Oncogenes and Tumor Angiogenesis: Differential Modes of Vascular Endothelial Growth Factor Up-Regulation in ras-transformed Epithelial Cells and Fibroblasts

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ABSTRACT

A possible link between oncogenes and tumor angiogenesis has been implicated by the finding that expression of various oncogenes, particularly mutant ras, can lead to a marked induction of a potent paracrine stimulator of angiogenesis, vascular endothelial growth factor (VEGF). We sought to determine how oncogenic ras induction of VEGF is mediated at the molecular level and whether the mechanisms involved differ fundamentally between transformed epithelial cells and fibroblasts. Our results suggest that in a subline (called RAS-3) of immortalized nontumorigenic rat intestinal epithelial cells (IEC-18) that acquired a tumorigenic phenotype upon transfection of mutant ras, up-regulation of VEGF occurs in the absence of an autocrine growth factor circuit. The expression of VEGF mRNA and protein by RAS-3 cells was strongly suppressed in the presence of LY294002, an inhibitor of phosphatidylinositol 3'-kinase, but remained largely unaffected in the same cells treated with an inhibitor (PD98059) of mitogen-activated protein/extracellular signal-regulated kinase kinase 1 (MKK/MEK-1). This is consistent with the observation that overexpression of a constitutively activated mutant of MEK-1 (AN3/S222D) in the parental IEC-18 cells did not result in up-regulation of VEGF production. The impact of mutant ras on VEGF expression was also significantly amplified at high cell density, conditions under which RAS-3 cells became less sensitive to LY294002-induced VEGF down-regulation.

In marked contrast to cells of epithelial origin, ras-transformed murine fibroblasts (3T3RAS) up-regulated VEGF in a manner that was strongly inhibitable by MEK-1 blockade (i.e. treatment with PD98059), whereas these cells were relatively unaffected by treatment with the phosphatidylinositol 3'-kinase inhibitor LY294002. In addition, VEGF was up-regulated by 2–3-fold in NIH3T3 cells overexpressing mutant MEK-1. Collectively, the data suggest that the stimulatory effect of mutant ras on VEGF expression is executed in a nonautocrine and cell type-dependent manner and that it can be significantly exacerbated by physiological/environmental influences such as high cell density.

INTRODUCTION

Historically, the major functional emphasis on oncogenes as contributors to tumor development has been on their impact on promoting aberrant cellular mitogenesis. However, we and Grugel et al. (1, 2) first suggested that oncogenes, such as mutant ras, may also have an important impact on tumor formation and growth through an indirect mechanism, namely, by driving tumor angiogenesis. Thus, transfection of mutant ras oncogenes into immortalized nontumorigenic rodent fibroblasts (2) or epithelial cells (1) resulted in a strong induction of VEGF,4 also known as vascular permeability factor. VEGF is a ubiquitous and potent stimulator of tumor angiogenesis with no known direct (autocrine) tumor cell growth-promoting activity (3). We also found that genetic disruption of the single mutant K-ras allele in two different human colorectal carcinoma cell lines (4) was associated with a significant suppression of VEGF expression (1) and loss of tumorigenic competence (1, 4). The latter effect could be duplicated by antisense-mediated down-regulation of VEGF in K-ras-expressing human colon cancer cells (5). Moreover, embryonic stem cells (6) in which the VEGF gene has been disrupted have grossly impaired tumor-forming capacity, even after transfection with oncogenic ras (7), as do VEGF−/− mouse embryo fibroblasts harboring an activated ras oncogene (8). Collectively, these studies suggest that VEGF expression may be a necessary but insufficient mediator (5, 9) of the tumorigenic function of mutant ras oncogenes. These results also appear to be consistent with in vivo studies in which the expression of a ras oncogene is conditionally switched off in growing tumors (10).

The exact mechanisms by which mutant ras exerts this impact on VEGF expression are not well understood. Our desire to study this relationship was based, in part, on the prediction that it may be possible to identify new targets for inhibitors of oncoprotein signal transduction that could obliterate tumor angiogenesis, albeit in an indirect manner. In this regard, we showed that ras farnesyltransferase inhibitors (Ras FTIs) can block VEGF expression in ras-transformed cells (1), a result that was recently confirmed in ras-transformed human keratinocyte (HaCAT) cell line (11). Thus, signal transduction inhibitors such as ras FTIs may bring about a part of their antitumor activity by inhibiting expression of proangiogenic growth factors such as VEGF (1, 12, 13), an effect that could contribute to the drug’s overall antitumor efficacy in vivo, but not in vitro.

