Vascular endothelial growth factor receptor-1 mediates migration of human colorectal carcinoma cells by activation of Src family kinases

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Vascular endothelial growth factor (VEGF) is the predominant pro-angiogenic cytokine in human malignancy, and its expression correlates with disease recurrence and poor outcomes in patients with colorectal cancer. Recently, expression of vascular endothelial growth factor receptors (VEGFRs) has been observed on tumours of epithelial origin, including those arising in the colon, but the molecular mechanisms governing potential VEGF-driven biologic functioning in these tumours are not well characterised. In this report, we investigated the role of Src family kinases (SFKs) in VEGF-mediated signalling in human colorectal carcinoma (CRC) cell lines. Vascular endothelial growth factor specifically activated SFKs in HT29 and KM12L4 CRC cell lines. Further, VEGF stimulation resulted in enhanced cellular migration, which was effectively blocked by pharmacologic inhibition of VEGFR-1 or Src kinase. Correspondingly, migration studies using siRNA clones with reduced Src expression confirmed the requirement for Src in VEGF-induced migration in these cells. Furthermore, VEGF treatment enhanced VEGFR-1/SFK complex formation and increased tyrosine phosphorylation of focal adhesion kinase, p130 cas and paxillin. Finally, we demonstrate that VEGF-induced migration is not due, at least in part, to VEGF acting as a mitogen. These results suggest that VEGFR-1 promotes migration of tumour cells through a Src-dependent pathway linked to activation of focal adhesion components that regulate this process.

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Three distinct receptor tyrosine kinases bind vascular endothelial growth factor (VEGF): vascular endothelial growth factor receptor (VEGFR)-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4). Vascular endothelial growth factor receptor 1 and VEGFR-2 are expressed primarily on vascular endothelial cells, while VEGFR-3 is expressed primarily on lymphatic endothelial cells and regulates lymphangiogenesis (Irrthum et al, 2000). Vascular endothelial growth factor receptor 2 is believed to be the major mediator of angiogenesis in human malignancy, as it regulates activation of downstream effector molecules such as phosphoinositide 3-kinase and AKT, and potentiates endothelial differentiation, DNA synthesis and proliferation (Waltenberger et al, 1994; Meyer and Rahimi, 2003). Vascular endothelial growth factor receptor 1 appears to function as a VEGF 'sink' during developmental vasculogenesis, but may contribute to angiogenesis in pathologic states such as ischaemia or malignancy (Hiratsuka et al, 1998, 2001; Carmeliet et al, 2001; Hayashibara et al, 2001).

In addition to their expression on endothelial cells, VEGFRs are also expressed on cells of haematopoietic origin and have recently been demonstrated on a variety of tumour types, including prostate, ovarian, melanoma, non-small-cell lung, pancreatic and colon (Decaussin et al, 1999; Ferrer et al, 1999; Masood et al, 2001). While the functions of VEGFRs on tumour cells are not completely understood, the concomitant production of VEGF and VEGF expression by tumour cells suggests the possibility that these receptors mediate biologic functions in tumour cells. Indeed, in cancers of the breast and skin (melanoma) and in some leukaemias, VEGF/VEGFR autocrine signalling loops have been identified (Bellamy et al, 1999; Dias et al, 2000, 2001; Lacal et al, 2000), but the elucidation of the contribution of individual VEGF/VEGFR family members to biologic functions mediated by individual receptors is only beginning to emerge. In a recent work, Wang et al (2004) demonstrated differential regulation of lymphoma xenografts utilising species-specific receptor antibodies to VEGFR-1 and VEGFR-2. In that study, targeting tumour-associated VEGFR-1 (human xenografted cells) increased apoptosis and diminished tumour growth, while targeting host (i.e. murine) VEGFR-2 diminished microvascular density (Wang et al, 2004). Also, inhibition of VEGFRs with a synthetic binding antagonist inhibited growth in xenograft models of colon and other tumours (Ueda et al, 2005). Individual receptor ligands may elicit different biologic responses as well. In PIGF−/− mice,
VEGF-B (another VEGFR-1 ligand) failed to rescue vascular
development, suggesting that differential responses occur depending
on which ligand binds the receptor in vivo (Carmeliet et al, 2001).

