after transfection and were assayed for luciferase activity using the Dual-luciferase reporter assay system (Promega, WI). For measuring VEGF promoter activity of the stable DU145-Si-Id-1 transfectants and the vector control, the cells were co-transfected with pGL-V109 and pRL-CMV-Luc vector. Firefly luciferase activity was measured at 48 h after transfection and the reading was then normalized with the Renilla luciferase activity, which served as the internal control for transfection efficiency. Each experiment was performed at least three times in duplicate wells and each data point represented the mean and SD. The percentage increase in luciferase activity of the pcDNA-Id-1 transfected cells or the DU145-Si-Id-1 transfectants was calculated relative to that of the vector controls. The mean percentage increase (or decrease) in luciferase activity was presented as the final result and the means of the assays was used as error bars.

Western blotting

Cells, set in six-well plates for VEGF ELISA analysis, were lysed and proteins were extracted using the method described previously (2). By electrophoresis on 12.5% SDS–polyacrylamide gel for aliquot of 20 μg of protein was separated and blotted onto polyvinylidene difluoride membranes (Amersham, Piscataway, NJ). The membranes were blocked with 10% non-fat milk in TBS for 1 h at room temperature. After washing with TBS-T, the blots were incubated with primary antibody against Id-1 (1:1000, C20, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h, and horseradish peroxidase-conjugated secondary antibody against rabbit IgG (1:3000; Amersham) for a further 1 h. Immunoreactive signals were detected using the ECL plus western blotting system (Amersham).

Capillary tube formation assay

LNCaP, LNCaP-pBabe and LNCaP-Id-1 transfectants were plated into six-well plates (2 × 10⁵ cells/well) in RPMI containing 5% FCS. After 48 h, the medium was removed and fresh medium (RPMI containing 1% FCS) was added. The cells were allowed to grow for 24 h until they were ~80% confluent. The growth medium was collected and filtered for the treatment of HUVECs. In vitro angiogenesis assay on HUVECs was performed using the In vitro Angiogenesis Assay Kit (Chemicon International, Temecula, CA) according to the manufacturer’s instructions. Briefly, HUVECs (1 × 10⁵ cells/well) were seeded onto polymeric three-dimensional collagen gel (ECMatrix, Chemicon International, Temecula, CA) in 96-well plates in the growth medium of LNCaP, LNCaP-pBabe and LNCaP-Id-1 transfectants. RPMI medium (1% FCS) containing recombinant human VEGF (20 ng/ml; R&D Systems) and RPMI only were also used to culture HUVECs as positive and negative controls, respectively. Tube formation in each treatment was inspected under an inverted light microscope after incubation for 8 h at 37°C and the images were captured at a single level beneath the monolayer. Tube formation ability was quantified by counting the total number of cell cluster and branch under three 4× magnified fields per well. The results were expressed as the mean percentage of branching over total cell clusters.

For VEGF inhibition assay, Flk-1 kinase inhibitor (40 μM, SU1498) (Calbiochem, San Diego, CA) was added to the growth medium of LNCaP.
and its transfectants and tube formation ability was determined as described above.

**Northern blot**

Cells of LNCaP, DU145 and their transfectant clones were seeded on 10 cm tissue culture dishes for 24 h, and the medium was then replaced with RPMI supplemented with 1% FCS. Forty-eight hours later, cells were lysed for total RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA) using the procedure provided by the manufacturer. mRNA was isolated from total RNA using the Oligotex mRNA kit (Qiagen, Valencia, CA) and 8 µg of mRNA was then resolved by denaturing formaldehyde electrophoresis and transferred onto a nylon membrane by capillary blotting. The blot was prehybridized in ExpressHyb Hybridization solution (BD Clontech, Bedford, MA) at 68°C for 2 h and then hybridized overnight with the 32P-labelled cDNA probe complementary to the VEGF mRNA sequence (21). The blot was then washed twice with SSC (0.15 M NaCl and 0.015 M sodium citrate) and then exposed to autoradiographic film for visualization. Equal loading of the mRNA was confirmed by the visualization of 18S and 28S with ethidium bromide staining of the gel.