One of the best-characterized signaling pathways directly activated by ras involves a cascade of interactions comprising Raf-1, MAPK kinase, or MEK, and its substrates, MAPKs, also known as ERK1 and ERK2. Expression of constitutively active mutants of Raf-1 or MEK in mouse fibroblasts can result in oncogenic transformation resembling that induced by oncogenic ras itself (14). This linear epistasis of different oncogenic proteins has been interpreted as evidence that theraf/MEK/MAPK cascade plays a central role in ras-driven tumorigenesis (15). However, more recent studies, have suggested that a number of other ras-activated pathways contribute significantly to oncogenic cellular transformation (15). These include the activity of the multi-molecular complex containing rac-1 and NADPH-oxidase, which is thought to be responsible for the generation of high levels of ROIs and...

4 The abbreviations used are: VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; TSP-1, thrombospondin 1; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; ROI, reactive oxygen intermediates; NAC, N-acetylcysteine; FBS, fetal bovine serum; PI3K, phosphatidylinositol 3'-kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; MBF, myelin basic protein; FTI, farnesyl transferase inhibitor; NDGA, nordihydroguaiaretic acid; L-NMMA, i-N-nitroarginim methyl ester.

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for stimulation of gene transcription and mitogenesis in mutant H-ras-transformed fibroblasts (16). Further emphasis on pathways other than rasraf/MAPK has become apparent with the observation that activity of P38 is required for ras-dependent transformation and that the catalytic subunit of this enzyme (p110) can synergize with activated Raf-1 (17, 18). Moreover, it has been shown that constitutive activation of the Raf-1/MAPK cascade fails to recapitulate the transforming effect of mutant ras on morphology, anchorage-independent growth in soft agar, and tumorigenicity in vivo in rodent epithelial cells (19). In such cells, unlike in fibroblasts (20), many features of malignant transformation can be mediated in an indirect, autocrine fashion by exuberant, ras-dependent overproduction of TGF-α (19, 21, 22). In this respect, it is noteworthy that TGF-α itself is a strong inducer of VEGF production via activation of the EGFR (13, 23–25).

Both oncogenic and certain physiological/regulatory influences can affect VEGF expression at multiple levels. This includes regulation of gene transcription (26–29), mRNA stability (30–33), the rate of mRNA translation (34–37), secretory pathways, and alternative splicing, as well as by expression of and heterodimerization with other members of the same growth factor family, e.g., placenta growth factor (38). In theory, each of these levels of regulation could be open to influences by mutant ras.

The purpose of the present study was to examine the contribution to VEGF up-regulation of some of the possible signaling cascades triggered by mutant H-ras and to determine whether they may differ in relative importance between epithelial and fibroblastic cells. Our results show that the mode of VEGF induction by activated ras is cell type specific and can be largely dissociated from the respective impact of this oncogene on cellular mitogenesis.

MATERIALS AND METHODS

Cells and Culture Conditions. The origin of cell lines used and their growth requirements have been described elsewhere in detail (1, 14). Briefly, immortalized rat intestinal epithelial cells (IEC-18) and their derived transfectants were cultured in α-MEM supplemented with glucose, insulin, and 5% FBS. NIH3T3 mouse fibroblasts and their sublines were maintained in DMEM with 10% FBS (1). The mutant H-ras-transformed NIH3T3 cell line (3T3RAS) consists of a pool of over 100 clones transfected with the expression vector (pSV2Ras3) that encodes V12 mutant of human H-ras proto-oncogene. The same vector was used to generate the IEC-18-derived series of H-ras transfectants. All transfections were carried out by using lipofectin reagent according to the recommendations of the manufacturer (Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s recommendations. RNA was resolved on 1% agarose gel and transferred to a Hybond N+ (Amersham Canada Limited, Oakville, Ontario, Canada) hybridization membrane. The hybridizations with 32P-labeled probes were carried out at 65°C after which the blots were washed while monitoring their radioactivity. The VEGF/vascular permeability factor probe spanning the 200-bp sequence common for all VEGF isoforms was a generous gift of Dr. Brygida Berse and Dr. Harold Dvorak (Beth Israel Hospital, Boston, MA). The 1.29-kb TSP-1 probe was prepared from pGEM2 vector encoding human thrombospondin-1 (American Type Culture Collection, Rockville, MD). To ensure equal loading, the gels were stained with ethidium bromide and photographed, and/or the membranes were subsequently probed for glyceraldehyde-3-phosphate dehydrogenase or for 28S ribosomal RNA.