Signalling pathways mediated by VEGFRs in both endothelial
and tumour cells are now being delineated. In endothelial cells, the
Src family kinases (SFKs), Src and Yes are required for VEGF-
induced vascular permeability and survival (Elicieri et al, 1999).
Likewise, VEGF-induced Src activation and signalling has been
reported in Kaposi’s sarcoma cells (Munshi et al, 2000). However,
the possibility that VEGFRs require SFKs to mediate their biologic
effects in other tumours has not been established.

When bound to receptor protein tyrosine kinases, SFKs become
activated. Activation of SFKs has been implicated in progression
and metastasis of a variety of solid tumours (reviewed by Summy
and Gallick, 2003). Experiments in colorectal cancer have
demonstrated increased Src kinase activity with progression from
adenoma to dysplasia to carcinoma and, finally, to metastatic
disease (Talamonti et al, 1993), suggesting a role for Src in
regulating colon tumour progression. Additionally, Src kinase
activity and VEGF have both been associated with poor prognosis
in patients with advanced colorectal cancer (Takahashi et al, 1997;
Werther et al, 2000; Allgayer et al, 2002). Thus, the potential of
VEGFRs to signal through SFKs in colorectal cancer may be of
clinical significance.

The purpose of this study was to determine whether SFKs must
be activated in order for VEGFRs to mediate biologic effects. We
tested the effects of VEGF signalling through SFKs on cellular
migration and proliferation in human colorectal cancer cells. We
found that migration, but not proliferation, was significantly
increased, suggesting the potential role of VEGFR-1 expression in
tumour progression in these cells.

MATERIALS AND METHODS

Cell culture

HT29 cells derived from a human colon adenocarcinoma were
maintained as a subconfluent monolayer in Dulbecco’s modified
Eagle’s medium (DMEM/F12) with Earle’s salts and 2 mM
glutamine (Life Technologies, Inc., Grand Island, NY, USA)
supplemented with 10% foetal bovine serum (HyClone Labora-
tories, Logan, UT, USA) without antibiotics and incubated in 5%
CO2/95% air at 37°C. Highly metastatic KM12L4 human colorectal
carcinoma (CRC) cells were kindly provided by IJ Fidler, PhD,
DVM (The University of Texas MD Anderson Cancer Center,
Houston, TX, USA). Cells were cultured and maintained in
minimal essential medium (MEM) supplemented with 10% foetal
bovine serum (HyClone Laboratories), 2 mM l-glutamine and nonessential amino acids,
and incubated in 5% CO2/95% air at 37°C.

Creation of siRNA expression plasmids silencing Src gene expression

SiRNA expression plasmids were created using the Ambion pSilencer 1.0-U6 (Austin, TX, USA) according to the manufac-
turer’s directions. c-Src specific target sequences were designed using the Ambion siRNA web design tool. The two target
sequences utilised were (52–71 bp) 5’-AACAGAG CAAGCC
CAAGGAT-3’ and (226–244 bp) 5’-AAGCTTGGAGGGCTT
CAAC-3’. Oligonucleotides corresponding to these sequences with
flanking Apal (5’) and EcoR1 (3’) ends were purchased from
Invitrogen/Life Technology (Carlsbad, CA, USA) and ligated into
the expression plasmid at compatible sites. Constructs were
confirmed by DNA sequencing. HT29 cells were then transfected

with 500 ng of each siRNA plasmid and 100 ng of pcDNA G418
resistance promoter-less plasmid for selection of transfectants.
Cells were then grown in selective media containing G418 as
described previously (Ahmad et al, 2001). Negative controls were
transfected with empty vector target sequences and pcDNA
plasmids at identical concentrations. Total c-Src expression levels
in siRNA clones were determined by Western blot analysis.

Inhibitors/recombinant growth factors

The novel, selective, potent Src kinase inhibitor, AP23464 (O’Hare
et al, 2004; Brunton et al, 2005; Corbin et al, 2005; Summy et al,
2005), or the commercially available Src kinase inhibitor, 4-amino-
5-(4-chlorophenyl)-7-(4-butyl)pyrazolo(3,4-d)pyrimidine
(PP2) (Calbiochem, San Diego, CA, USA) suspended in 1% dimethyl
sulphoxide (DMSO) at desired concentrations and the VEGFR-1
monoclonal blocking antibody IMC-18F1 were used in this study.
The recombinant human growth factors VEGF-A and VEGF-B
(R&D Systems, Minneapolis, MN, USA) were used at 10 and
50 ng ml−1, respectively, unless noted otherwise.