**VEGF RT–PCR**

Total RNA was isolated using Trizol reagent according to the manufacturer’s protocol (Invitrogen). cDNA was synthesized using the SuperScript First Strand Synthesis System (Invitrogen) and was then amplified by PCR with VEGF specific primers (VEGF-e: 5'-CTTGTATCGTTCCTCCG-3' and VEGF-r: 5'-CGAAGTGTTGGAATTCGGA-3') (22). PCR cycling protocol was as follows: 30 cycles of 1 min at 95°C, 30 s at 95°C, 30 s at 55°C, 1 min at 72°C and 10 min at 72°C. Glyceraldehyde 3-phosphate dehydrogenase was amplified as an internal control. The PCR products were electrophoresed on a 2% agarose gel and analysed using a gel documentation system.

**Bromodeoxyuridine (BrdU) staining**

Detailed experimental procedures were described in our previous studies (23). Briefly, HUVECs (6000 cells/well) were grown on Chamber slides in EGM2 for 24 h. The culture medium was removed and replaced with the growth medium of LNCaP and its transfectants, 20 ng/ml rhVEGF in RPMI with 1% FCS, EGM2 and RPMI containing 1% FCS, respectively, for incubation medium of LNCaP and its transfectants when compared with the vector control (Figure 1C). To further confirm that the VEGF gene expression was upregulated by Id-1, we performed northern blotting as well as semi-quantitative RT–PCR to quantify the mRNA level of the VEGF. As shown in Figure 1D and E, VEGF transcript was upregulated in the Id-1 transfectant, indicating that ectopic Id-1 expression led to increased transcriptional activation of the VEGF gene.

**Immunohistochemistry**

The establishment of CWR22 and CWR22R human prostate xenografts has been described previously (24,25). Both the xenografts have been successfully propagated with androgen supply in male nude mice (for CWR22) or castrated male nude mice (for CWR22R) in our laboratory. Immunohistochemical staining was performed on 4 µm sections from the formalin-fixed, paraffin-embedded CWR22 and CWR22R tumour specimens using the standard avidin–biotin complex procedure as described in our previous studies (13,14). The primary and secondary antibodies used against Id-1 and VEGF have been described in Western blotting section. Photos were taken under 400× magnifications.

**Detection of apoptotic cells by 4',6-diamidino-2-phenylindole (DAPI) staining**

Apoptotic cells were detected by DAPI nuclear staining according to the protocol described previously (8). Briefly, HUVECs were plated onto a 12 mm cover glass and the rhVEGF was added alone or together with SU1498. They were fixed in ice-cold acetone and methanol (1:1), washed with phosphate-buffered saline, and then stained with DAPI for 5 min. The stained cells were examined under a fluorescent microscope.

**Results**

**Ectopic expression of Id-1 leads to upregulation of VEGF in prostate cancer cells**

Previously, it was reported that VEGF protein expression was relatively low in LNCaP cells compared with the androgen-independent cell lines such as DU145 (26). In addition, previously, we have found that Id-1 protein was also undetectable in LNCaP cells under serum-free culture conditions (2). In this study, we investigated if ectopic Id-1 expression in LNCaP cells could lead to the upregulation of VEGF secretion. Using four transfectant clones (Id-1-C1 to C4) generated from a previous study (2) (Figure 1A), we quantified the VEGF protein in the culture media of the parental cells, the vector control (pBabe) and the Id-1 expressing clones (C1 to C4) using ELISA method. As shown in Figure 1B, compared with LNCaP cells, there was ~40–80% increase in VEGF protein concentrations in the Id-1 transfectants with actual concentrations ranging from 19.5 to 34.5 ng/ml, while the VEGF level was similar to the parental line in pBabe cells, indicating that ectopic Id-1 expression led to increased secretion of VEGF in LNCaP cells. To investigate whether the increased VEGF secretion in the Id-1 transfectants was due to gene activation at the transcriptional level, we then co-transfected the Id-1 expression vector (pCDNA-Id-1) together with a luciferase reporter containing the VEGF promoter in LNCaP cells and generated transient transfectants. We found that the VEGF promoter activity was increased by >2-fold in the Id-1 transfectants when compared with the vector control (Figure 1C).

