Mutant ras Oncogenes Upregulate VEGF/VPF Expression: Implications for Induction and Inhibition of Tumor Angiogenesis

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Abstract

The growth of solid tumors in vivo beyond 1–2 mm in diameter requires induction and maintenance of an angiogenic response. This can occur through the release of various angiogenic growth factors from tumor cells. One such factor is vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), a secreted and specific mitogen for vascular endothelial cells. We show that one of the most commonly encountered genetic changes detected in human cancer, i.e., expression of mutant ras oncogenes, is associated with marked up-regulation of VEGF/VPF in transformed epithelial cells. Thus, elevation of the levels of both VEGF/VPF mRNA and secreted functional protein were detected in human and rodent tumor cell lines expressing mutant K-ras or H-ras oncogenes, respectively. Genetic disruption of the mutant K-ras allele in human colon carcinoma cells was associated with a reduction in VEGF/VPF activity. Furthermore, pharmacological disruption of mutant RAS protein function in H-ras transformed rat intestinal epithelial cells by treatment with L-739,749 (a protein farnesyltransferase inhibitor) caused a significant suppression of VEGF/VPF. The results suggest that dominantly acting ras oncogenes may contribute to the growth of solid tumors in vivo not only by a direct effect on tumor cell proliferation but also indirectly, i.e., by facilitating tumor angiogenesis. Hence, pharmacologically targeting mutant ras oncogenes could conceivably suppress solid tumor growth in vivo, in part, by inhibiting tumor-induced angiogenesis.

Introduction

The onset and maintenance of a blood supply to solid tumors requires the formation of new blood vessel capillaries from the existing host vasculature, a process known as tumor angiogenesis. This process is essential for solid tumors to grow beyond microscopic sizes, i.e., 1–2 mm in diameter (1), and is thought to be mediated by a number of either direct or indirect acting growth factors, many of which are produced by tumor cells (2). Among growth factors which have a direct effect on vascular endothelial cell proliferation and motility, VEGF, also known as VPF, has attracted the most experimental interest over the last five years (3–5). VEGF/VPF exists in four molecular isoforms, two of which are secreted, and binds to specific receptor tyrosine kinases (e.g., KDR/flk-1 and flt-1) found in a particularly high concentration in activated endothelial cells associated with angiogenesis (5). VEGF/VPF is expressed by the tumor cell parenchyma of a wide variety of solid tumors, e.g., breast, kidney, colon, ovarian and bladder carcinomas, brain tumors, and melanomas (3, 4, 6). Experiments utilizing gene transfection methods (7, 8) and monoclonal neutralizing antibodies to VEGF/VPF (9, 10) have established strong evidence for a contribution of VEGF/VPF to the progressive growth of solid tumors through its effects on promoting tumor angiogenesis.

One interesting feature of VEGF/VPF expression in solid tumors is the induction of its expression in regions of hypoxia (11), a process that at the cellular level appears to involve the activation of c-src and raf kinases (12). Therefore, it is conceivable that activation of the RAS pathway, including activating mutations of the ras gene itself, would lead to overexpression of VEGF and to stimulation of angiogenesis. Surprisingly, however, very little is known about this possible pro-angiogenic effect of mutant ras oncogenes. The main purpose of this study, therefore, was to evaluate whether expression of mutant ras oncogenes, the most common dominantly acting oncogene detected in human cancers, is associated with altered expression of VEGF/VPF. We focused our analysis on transformed intestinal epithelial cells because colon cancers are known to have a high frequency of ras mutations (13) and express high levels of VEGF/VPF (4, 10). Evidence for an inducing effect of activated ras on VEGF/VPF expression was indeed obtained. As such, this suggests that in addition to their direct effects on tumor cell mitogenesis, mutant ras oncogenes may contribute to the development and growth of solid tumors in vivo by promoting VEGF/VPF-dependent angiogenesis. Hence, it is possible that targeting mutant ras oncogenes may inhibit the growth of mutant ras-expressing solid tumors in vivo, in part, by inhibition of angiogenesis. Preliminary evidence in support of this latter hypothesis was obtained by testing the effects of a protein farnesyltransferase inhibitor on levels of VEGF/VPF produced in vitro by mutant H-ras-transformed tumor cells.