Cell lysis

Cells in log growth phase at 50% confluence were serum starved
overnight and exposed to the VEGFR-1 blocking antibody IMC-
18F1 (20 μg ml−1), or AP23464 (1 μM) was added to culture media
for 1 h at 37°C. Recombinant VEGF-A or -B or PBS control was
then added at the desired concentration, and, at the desired time,
cells were rinsed twice with ice-cold PBS and then lysed with RIPA-
B lysis buffer (20 mM sodium phosphate, 150 mM NaCl, 5 mM
EDTA, 1% Triton X-100, 0.5% sodium deoxycholate) supplemented
with one tablet complete mini-EDTA protease inhibitor
cocktail (Roche Diagnostic, Manheim, Germany) and 1 mM
sodium orthovanadate (pH 7.4). Cells were harvested with the aid of a
rubber policeman, clarified by centrifugation at 13 000 g for 20 min
at 4°C, and prepared for Western blot analysis or immunoprecipi-
tation, as described previously (Windham et al, 2002).

Immunodepletion assay

Cloned cell lysates (250 μg protein) were incubated overnight
at 4°C with 10 μl anti-VEGFR-1 antibody (Oncogene Research
Products, Cambridge, MA, USA) or 10 μl antiserum IgG (Organon
Teknika, Durham, NC, USA). Immune complexes were formed by
the addition of 50 μl protein A:G agarose beads for 2 h at 4°C.
The remaining soluble lysates were centrifuged at 13 000 g for 2 min
and the supernatants were collected for immunoblotting.

Immunoprecipitation and recombinant complex kinase assay

Cloned cell lysates (500 μg protein) were incubated overnight
at 4°C with 10 μl of Src monoclonal antibody 327 (Oncogene
Research Products, Cambridge, MA, USA), 10 μl Yes antibody
1B7 (WAKO Biologicals, Richmond, VA, USA) or 6 μl focal
adhesion kinase (FAR) antibody (clone 2A7, Upstate Biotechno-
logy, Lake Placid, NY, USA). Immune complex kinase assays were
performed as described previously (Windham et al, 2002). Briefly,
immune complexes were formed by the addition of 10 μl of rabbit
antimouse IgG (Organon Teknika, Durham, NC, USA), 10 μl
protein G-Sepharose beads, and 10 μl of 10% (v/v) formalin-fixed
Pansorbin (Staphylococcus aureus, Cowan strain; Calbiochem, La Jolla, CA,
USA) for 60 min. Pellets were then washed three times in a buffer
consisting of 0.1% Triton X-100, 150 mM NaCl and 1 mM
sodium phosphate. Reactions were initiated at 22°C by the addition
of 10 μCi of [γ-32P]ATP, 10 mM Mg++ and 100 μM sodium ortho-
vanadate in 20 mM HEPES buffer to each sample. To analyse
phosphorylation of an exogenous substrate, 10 μg of rabbit muscle
enolase (Sigma-Aldrich, St Louis, MO, USA) was added to the