Tumorigenicity Assay. Cells were injected s.c. into nude mice in equal numbers, which, for different experiments, varied between 1 × 106 and 5 × 106 cells/injection. Tumor growth was monitored by measuring two perpendicular diameters from which the tumor volume was calculated according to the protocol published previously (1, 13). Absence of a measurable tumor 3–10 months after injection was considered as “no take.”

VEGF Protein Quantitation. Production of VEGF was quantitated by an ELISA (R&D Systems Inc., Minneapolis, MN) specific for either human or mouse VEGF protein, as described by the supplier. The latter assay can also detect rat VEGF. The results were expressed as either an absolute concentration of VEGF (in pg/ml; i.e., VEGF production in pg/ml/106 cells/time of conditioning; 6–48 h) or as a percentage of change compared to the respective controls.

Mitogenesis Assays. The [3H]thymidine incorporation assay was conducted as described previously (1). Briefly, cells were plated at desired confluence (usually 5–10 × 105 cells/well) in the 96-well plate in 0.1 ml of medium and incubated with or without treatment for different lengths of time (6–18 h). The last 2 h of the incubation were conducted in the presence of 2 μCi of [3H]thymidine (Amersham) per well. The plates were frozen and thawed, and radioactive DNA was harvested onto the filter paper. The [3H]thymidine incorporation was quantitated by using the Betaplate (Pharmacia) scintillation counter.

MAPK Assay. The treatment was carried out as indicated. The cells were lysed in buffer containing 50 mM Tris (pH 7.5), 2% NP40, 5 mM sodium orthovanadate, 5 mM EDTA, 5 mM sodium P-nitrophenyl phosphate, 5 mM sodium fluoride, 50 mM sodium chloride, 50 μM aprotinin, and 50 μM leupeptin. MAPK (ERK2) was immunoprecipitated from the equivalent of 3 × 106 cells by overnight incubation with 1 μg of the anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with protein A-Sepharose. The precipitates were washed and incubated at 30°C for 30 min with 20 μl of kinase buffer containing 30 mM Tris (pH 7.3), 20 mM magnesium chloride, 2 mM manganese chloride, 10 mM ATP, 10 μCi of

\[ \text{BAY 11-7085, 20 μM, PP1, 20 μM; and rapamycin, 550 mM. Likewise,} \]

NDGA (Calbiochem-Novabiochem Corp. La Jolla, CA) from \textit{Larrea divaricata} was diluted in culture medium to a concentration of 20 μM from a concentrated stock prepared in DMSO. NAC was purchased from Calbiochem and used at the concentrations indicated, which were prepared from fresh 0.5 mM aqueous stock solutions and adjusted to the physiological pH with 1 N NaOH.

L-NAME (Biomol) was used at the working concentration of 10 μM, prepared from aqueous concentrated stock. The monoclonal neutralizing antibody that recognizes both human and rat EGFR (mR3), a generous gift of Dr. Tania Crozet (Centre for Molecular Immunology, Havana, Cuba), was used at a concentration of 50 μg/ml, and was previously demonstrated to oblate EGFR-dependent expression of VEGF in A431 cells, similar to results obtained using a different monoclonal anti-EGFR antibody, i.e., C225 (13). Most treatments were performed in the same assay medium (DME and 1% FBS), unless otherwise indicated. Conditioned medium was obtained by incubating cells with the growth medium or the assay medium for at least 24 h; then the medium was collected, spun, passed through a 0.22 μm filter, and assayed or used for further treatments.