Materials and Methods

Cell Lines and Culture Conditions. Spontaneously immortalized IEC-18 cells were originally derived from the newborn rat intestine as described by Quaranti and Isselbacher (14). This cell line and all of its derivatives were maintained as monolayers in MEM base medium supplemented with 5% FBS (GIBCO-BRL, Grand Island, NY), 4 mM L-glutamine, 20 mM glucose, 10 μg/ml insulin (Sigma Chemical Co., St. Louis, MO). All the transfectants were periodically treated with 200 μg/ml G418 (Geneticin; GIBCO-BRL). Derivation of clones ras-3, ras-4, and ras-7 constitutively expressing an activated c-H-ras human oncogene and clones src-2 and src-3 expressing oncogenic v-src were described previously (15, 16). Generation and properties of the IEC-18 clone called MTras transfected with activated c-H-ras under the control of the metallothionein-inducible promoter has been described elsewhere (16). In the latter case, the expression of H-ras was induced by addition of 100 μM ZnCl2 and 2 μM CdCl2 into the culture medium for 12–36 h. The H-ras-inducing effect of this treatment was confirmed by a visible change in cell morphology. Human colorectal cancer cell lines DLD-1 and HCT-116 are known to possess an activating mutation in one of their K-ras alleles. The derivation and characterization of the respective variant sublines (DKS-8 and Hkh-2) in which the activated K-ras gene was disrupted and, thus, rendered...
nonfunctional, was reported previously (17). All four human cell lines used in this study were maintained in DME medium supplemented with 10% FBS. HUVECs were purchased from Clonetics Corporation (San Diego, CA) and maintained in gelatinized tissue culture dishes in MCDB131 medium supplemented with 10% FBS, 5 ng/ml human recombinant basic fibroblast growth factor (Upstate Biotechnology, Inc., Lake Placid, NY), 10 ng/ml epidermal growth factor (Upstate Biotechnology, Inc.), and 10 units/ml heparin (GIBCO-BRL).

**Tumorigenicity Assay.** Cells were cultured to semi-confluence in their appropriate growth medium. The cultures were harvested by brief trypsin-EDTA (GIBCO-BRL) treatment, washed in serum-free medium, resuspended at the appropriate density, and injected s.c. into athymic 8–12-week-old nu/nu BALB/c mice. Typically, groups of five to seven animals were injected with either 2 × 10^6 cells/mouse/0.2 ml of IEC-18 cells (or their variants) or 10^7 cells/mouse/0.2 ml of human colorectal carcinoma cell lines. The resulting tumors were then inspected twice a week and measured by using a Vernier's calliper. Tumor volume (mm^3) was calculated by using the standard formula \( \text{volume} = \text{length} \times \text{width} \times \text{height} / 2 \). The experiments were terminated based on ethical considerations, and the mice were autopsied.

**Endothelial Cell Growth/Survival Stimulation Assay.** The respective rat or human immortalized cell lines were plated overnight in T-25 tissue culture flasks (Falcon) in their appropriate growth medium. The medium was subsequently removed, the monolayers were washed, and 3–5 ml of either ExCell 300 serum-free medium or fresh growth medium was added for a period of 24–96 h. The conditioned medium was collected and either frozen at −70°C or used immediately. HUVECs were plated overnight in their appropriate growth medium in 96-well tissue culture plates at the density of 5–7 × 10^3 cells/well. The medium was then removed, and the cells were washed twice and overlayed with 50–100 μl/well of MCDB131 medium containing 2–10% FBS. Under these conditions, the cells ceased proliferating and underwent apoptotic death within 48–96 h, unless appropriate growth factors were added. An equal volume of epithelial conditioned medium (containing or not containing endothelial growth factors) was added to HUVECs at different dilutions and incubated for 72–96 h. For the last 4–6 h of the incubation, the cells were pulsed with 2 μCi/well of [3H]thymidine (Amersham). The plates were then harvested, and the incorporated radioactivity was counted in a Betaplate (Pharmacia) liquid scintillation counter. The thymidine uptake was in good agreement with visual appearance and MTS (tetrazolium salt) analysis of the HUVEC growth and viability.