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Immunoblotting

Proteins (50 μg) from clarified cell lysates were separated via 8% SDS–PAGE and electroblotted onto polyvinylidene difluoride membranes (Amersham Corp., Chicago, IL, USA). The membranes were blocked with Tris-buffered saline/Tween (0.15% + 5% dried milk for 30 min at room temperature and probed with the desired primary antibody diluted 1:1000 in blocking buffer overnight at 4°C. Membranes were probed with antibodies to Src mAb#327 (Oncogene Research Products), Yes Ab 1B7 (WAKO Biologicals), phospho FAK Y397 (Biosource International, Camarillo, CA, USA), phospho FAK Y861 (Biosource International), FAK (BD Transduction, San Jose, CA, USA), Akt (5G3, Cell Signaling Technology, Beverly, MA, USA), phospho-Akt S473 (Cell Signaling Technology), p42/44 Erk MAPK (Cell Signaling Technology), phospho-p42/44 Erk (T202/Y204) (Cell Signaling Technology), VEGFR-1 (Oncogene Research Products), paxillin (Cell Signaling Technology), phospho-paxillin Y118 (Cell Signaling Technology), p130Cas (BD Transduction), phospho-p130Cas Y861 (Cell Signaling Technology), phosphotyrosine (4G10, Upstate, Lake Placid, New York, NY, USA) and vinculin (Sigma-Aldrich). Primary antibody incubation was followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad goat anti-mouse, sheep anti-rabbit or rabbit anti-goat; Bio-Rad laboratories, Hercules, CA, USA) diluted 1:3000 in blocking buffer for 1 h at room temperature. Proteins were visualised with electrochemiluminescence detection reagents (Perkin-Elmer, Boston, MA, USA) and detected with autoradiography.

Proliferation assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (M2128) (Sigma Chemical Corp., St Louis, MO, USA) was prepared by dissolving 5 mg M2128 in 1 ml PBS. The solution was protected from light and stored at 4°C. To determine proliferation, HT29 cells (1.5 × 10⁶ cells) were seeded into 96-well plates in quintuplicate and allowed to adhere overnight in 10% complete DMEM/F12. The medium was then removed and replaced with 0.2 ml of media containing VEGF-A supplemented with solvent control, IMC-18F1, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (M2128) (Sigma Chemical Corp., St Louis, MO, USA) was prepared by dissolving 5 mg M2128 in 1 ml PBS. The solution was protected from light and stored at 4°C. To determine proliferation, HT29 cells (1.5 × 10⁶ cells) were seeded into 96-well plates in quintuplicate and allowed to adhere overnight in 10% complete DMEM/F12. The medium was then removed and replaced with 0.2 ml of media containing VEGF-A supplemented with solvent control, IMC-18F1, or AP23464 and allowed to incubate at 37°C for 2 h. The media was removed carefully and the cells were solubilised in 0.2 ml DMSO. Plates were read using spectrophotometric analysis at a wavelength of 570 nm using the TECAN Genios plate reader and Magellan version 4.0 software. Results are representative of three independent experiments.

Migration assay

The modified Boyden chamber migration assay was used as described previously (Minard et al., 2005). Cells (2.5 × 10⁵ cells) were suspended in the upper well of the migration chamber (control inserts, 8 μm pore size; Becton-Dickinson, Bedford, MA, USA) in 0.5 ml of serum-free media. The lower chamber was filled with 0.75 ml of media with VEGF-A (10 ng ml⁻¹) supplemented with DMSO control, IMC-18F1 (20 μg ml⁻¹) or AP23464 (1 μM). After 72 h of incubation, the nonmigratory colon carcinoma cells on the upper filter surface were removed with a cotton swab, and cells that had migrated to the lower filter were fixed and stained with HEMA 3 (Biochemical Sciences, Swedenborg, NJ, USA) according to the manufacturer’s instructions. The migratory cells were counted under a microscope at ×100 magnification. Cell images were obtained using a Sony PXC-990 3CCD colour video camera (Sony of America, New York, NY, USA). Cells were counted in five random fields per insert in triplicate.

Statistical analyses

Statistical differences among groups were examined using the two-tailed Student’s t-test. P < 0.05 was considered statistically significant.

RESULTS

VEGF induces SFK activation in human CRC cells

A variety of growth factors are known to induce SFK activation in endothelial cells. We examined HT29 and KM12L4 human CRC cells to determine whether Src and Yes, the SFK members principally expressed in these cells (Cartwright et al., 1989; Park et al., 1993), are activated by VEGFA. As measured by immune complex kinase assay, treatment of HT29 cells with VEGF-A (10 ng ml⁻¹) increased both Src and Yes kinase activity in a time-dependent manner (Figure 1A). Maximal activation (~2.5-fold increase) was observed within 15 min, which is consistent with Src activation by a number of ligands. Src expression did not change during the time period analysed, consistent with our postulate that VEGF-A increases specific activity of these SFKs. As shown in Figure 1B, VEGF-A also activates SFKs in a dose-dependent manner (Figure 1B). Maximal activation (~2.5-fold increase) was observed within 15 min, which is consistent with Src activation by a number of ligands. Src expression did not change during the time period analysed, consistent with our postulate that VEGF-A increases specific activity of these SFKs. As shown in Figure 1B, VEGF-A also activates SFKs in a dose-dependent manner (Figure 1B).
manner in HT29 cells, with maximal kinase activation observed at 25 ng ml\(^{-1}\). Likewise, treatment of KM12L4 human CRC cells with VEGF-A (10 ng ml\(^{-1}\)) induced a similar increase in SFK activation (Figure 1C) as that observed in HT29 cells.