Northern Blotting. Polyadenylated mRNA was prepared by SDS oligoethyleneoxymethylidylic acid method as described previously (1). Total RNA was prepared by using Trizol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer’s recommendations. RNA was resolved on 1% agarose gel and transferred to a Hybond N+ (Amersham Canada Limited, Oakville, Ontario, Canada) hybridization membrane. The hybridizations with 32P-labeled probes were carried out at 65°C after which the blots were washed while monitoring their radioactivity. The VEGF/vascular permeability factor probe spanning the 200-bp sequence common for all VEGF isoforms was a generous gift of Dr. Brygida Berse and Dr. Harold Dvorak (Beth Israel Hospital, Boston, MA). The 1.29-kb TSP-1 probe was prepared from pGEM2 vector encoding human thrombospondin-1 (American Type Culture Collection, Rockville, MD). To ensure equal loading, the gels were stained with ethidium bromide and photographed, and/or the membranes were subsequently probed for glyceraldehyde-3-phosphate dehydrogenase or for 28S ribosomal RNA.

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\[ \text{5 A. Viloria-Petit, T. Crozet, and R. S. Kerbel, unpublished observation.} \]
of VEGF mRNA was up-regulated, although both oncogenes produce a similar type of cellular transformation (44). Thus, VEGF mRNA was up-regulated severalfold in both H-ras (RAS-3 and RAS-4) and v-src (SRC-3 and SRC-4) transfectants (Fig. 1A), in agreement with results obtained previously by us and others (1, 45–47). In contrast, levels of TSP-1 transcript followed an oncogene-specific pattern of expression, i.e., it became virtually nondetectable in all stable cell lines transfected with oncogenic ras, whereas clones harboring v-src retained considerable levels of TSP-1 expression (Fig. 1A). Despite its known angiogenesis inhibitory properties in fibroblasts (48, 49), expression of TSP-1 did not obliterate the tumorigenic capacity of VEGF-producing v-src transfectants of IEC-18 cells, all of which formed aggressive and highly angiogenic tumor outgrowths upon injection of the cells into nude mice (Fig. 1B). Tumors also formed after injection of H-ras transfectants, but not after injection of parental IEC-18 cells, or their two independent clonal derivatives, IEC-18/4A and IEC-18/4B (engineered to express TGF-α). It is noteworthy that differential levels of TSP-1 expression correlated with and could potentially account for somewhat delayed tumor take in the case of v-src-transformed IEC-18 cells as compared to their counterparts harboring oncogenic ras (Fig. 1B). In this context, however, up-regulation of VEGF seems to be an angiogenesis-related change that is more closely associated with oncogenic transformation of epithelial cells.

**Up-Regulation of VEGF in ras-transformed Epithelial Cells Is Not Mediated by an Autocrine Growth Factor Stimulation.** It has been demonstrated recently that in rodent epithelial cells, many characteristics of ras-induced transformation are mediated by up-regulation of and autocrine response to TGF-α (21, 22). Because this growth factor is also known as a potent inducer of VEGF expression (25), it is reasonable to anticipate that it could be involved in the mechanism of ras-induced VEGF up-regulation. Furthermore, we noticed that expression of VEGF mRNA (Fig. 2A) and protein (data not shown) is considerably elevated with increasing cell density, but only in ras-transformed IEC-18 cells (RAS-3), and not in the nontumorigenic parental IEC-18 counterparts (Fig. 2A). An explanation for this finding could lie in the existence of a growth factor-mediated autocrine stimulation (50) possibly involving an oncogenic ras-induced growth factor (e.g., TGF-α). However, there are several facts that argue against such an interpretation. First, addition of the mR3 neutralizing anti-EGFR antibody, which blocks binding of TGF-α to the rat EGFR, did not diminish VEGF expression by RAS-3 cells (Fig. 2B), nor did direct neutralization of the growth factor itself (data not shown). Furthermore, treatment of RAS-3 cells with genistein, a tyrosine kinase inhibitor also known to block EGFR activity, failed to abrogate VEGF up-regulation; in fact, it led to a marked increase in expression of this angiogenic growth factor (Fig. 2C; Table 1). Because inhibition of phosphatase activity by okadaic acid treatment brought about the opposite effect (Table 1), it is possible that the impact of mutant ras on VEGF expression is, in fact, negatively regulated by events involving tyrosine phosphorylation. Finally, addition of the RAS-3-conditioned medium to parental IEC-18 cells did not result in any detectable transfer of VEGF up-regulation (Fig. 2D). Interestingly, a slight increase in VEGF mRNA was observed in RAS-3 cells in the presence of their own conditioned medium as compared to the situation when the cells were exposed to control culture medium or IEC-18-conditioned medium (Fig. 2D). This observation suggests that RAS-3 cells may display some degree of hypersensitivity to their own