**Inactivation of Id-1 leads to downregulation of VEGF in androgen-independent prostate cancer cells**

It was reported that both VEGF and Id-1 protein levels were high in an androgen-independent prostate cancer cell line DU145 (8,26). To further confirm the association between Id-1 and VEGF, we then transfected a vector containing the Si-Id-1 into DU145 cells to study if the downregulation of Id-1 could lead to inhibition of VEGF protein secretion. As shown in Figure 2A, the stable transfectants (C1 to C4) showed decreased Id-1 protein expression (up to 80% decrease), compared with the parental DU145 cells and the vector control (ssCon) cells. Using ELISA assay, we also found that downregulation of Id-1 was associated with decreased secretory VEGF protein in the cell culture medium (Figure 2B). In addition, when we transfected the VEGF reporter construct into the Si-Id-1 and vector transfectants, decreased VEGF promoter activity was observed in the Si-Id-1 transfectants (up to 90% decrease) compared with the vector controls (Figure 2C). In addition, mRNA of VEGF was also downregulated in the Si-Id-1 transfectant of DU145 cells (Figure 2D and E). These results further suggest the association between Id-1 and VEGF secretion in prostate cancer cells.

**Effect of secretory VEGF on angiogenesis in HUVECs**

Since the function of VEGF in tumour metastasis is mainly determined by its ability to promote new blood vessel formation, we investigated if the VEGF protein secreted by the Id-1 expressing cells had any effect on HUVECs in terms of proliferation and angiogenic ability by treating the HUVECs with cell culture media generated from Id-1 negative cells (LNCaP and pBabe) as well as from Id-1 positive cells (Id-1 clones as well as a pool of Id-1 transfectants) (Figure 3A). We then studied the alterations of cell morphology, capillary tube formation and proliferation of endothelial cells. As shown in Figure 3B, after culturing the HUVECs in the medium of Id-1 transfectants for 48 h, these cells showed elongated morphology and the ability to form cell–cell networking, which was also observed in the same cells treated with the...
recombinant human VEGF (rhVEGF, 20 ng/ml; equivalent to the lowest VEGF concentration produced in the Id-1 transfectants). In contrast, the cells cultured in the RPMI medium, or media generated from LNCaP and pBabe cultures showed the typical round and scattered morphology. In addition, with the use of the culture medium from Id-1 transfectants the ability of HUVECs to form tube-like structures was enhanced by up to 3-fold. This effect was similar to the one induced by similar concentrations of rhVEGF (Figure 3C and D). Furthermore, the proliferation rate of the HUVECs grown in the media generated from Id-1 expressing cells was up to 3-fold higher than the cells cultured in the media from LNCaP and pBabe cells (Figure 3E). Taken together, these results showed that the secretory VEGF by the Id-1 expressing LNCaP cells was able to promote proliferation and angiogenesis in HUVECs.

Effect of VEGF receptor Flk-1 kinase inhibitor, SU1498 and VEGF neutralizing antibody, on Id-1-induced angiogenesis

To further confirm that the Id-1-induced VEGF secretion was responsible for the angiogenic effect on HUVECs, we treated
the prostate cancer cell culture media with an inhibitor of the VEGF receptor Flk-1, SU1498. As shown in Figure 4, the addition of SU1498 (40 μM) in the culture medium of Id-1 transfectants or in the medium that is supplemented with hrVEGF led to the disappearance of the tube-like structure (Figure 4A and B) and decrease in the BrdU incorporation rate (Figure 4C) in HUVECs without the cell viability being affected (Figure 4D). The same effect was observed when the VEGF neutralizing antibody (+neut Ab) was added into the prostate cancer cell culture media, indicating that the inhibition of VEGF function reverses the angiogenic effect induced by Id-1 in prostate cancer cells.