**Measurement of Human VEGF Protein Levels in Conditioned Medium (ELISA).** A commercially available human VEGF ELISA kit (R&D Systems, Inc., Minneapolis, MN) was used to quantitate the levels of VEGF in conditioned medium obtained from the DLD-1 and HCT-116 human colorectal carcinoma cell lines and their respective K-ras knockout sublines, according to manufacturer's instructions. This reagent, in our hands, does not detect rat VEGF.

**Detection of VEGF/VPF Activity in Conditioned Medium by Anti-VEGF/VPF Neutralizing Antibodies.** The effect of anti-VEGF/VPF neutralizing antibodies on the mitogenic activity of conditioned media was evaluated by the addition of either Ab 618 rabbit polyclonal antibody raised against human purified VEGF165 or Ab 463 rabbit polyclonal antibody raised against 26 amino acid peptides corresponding to the NH2-terminal sequence of human VEGF protein.4 Both antibodies specifically recognize human recombinant VEGF165 (R&D Systems, Inc.) at a final dilution of 1:50. However, only the Ab 618 antibody has a neutralizing effect on rat VEGF.

**Treatment of H-ras-transformed Cells with the Protein Farnesyltransferase Inhibitor L-739,749.** To determine if ras inhibitors can pharmacologically suppress VEGF/VPF production, treatment of mutant H-ras-transformed IEC 18 cells with the farnesyltransferase inhibitor L-739,749 (0.2–75 μM) was carried out as described elsewhere (18, 19). The effectiveness of the treatment was monitored by examining the time- and dose-dependent changes in the cell shape. Acquisition of a flat, epithelial-like morphology by H-ras transfectants was usually achieved within 24–96 h and at concentrations of the inhibitor ranging between 1 and 50 μM. The conditioned medium was subsequently collected and assayed against HUVECs at 1:1 dilution in MCDB131 medium (2.5% FBS). In some experiments, the drug containing conditioned medium was subjected to ultrafiltration in order to remove the drug. The medium was diluted 200–300-fold, reconstituted by using a centrifrip concentrator (Millipore) and then added to HUVECs as described.

**Northern Blotting.** Approximately 10^6 cells were used for the extraction of polyadenylated RNA by a standard SDS-oligoethoxymydic acid method. The RNA was then resolved on 1% agarose gel containing 6.6 M formaldehyde, transferred to Zeta Probe (Bio-Rad, Hercules CA) membrane, and hybridized at 65°C with a 32P-labeled cDNA probe containing 200 bp human VEGF sequence common for all four known isoforms of the VPF/VEGF protein (20) (a generous gift of Dr. Brygida Berse and Dr. Harold Dvorak, Beth Israel Hospital, Boston, MA). The amount of RNA loaded in each lane was evaluated by ethidium bromide staining of the gel before the transfer. The membranes were autoradiographed, and the intensity of the specific 3.7 and 4.5 kb VEGF/VPF signal was evaluated. Human HT 1080 fibrosarcoma cells were used as a positive control (21).