VEGF-induced SFK activation occurs through VEGFR-1 in HT29 cells

As reported previously, HT29 cells express VEGFR-1 but not VEGFR-2 or VEGFR-3 (Fan et al, 2005). To demonstrate VEGFR-1 activation and specificity of the monoclonal VEGFR-1 blocking antibody, IMC-18F1, tyrosine phosphorylation of VEGFR-1 was determined by Western blotting. As presented in Figure 2A, VEGF-A treatment resulted in marked increase in tyrosine phosphorylation of a 180 kDa cellular protein, which was effectively blocked by pretreatment with IMC-18F1, confirming the ability of IMC-18F1 to inhibit VEGFR-1 activation without altering VEGFR-1 expression levels. Next, to determine whether activation of SFKs was mediated by VEGFR-1, SFK activity with VEGF-A (ligand for both VEGFR-1 and -2) and the VEGFR-1 specific ligand VEGF-B was examined in the presence or absence of IMC-18F1. As shown in Figure 2B and C, VEGF-A and -B induced similar SFK activation at the doses indicated; however, pre-incubation of HT29 cells with IMC-18F1 (20 μg ml\(^{-1}\)) abrogated Src and Yes activation by both VEGF-A and -B, demonstrating that functional VEGFR-1 is required for VEGF-mediated SFK activation.

Increased activity of SFKs usually results from direct association with the cognate receptor. To determine whether VEGF induces SFK/VEGFR-1 complexes, co-immunoprecipitation experiments were performed with VEGF stimulation in the presence or absence of VEGFR-1 blocking antibody. As demonstrated in Figure 2D, VEGF greatly enhanced SFK/VEGFR-1 complex formation, and this association was effectively blocked by pretreatment with IMC-18F1. Immune complexes were not detectable when using an irrelevant IgG antibody (data not shown). Taken together, these results suggest that increased SFK activity may result from direct interaction with VEGFR-1, though other mechanisms might account for increased Src activity as well.

VEGF induces migration of human CRC cells

Src family members have been implicated in numerous biologic activities, including proliferation and migration (reviewed by Brown and Cooper, 1996; Thomas and Brugge, 1997). To identify the potential biologic effects mediated by VEGFR-1 expression, we first assessed cellular migration in response to VEGF-A, utilising a modified Boyden chamber as described in Materials and Methods (Figure 3). As demonstrated in Figures 3B and D, VEGF-A treatment of HT29 and KM12L4 human CRC cells resulted in a five- and three-fold increases in cellular migration, respectively, over nontreated control cells (P<0.001). Pharmacologic blockade of VEGFR-1 with IMC-18F1 or Src kinase inhibition with AP23464 or PP2 completely inhibited VEGF-induced migration in both cell lines (P<0.001), again demonstrating the requirement of SFK activation through VEGFR-1 in this process.

Effects of Src-targeted siRNA on VEGF-induced migration of CRC

To independently confirm the requirement for Src in mediating VEGF-A-induced migration, the ability of this ligand to affect
migrating in HT29 clones reduced in Src by stable expression of an antisense expression vector was determined. As shown in Figure 4A, two independent clones (siRNA cl.18 and 23) were reduced by more than 80% in Src expression. These cells were considerably reduced in their migratory abilities (Figure 4B), consistent with Src being important in cellular migration, and addition of VEGF-A did not increase migratory capability of these cells (Figure 4C), providing further evidence that VEGF mediates migration through Src activation. Basal proliferation of these cells determined by MTT assay did not differ significantly from nontransfected parental cells (data not shown).