**Correlation of Id-1 and VEGF expression in human prostate cancer xenografts**

To further confirm the association between Id-1 and VEGF in prostate cancer, we studied the expression of Id-1 and VEGF in human xenografts, CWR22 and CWR22R, which are recently established prostate cancer models and have been shown to mimic the clinical conditions in recurrent prostate cancer patients (24,25). Previously, we found that CWR22R, the androgen-independent derivative of the original androgen-dependent CWR22 xenograft, had much higher levels of Id-1 expression (14). In this study, using immunohistochemistry, we observed (Figure 5A) that the increased Id-1 protein

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**Fig. 2.** Suppression of VEGF expression in Si-Id-1 transfectants. (A) Id-1 expression in stable DU145 transfectants expressing the Si-Id-1 (C1–C4) demonstrated by western blotting. (B) Secretory VEGF protein in the culture media of Si-Id-1 transfectants, the control (ssCon) and the parental cells examined by ELISA. (C) VEGF promoter activity measured by luciferase assay. pGL-V109 and pRL-CMV-Luc vectors were co-transfected into DU145ssCon and the Si-Id-1 transfectants. Luciferase activity was measured 48 h after transfection. Results represent three independent experiments. (D and E) mRNA level of VEGF in vector control and Si-Id-1 transfectant demonstrated by semi-quantitative RT–PCR and northern blotting. Note that downregulation of Id-1 in DU145 cells leads to decreased VEGF secretion, promoter activity and mRNA level.
expression in the CWR22R tumour (panel 2) was associated with higher levels of VEGF expression (panel 4) when compared with the CWR22 tumour specimens (panels 1 and 3). Recently, it was reported that increased Id-1 mRNA expression was found in prostate cancer tissues after the hormone ablation treatment (27). In this study, using the CWR22 nude mice model, we investigated whether the Id-1 protein expression in the prostate cancer cells was associated with VEGF levels, before and after the surgical castration. As shown in Figure 5B, Id-1 protein expression was notably
increased in the CWR22 xenograft 24 h after castration of the nude mice compared with the tumours generated from uncastrated mice (panels 1 and 2). We also found that the increased Id-1 expression in the tumours from castrated mice was associated with high levels of VEGF protein expression (panel 4) compared with the tumours from uncastrated mice (panel 3). These findings confirm the results generated from prostate cancer cell lines and further suggest the association between Id-1 and VEGF.

Discussion

Recently, more and more evidence supports the role of Id-1 as an oncogene which promotes tumourigenesis through inactivation of tumour suppressing (i.e. p16INK4a/pRB) (2) and activation of growth promoting (i.e. MAPK) (15) pathways. However, the molecular basis responsible for the function of Id-1 in tumour progression is not clear. In this study, we have demonstrated that increased Id-1 expression leads to increased VEGF protein secretion through activation of the VEGF gene (Figures 1 and 2). The active form of VEGF in turn stimulates endothelial cell proliferation and promotes blood vessel formation (Figure 3). The association between Id-1 and VEGF was also confirmed on prostate cancer xenografts, the increased Id-1 expression was correlated with the upregulation of VEGF in nude mice (Figure 5). Our results provide a novel molecular basis for the metastatic progression of prostate cancer and indicate that inactivation of Id-1 may be a new strategy in the treatment of metastatic prostate cancer through suppression of tumour angiogenesis.

Previously, impaired angiogenesis has been reported in Id-1 deficient mice as well as breast cancer xenografts (16–18). However, the present study is the first to demonstrate the direct correlation of Id-1 with VEGF-induced angiogenesis in human cancer cells. Several novel indications have been generated in this study. First, the results that ectopic Id-1 expression in LNCaP cells led to the increase in both VEGF protein secretion and its gene promoter activity indicate that upregulation of Id-1 alone in prostate cancer cells activates the VEGF gene expression through transcriptional activation (Figure 1). These results were further confirmed when a Si-Id-1 vector was transfected into DU145 cells that led to the downregulation of Id-1 alone in prostate cancer cells activates the VEGF gene expression through transcriptional activation (Figure 1). This study demonstrates that the secretory VEGF produced by the Id-1 transfectants in the culture medium induced endothelial angiogenesis, indicating that the Id-1-induced VEGF...
that increased VEGF expression is associated with prostate androgen independent cells (14). Recently, it was reported androgen-sensitive LNCaP cells leads to its progression into cancer cells (13). In addition, ectopic Id-1 expression in the increase with increased Gleason grade in human prostate cancer in the animal model (28) and Id-1 expression levels associated when normal cells progress from hyperplasia to prostate cancer xenografts CWR22 and CWR22R. (A) Differential expression of Id-1 and VEGF in CWR22 and CWR22R xenografts before and after castration. Note that increased Id-1 expression is associated with high levels of VEGF protein in human prostate cancer xenografts. Results represent xenografs from five nude mice. See online Supplementary material for a colour version of this figure.