**Results**

**Up-Regulation of VEGF/VPF in Immortalized Rat Intestinal Epithelial Cells by Mutant H-ras.** The first system we used to test the relationship of mutant ras oncogenes and VEGF/VPF production was the cell line known as IEC-18 and several ras transfectants obtained from this line (14–16). The parental line represents a clone of an immortalized population of rat intestinal epithelial cells. The properties of this cell line (14) and the ras transfectants have been described in detail elsewhere (14–16). As shown in Fig. 1A, the parental population is non-tumorigenic (2 × 10^6 cells were injected s.c.), whereas three mutant H-ras transfectant sublines known as ras-3, ras-4, and ras-7 are all highly tumorigenic, giving rise in a rapid manner to highly vascularized solid tumors. Furthermore, the ras transfectant tumorigenic sublines were found to secrete into conditioned media an activity, or activities, that stimulated the proliferation of HUVECs, as shown in Fig. 1B. Such activity was also detected in the conditioned medium of tumorigenic v-src-transfected clones of IEC-18 cells called src-2 and src-3 (Fig. 1B). The identity of this activity was initially unknown, but VEGF/VPF was implicated on the basis of two observations: (a) IEC-18 did not express appreciable quantities of VEGF/VPF mRNA as assessed by Northern blotting (Fig. 1C), whereas the ras-3 and ras-7 sublines expressed high levels of that transcript (this is also true for ras-4, src-2, and src-3 cell lines; data not shown); (b) in the IEC-18 transfectant, in which the expression of the mutant H-ras was under the control of a metallothionein-inducible promoter (Fig. 1C, right panel), upon addition of the inducer (zinc and cadmium ions) there was a marked up-regulation of VEGF/VPF mRNA, as well as a parallel increase in secretion of HUVEC-stimulating activity to conditioned medium by these cells (data not shown). To obtain a formal demonstration that at least a portion of the mitogenic activity was due to VEGF/VPF, we undertook antibody neutralization assays. As shown in Fig. 2, polyclonal anti-VEGF/VPF-neutralizing antibody (Ab 618), but not the control antibody (Ab 463), was able to substantially suppress the mitogenic activity for HUVECs found in conditioned medium of both H-ras- and v-src-transformed IEC 18 cells. Indeed, the levels of mitogenesis-inducing activity were brought down to those observed in the parental IEC 18 cell line.

**VEGF/VPF Expression in Human Colon Cancer Cell Lines Containing an Intact or Disrupted Mutant K-ras Allele.** The second system we used to confirm the regulatory effects of mutant ras on VEGF/VPF consisted of two human colon cancer cell lines, HCT 116 and DLD-1, each of which contained a mutant K-ras allele, and two respective sublines in which this mutant allele has been disrupted and, thereby, inactivated by targeted homologous recombination (17). In this regard, it is known that a very high frequency of human colorectal cell lines and tissue specimens express VEGF/VPF (10, 21). The
parent DLD-1 and HCT-116 cell lines grow progressively in nude mice, whereas the mutant K-ras disrupted sublines do not (17). This was confirmed and is shown in Fig. 3A. As shown in Fig. 3B, the disrupted mutant K-ras sublines show a several-fold decrease of VEGF/VPF mRNA. This result was reflected by a commensurate decrease in VEGF/VPF protein detected in the conditioned medium by an ELISA assay, where between a 4- and 5-fold decrease of protein was observed in the K-ras-disrupted DLD-1 and HCT 116 sublines (Fig. 3C). Moreover, the conditioned medium of the parent cell lines was significantly more active in inducing mitogenesis in HUVECs than was the disrupted sublines (data not shown).

Effect of the Protein Farnesyltransferase Inhibitor L-739,749 on VEGF/VPF Expression by H-ras-transformed IEC 18 Cells. From the above results in which genetic disruption of mutant K-ras alleles is associated with a marked decrease in VEGF/VPF mRNA and protein, it follows that pharmacologically inhibiting the expression or function of mutant RAS proteins in cancer cells, e.g., by treatment with farnesyltransferase inhibitors (22, 23), could conceivably suppress the growth of large, established ras-positive solid tumors in vivo (19, 24), through inhibition of angiogenesis, in addition to their direct suppressive effects on tumor cell mitogenic activity.