**VEGF activates FAK, p130\(\text{cas}\) and paxillin in HT29 cells**

In epithelial and fibroblast cells, migration is regulated, in part, by activation of FAK. Recent studies in endothelial cells have implicated FAK as required for VEGFR-1-induced tubulogenesis (Maru et al, 2001). Src/FAK activation then leads to phosphorylation of both paxillin and p130\(\text{cas}\). To determine if FAK were activated upon treatment of HT29 cells with VEGF, both FAK immune complex kinase assays and Western blot analysis for specific FAK phosphorylation sites were performed as described in Materials and Methods. As presented in Figure 5A, VEGF treatment of HT29 cells increased both autophosphorylation of FAK and phosphorylation of the exogenous substrate enolase two-fold at 30 min. As enolase phosphorylation may also result from Src being immunoprecipitated in the immune complexes, we directly examined phosphorylation of Y861 and Y937 in response to VEGF stimulation of HT29 cells. Phosphorylation of Y861, and to a lesser extent Y937, was increased, and these increases were blocked by prior addition of IMC-18F1. These findings are consistent with previous experimental work in VEGFR-1-overexpressing fibroblasts (Maru et al, 2001) and suggest crosstalk between VEGFR-1 and FAK in HT29 cells. As shown in Figure 5B and C, VEGF treatment of HT29 cells also increased tyrosine phosphorylation of both paxillin and p130\(\text{cas}\). Maximal phosphorylation occurred within 15 – 30 min, consistent with the kinetics of Src and Yes activation. Pretreatment of HT29 cells with IMC-18F1 effectively blocked FAK, paxillin and p130\(\text{cas}\) phosphorylation, confirming the requirement of VEGFR-1 for VEGF-induced activation of these substrates. Together, these results suggest that a VEGFR-1/SFK complex interacts with components of focal adhesions, thus mediating cellular migration in HT29 cells.

**VEGF does not induce proliferation in HT29 cells**

Finally, to determine if VEGF-induced migration could be accounted for, at least in part, by VEGF acting as a mitogen, we...
examined the effect of VEGF-A stimulation on proliferation of HT29 cells. As shown in Figure 6, exogenous VEGF-A had no effect on HT29 cell proliferation, as determined by MTT assay. Likewise, pretreatment of HT29 cells with IMC-18F1 did not decrease proliferation. Consistent with these findings, VEGF-A treatment of HT29 cells induced minimal to no activation of Erk 1/2 or Akt, as assessed by Western blotting for phosphorylated (active) forms of these signalling enzymes (data not shown).

Figure 5 Effects of VEGF on phosphorylation of FAK, p130<sup>Cas</sup> and paxillin in CRC. Serum-starved HT29 cells at 50% confluency were pretreated with the VEGFR-1 blocking antibody (IMC-18F1) or PBS control for 1 h and were untreated (0) or stimulated with VEGF-A for 10, 15 and 30 min. Cell lysates were immunoprecipitated with anti-FAK antibody and subjected to immune complex kinase assay with enolase as an exogenous substrate or subjected to Western blotting with anti-phospho-FAK Y861, anti-phospho-p130 cas Y397 or anti-FAK antibodies as indicated (A), run on SDS–PAGE and subjected to Western blotting with anti-phospho-p130<sup>Cas</sup> or anti-p130<sup>Cas</sup> antibodies (B) or subjected to Western blotting with anti-phospho-paxillin or anti-paxillin antibodies (C). Western blot for vinculin is included to demonstrate equivalent protein loading.

Figure 6 Effects of VEGF on CRC proliferation. HT29 cells (2 × 10<sup>3</sup>) in 96-well plates in serum-free media were untreated or treated with VEGF-A (10 ng ml<sup>–1</sup>) in the presence of AP23464 (1 μm) or IMC-18F1 (20 μg ml<sup>–1</sup>) and proliferation was determined by MTT assay as described in Materials and Methods. Bars represent standard error of the mean. Results are representative of three independent experiments.