expression is functionally active, which promotes endothelial proliferation and their ability to form new blood vessels. Third, inhibition of VEGF function by the addition of an Flk-1 kinase inhibitor (SU1498) or the VEGF neutralizing antibody blocked the Id-1-induced endothelial proliferation and reduced tube formation ability (Figure 4A), further suggesting that the active VEGF protein secreted by the Id-1 expressing cells is responsible for the stimulatory effect on endothelial cells. The results that showed the increased Id-1 expression was associated with the upregulation of VEGF in human xenografts in nude mice (Figure 5) further support the results generated from in vitro cell lines. It is possible that the upregulation of Id-1 in the less aggressive prostate cancer cells may provide an autocrine signal to stimulate the VEGF expression, which is then secreted extracellularly to provide growth factors necessary for the formation of blood vessels for ectopic growth of the cancer cells. Previously, we have found that Id-1 is upregulated when normal cells progress from hyperplasia to prostate cancer in the animal model (28) and Id-1 expression levels increase with increased Gleason grade in human prostate cancer cells (13). In addition, ectopic Id-1 expression in the androgen-sensitive LNCaP cells leads to its progression into androgen independent cells (14). Recently, it was reported that increased VEGF expression is associated with prostate tumour progression from androgen-dependent to androgen-independent growth in an orthotopic prostate cancer animal model (29). In combination with the evidence presented in this study, these results suggest that the upregulation of Id-1 in prostate cancer may be a key factor not only in promoting prostate carcinogenesis but also in tumour progression. One of the molecular mechanisms in this process is through the activation of the VEGF gene. Recent evidence from studies on breast cancer also supports this hypothesis. For example, it was reported that ectopic expression of Id-1 in estrogen sensitive breast cancer cells led to reduced estrogen sensitivity (9). By the downregulation of Id-1 using antisense technology, it was possible to prevent the metastasization of breast cancer cells to the lungs of the experimental animals, although the role of Id-1 in angiogenesis was not studied (18). In addition, recently, specific response elements were found in the Id-1 promoter to the bone morphogenetic proteins (BMPs) (30), which are frequently upregulated in prostate cancer cells and are important for the formation of bone metastasis (31). By the treatment of prostate cancer cells with BMPs, it was possible to induce VEGF expression at both transcriptional and protein levels (32). In combination with the results demonstrated in this study, it is possible that in prostate cancer, increased BMP may be able to activate Id-1 through transcriptional activation which in turn stimulates VEGF expression leading to bone metastatic growth. This hypothesis may also explain why bone metastasis is the common site for the ectopic growth of prostate cancer. Furthermore, increased Id-1 expression has been associated with advanced tumour stage as well as poor survival in several types of human cancer such as cervical and ovarian cancers (11,33), it is also possible that the positive role of Id-1 in cancer progression and metastasis may not be a specific event in prostate cancer but a general phenotype in human cancer. However, further studies are necessary to confirm this hypothesis.

In summary, we have demonstrated the positive role of Id-1 in prostate cancer angiogenesis through the activation of VEGF. Our results not only provide a molecular basis for the function of Id-1 in cancer progression but also suggest a novel therapeutic target in the treatment of androgen-independent metastatic prostate cancer through the inactivation of the Id-1 gene.

Supplementary material
Supplementary material is available online at: http://carcin.oxfordjournals.org/

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References