As a first step toward evaluating whether pharmacological inhibitors of mutant RAS proteins may function as de facto antiangiogenesis agents in vivo, we tested the effects of L-739,749, a protein farnesyltransferase inhibitor known to be effective in suppressing the growth in vitro (18) and in vivo, of mutant ras-transformed cells (19, 24). We selected H-ras-transformed IEC 18 clones as the target cell population because L-739,749 is much more active against mutant H-ras-expressing cells than are mutant K-ras-positive cells\(^5\) similar to other currently used experimental farnesyltransferase inhibitors such as BZA-5B (25–27). As shown in Fig. 4, L-739,749 treatment of the parental IEC 18 cells had no effect on conditioned medium activity. In marked contrast, the HUVEC growth stimulatory activity of conditioned medium from IEC-ras-4 cells was suppressed 50–90% by drug treatment. This was not a direct effect of the drug on endothelial cells because the suppression was still evident when the inhibitor was removed from the conditioned medium (by ultrafiltration), and, moreover, treatment of HUVECs with L-739,749 in the presence of VEGF/VPF had only a minor effect on cell proliferation (data not shown). Thus, the results obtained by pharmacological and genetic disruption of mutant RAS function or expression are consistent and implicate mutant ras oncogenes as regulators of VEGF/VPF production.

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\(^5\) A. Oliff, personal communication.
**MUTANT ras ONCOGENES UPREGULATE VEGF/VPF EXPRESSION**

Colonic epithelial cells. Mutant ras genes may exert their up-regulatory effect on VEGF/VPF expression via direct transcriptional control of the VEGF/VPF gene(s). This is because it is known that the (human) VEGF/VPF promotor contains four potential AP1 sites (30), through which the ras signal transduction pathway is known to mediate transcriptional regulation of certain genes (31, 32).

Our results could add a new dimension to understanding how mutant ras oncogenes can contribute to tumor development and progression. The impact of ras mutations is generally regarded to be the result of their effects on directly promoting tumor cell proliferation (31, 32). However, a potentially powerful indirect effect on promoting tumor cell growth could also be achieved if oncogenic ras alleles also stimulated tumor angiogenesis, as first indicated by Thompson et al. (33) in a three-dimensional organ culture system. Such an effect would go undetected if such genetic changes are studied primarily in tissue culture using pure populations of tumor cells, which has often been the case (Ref. 33 is a conspicuous exception). It follows that inhibiting the function of mutant ras alleles in cancer cells, e.g., by treatment with farnesyltransferase inhibitors (18, 26), may suppress the growth of such (solid) tumors in vivo (19), in part through inhibition of angiogenesis, and not just by their direct suppressive effects on cell mitogenic activity. This could also help explain the paradox of why such inhibitors can retard the growth of tumors in nude mice to a greater extent than could a conventional cytotoxic drug such as doxorubicin, despite being only antiproliferative and not toxic, to the same tumor cells in monolayer cell culture (19). Indeed, even extremely large, established mammary gland or salivary gland carcinomas, reaching one-third of the body weight of ras transgenic mice, can undergo total visible regressions by continuous 21-day treatment with a farnesyltransferase inhibitor (24). In this.

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**Discussion**

Bouck and others (2, 28, 29), including ourselves, have hypothesized previously that the transforming capacity of ras oncogenes and perhaps other oncogenes or tumor suppressor genes would likely encompass up-regulation of pro-angiogenic activities required for expression of the tumorigenic phenotype. Consistent with this hypothesis, our present results suggest a possible role for mutant ras oncogenes in regulating the expression of the angiogenic growth factor known as VEGF/VPF in transformed rodent or human intestinal/colon and colorectal epithelial cells. Mutant ras genes may exert their up-regulatory effect on VEGF/VPF expression via direct transcriptional control of the VEGF/VPF gene(s). This is because it is known that the (human) VEGF/VPF promotor contains four potential AP1 sites (30), through which the ras signal transduction pathway is known to mediate transcriptional regulation of certain genes (31, 32).