Figure 7 Model by which Src and VEGF contribute to tumour progression and metastasis through activities in both tumour and tumour-associated endothelial cells. In tumour cells (left) Src activation is frequent, resulting from overexpression of growth factor receptors, FAK and deregulated transcription (A). Src activation increases VEGF expression from tumour cells (B), leading to binding of VEGFR-1 (C), association with and further activation of Src (D), and the subsequent autocrine loop contributes to tumour cell migration through FAK activation (E). Vascular endothelial growth factor produced by tumour cells also binds VEGF receptors on endothelial cells (F), leading to association and activation of Src in these cells (G) and leading to increased permeability through VE cadherin phosphorylation affecting tumour cell extravasation (H), a process critical for permeability and tumour cell extravasation (I). Thus, inhibitors of VEGF, VEGFR-1 and Src may have therapeutic efficacy due to their effects in both normal and tumour cells.

DISCUSSION

Our findings of VEGF-induced SFK activation and enhanced cellular migration in human CRC cells demonstrate that functional VEGFR-1 mediates intracellular signalling and biologic behaviour in these cells. Src family kinase activation in response to VEGF has been observed in cells of endothelial origin expressing VEGFRs, and SFK members have been shown to associate with VEGFRs upon activation (Chou et al, 2002). In contrast to work in endothelial cells where Src preferentially associates with VEGFR-2 and Yes and Fyn with VEGFR-1, we observed Src and Yes kinase complex formation with VEGFR-1 upon VEGF stimulation, suggesting that at least in these tumour cells, association of SFKs with VEGFR-1 may be more promiscuous than in normal endothelial cells.

The ability of VEGF to mediate particular biologic functions appears to be cell type specific, as does expression of VEGF receptor subtypes. Many cell types exhibiting increased proliferation in response to VEGF express both VEGFR-1 and -2, or are of nonepithelial origin. The significance of aberrant expression of VEGF-1 alone on tumours of epithelial origin remains unclear, but a recently published work found a similar lack of proliferation in VEGF-stimulated colon cancer cells expressing VEGFR-1 (Fan et al, 2005).

Interestingly, we observed a robust increase in cellular migration and tyrosine phosphorylation of FAK, paxillin and p130<sup>Cas</sup> in CRC cells with VEGF stimulation, which appears to require VEGFR-1. This is consistent with prior reports suggesting that VEGFR-1 regulates cellular migration, while VEGFR-2 mediates activation of the MAPK pathway and cellular proliferation (Barleon et al, 1996). Further, our finding of VEGF-induced FAK phosphorylation, resulting in its increased activation, is consistent with previous studies on VEGFR-1-overexpressing fibroblasts (Maru et al, 2001). This result suggests an interaction between VEGFR-1 and FAK in HT29 cells. Whether this interaction is direct or indirect remains to be determined.

Vascular endothelial growth factor is a pleuripotent cytokine that induces angiogenesis, proliferation, migration, differentiation and vascular permeability in endothelial cells (Leung et al, 1989; Millauer et al, 1993; Neufeld et al, 1999). It is secreted by most, if not all solid tumours, including those arising in colon, where it is
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Corbin AS, Demehri S, Griswold IJ, Wang Y, Metcalf III CA, Theyden MN, 2004), further study of VEGF/VEGFR interactions in endothelial and, perhaps, tumour cells is warranted (Figure 7). Here we have shown that VEGFR-1 participates in intracellular signal transduction and mediates biologic activity, specifically cellular migration, in human CRC cells. The expression of this receptor protein tyrosine kinase in tumour cells may further amplify signalling pathways already activated in colon tumour cells. Thus, VEGF-mediated signalling offers novel targets for therapeutic interventions, either by targeting the receptor itself or important downstream mediators, such as Src. As Src activity has been shown to regulate VEGF expression, particularly under hypoxic conditions, in CRC cells (Ellis et al, 1998), the existence of an autocrine signalling loop capable of enhancing tumour survival and progression is intriguing. Delineating the mechanisms by which individual receptor tyrosine kinases mediate biologic activity in tumour cells is crucial for defining tumour-specific targets for optimal therapy. As recent work has demonstrated the presence of VEGFR-1 in multiple colon cancer cell lines and in both primary and metastatic tumour samples (Fan et al, 2005), VEGFR-1 may be a relevant clinical target, not only for its expression in endothelial cells but also for its expression/function in tumour cells.

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