**RESULTS**

**Inverse Relationship between Expression of VEGF and TSP-1 in H-ras-transformed Intestinal Epithelial Cells.** Transfection of a mutant H-ras oncogene into the immortal but nontumorigenic rat intestinal epithelial cell line IEC-18 resulted in cellular transformation and expression of tumorigenic and angiogenic properties (1, 41). Whereas it is generally believed that sustained tumor angiogenesis occurs as a result of a shift in balance between various stimulatory and inhibitory influences (42, 43), it is not clear whether different transforming oncogenes produce a unique pattern or common pattern of such molecular changes. In this regard, we observed that in IEC-18 cells, the impact of mutant ras on expression of angiogenesis regulators such as VEGF (an angiogenesis stimulator) and TSP-1 (an endogenous angiogenesis inhibitor) is different from that of the v-src oncogene (Fig. 1A), although both oncogenes produce a similar type of cellular transformation (44). Thus, VEGF mRNA was up-regulated severalfold in both H-ras (RAS-3 and RAS-4) and v-src (SRC-3 and SRC-4) transfectants (Fig. 1A), in agreement with results obtained previously by us and others (1, 45–47). In contrast, levels of TSP-1 transcript followed an oncogene-specific pattern of expression, i.e., it became virtually nondetectable in all stable cell lines transfected with oncogenic ras, whereas clones harboring v-src retained considerable levels of TSP-1 expression (Fig. 1A). Despite its known angiogenesis inhibitory properties in fibroblasts (48, 49), expression of TSP-1 did not obliterate the tumorigenic capacity of VEGF-producing v-src transfectants of IEC-18 cells, all of which formed aggressive and highly angiogenic tumor outgrowths upon injection of the cells into nude mice (Fig. 1B). Tumors also formed after injection of H-ras transfectants, but not after injection of parental IEC-18 cells, or their two independent clonal derivatives, IEC-18/4A and IEC-18/4B (engineered to express TGF-α). It is noteworthy that differential levels of TSP-1 expression correlated with and could potentially account for somewhat delayed tumor take in the case of v-src-transformed IEC-18 cells as compared to their counterparts harboring oncogenic ras (Fig. 1B). In this context, however, up-regulation of VEGF seems to be an angiogenesis-related change that is more closely associated with oncogenic transformation of epithelial cells.

**Fig. 1.** Differential patterns of VEGF and TSP-1 mRNA expression in epithelial cells transformed with oncogenic forms of ras or src. A, simultaneous up-regulation of VEGF and down-regulation of TSP-1 in sublines of IEC-18 cells expressing mutant H-ras (RAS-3, RAS-4; left panel). Up-regulation of VEGF without a change in TSP-1 levels in v-src-expressing IEC-18 cells (SRC-3, SRC-4; right panel). Polyadenylated RNA preparation was analyzed as described in "Materials and Methods." B, relative tumor-forming capacity of various IEC-18 sublines on s.c. injection (2 x 10⁶ cells were injected per mouse, five mice per group). Tumor take was 100% for both H-ras or v-src-transformed cell lines (RAS-3, RAS-4, SRC-3, and SRC-4), respectively. No tumors were observed in the case of IEC-18 parental cells or two clones derived thereof by transfection with a human TGF-α expression vector (21) for up to 5 months, at which point the experiment was terminated.
autocrine growth factors, to which IEC-18 cells are unresponsive. However, taken together, these results argue for a more direct (non-autocrine) linkage between expression of mutant ras and up-regulation of VEGF in transformed intestinal epithelial cells.