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regard, our results showing treatment of H-ras-transformed IEC 18 cells with L-739,749 can suppress VEGF/VPF production in vitro are quite pertinent because this implies a possible antiangiogenic function for this type of drug. It will be of interest, therefore, to confirm whether inhibitors of mutant ras can indeed function as de facto anti-angiogenesis agents in an in vivo context.

Our results may also be pertinent to helping explain why ‘knocking out’ the function of a single dominant oncogene or replacing a defective tumor suppressor gene with the wild-type form (34, 35), in a tumor cell population can completely revert the tumorigenic phenotype of the cells, despite the presumed presence of a number of other different oncogenes or tumor suppressor genes in the same cells. As Bouck (28) has emphasized, the loss of the ability to form a solid tumor in vivo could arise if reversing or nullifying the single genetic ‘defect’ leads to a significant loss in the angiogenesis-inducing ability by the tumor cells. This in turn may explain why the tumor-forming ability in vivo can be restored in such situations by cooinoculation of the cells with Matrigel (36) because this is known to stimulate angiogenesis through (as yet) unknown mechanisms (37, 38). This is somewhat similar to our results in which we have found that Matrigel coinoculation can partially “rescue” the tumorigenic phenotype of carcinoma cell lines containing a disrupted mutant K-ras allele.6

Our results may also have relevance to explaining the timing of activating ras mutations during the progression of many types of solid tumor such as colon and pancreatic carcinomas (13). Such mutations generally coincide with the stage of rapid three-dimensional tumor growth (13). Such solid growths would require angiogenesis to grow beyond 1—2 mm in diameter. Indeed, this may help explain the much higher frequency of K-ras mutations detectable in polyplody forms of colon tumors compared to the flat superficial forms of the disease (39, 40).

It is noteworthy that although oncogenic ras seems to play a major role in the up-regulation of VEGF production, other dominantly acting oncogenes and/or tumor suppressor genes must surely be involved as well. This is implied by our results with v-src-transformed IEC 18 cells (Fig. 1) and by the finding that virtually all colon carcinomas tested expressed VEGF/VPF (10, 21), and yet it is well known that only about 50% of such cancers have a mutant K-ras allele (31, 32). In some cases, up-regulation of VEGF/VPF in solid tumors can be explained by transient environmental effects such as hypoxia (12) or exposure to various growth factors or hormones (3, 4). Oncogenic activation of genes involved in the signal transduction pathways mediating these effects could alter the constitutive levels of VEGF/VPF and, thus, augment tumor angiogenesis in a more stable manner. In summary, our results indicate the existence of a causative relationship between activation of ras oncogenes and up-regulation of the angiogenic factor VEGF/VPF. This relationship has potentially important implications for understanding the ways in which mutant ras alleles contribute to solid tumor development and progression, how angiogenesis can be induced and regulated in solid tumors, and for the rationale of specifically targeting mutant ras genes as a means of cancer treatment. A similar situation can be found in the ways whereby inactivation of the normal function of wild-type tumor suppressor genes may contribute to the development and progressive growth of solid tumors. Thus mutant p53 tumor suppressor genes may influence the growth of solid tumors not just by their direct effects on cell growth (and survival) but also indirectly by virtue of their ability to alter the expression of some crucial inhibitors of angiogenesis, such as thrombospondin (41) and GD-AIF (42). The combination of inactivation of multiple tumor suppressor genes and activation of different dominantly acting oncogenes may “drive” tumor angiogenesis in a manner analogous to directly enhancing tumor cell proliferation.

Acknowledgments

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Note Added in Proof

Since completion of this work, we became aware of another study showing the v-ras oncogene can induce VEGF/VPF expression (S. Grugel, G. Finkenzeller, C. Weindel, B. Barleon, and D. Marme. Both v-H-ras and v-raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells. J. Biol. Chem., 270: 25915—25919, 1995).

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