**Dissociation of MEK/MAPK Activity from Up-Regulation of VEGF in H-ras-transformed Epithelial Cells.** Because the raf/MEK/MAPK pathway is thought to play a central role in oncogenic ras-dependent transformation (14, 51–54), it was logical to examine its potential contribution to the up-regulation of VEGF expression in an epithelial cell context. However, several independent clones (11A-4.2, 11A-2.3, and 11A-2.2) of IEC-18 cells engineered to overexpress the constitutively active mutant of MEK-1 (ΔN3/S222D) did not display any appreciable increase in VEGF production as compared to cells expressing wild-type MEK-1 (wt-4.4) or to the parental (IEC-18) population (Fig. 3A). Treatment of these various sublines as well as ras-transformed IEC-18 cells (RAS-3) with a pharmacological inhibitor of MEK-1 activity (PD98059) did not result in abrogation of VEGF secretion into the condition medium (Fig. 3A). Despite only a slight reduction in expression of VEGF protein and mRNA in RAS-3 cells treated with PD98059 (Fig. 3, A and D, respectively), this drug was highly effective in suppressing both MAPK enzymatic activity and cellular mitogenesis (Fig. 3, B and C, respectively). This is illustrated by an up to 6-fold reduction in MAPK-dependent phosphorylation of MBP within 1 h of the addition of PD98059 (Fig. 3B). In the presence of the drug (50 μM), this suppression of MAPK continued for at least 18 h (Fig. 3B), with a commensurate time-dependent decrease in the rate of DNA synthesis (Fig. 3C). Again, these obvious manifestations of PD98059 bioactivity stand in marked contrast to the unremarkable effect of this inhibitor on ras-dependent up-regulation of VEGF.

**Up-Regulation of VEGF by Mutant H-ras in Epithelial Cells Is Diminished on Inhibition of PI3K Activity.** PI3K has been recently shown to mediate many aspects of oncogenic ras-dependent transformation (17). This enzyme is constitutively activated in IEC-18 cells harboring mutant H-ras (55). We therefore decided to examine VEGF expression in RAS-3 cells in the presence or absence of a potent PI3K inhibitor known as LY294002 (56). Indeed, the drug profoundly down-regulated VEGF expression at both the mRNA (Fig. 3D) and protein (Fig. 4) levels. Interestingly, the combined effect of PD98059 and LY294002 was greater than that of the latter drug alone, suggesting that both respective pathways may show some degree of cooperation in regulation of VEGF (Fig. 3D). Although secretion of VEGF by RAS-3 cells into their conditioned medium could be largely inhibited in the presence of LY294002, the magnitude of this effect was significantly modified by external influences such as cell density (Fig. 4). Thus, in sparse cell cultures, the effect of PI3K inhibition was very pronounced, leading to reduction of VEGF levels by approximately 4–10-fold (at 4 and 20 μM LY294002, respectively), whereas in parallel confluent cultures, the corresponding values were only 5% and 60% inhibition (Fig. 4). This observation suggests that whereas oncogenic ras-induced up-regulation of VEGF expression is in itself dependent on PI3K activity, its cooperation with factors regulated by cell density (see Fig. 2A) is not. The addition of higher serum concentrations (10%) masked the resistance of confluent cells to LY294002 treatment.

![Fig. 2. Evidence for nonautocrine mechanism of VEGF up-regulation in epithelial cells harbouring mutant H-ras. A, up-regulation of VEGF at high cell density as a function of ras-transformation. Parental IEC-18 or H-ras transformed (RAS-3) cells were grown as either dense, (confluence >90%) or sparse (semiconfluent, 30–50%) cultures, and total RNA (25 μg/lane) was probed for VEGF expression. Selective, density-dependent up-regulation of VEGF by RAS-3 cells was also confirmed by protein analysis (data not shown). B, Ras-dependent up-regulation of VEGF mRNA is unresponsive to treatment with neutralizing antibody to EGFR (mAb) at 50 μg/ml. C, tyrosine kinase inhibitor, genistein (100 μM), is unable to down-regulate VEGF in RAS-3 cells (see Table 1). D, transfer of conditioned medium from RAS-3 cells failed to up-regulate VEGF in parental IEC-18 cells. Growth medium was incubated with respective cells lines for 24 h (CM IEC-18, CM RAS-3) and added to cultures of either IEC-18 or RAS-3 cells for an additional 24 h. Control medium (Con) was incubated in tissue culture dishes in the absence of cells.

**Table 1. Interference of pharmacological signal transduction inhibitors with ras-dependent expression of VEGF in epithelial and fibroblastic cells**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>VEGF mRNA (% control)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>RAS-3</td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>COX-2</td>
<td>525% 250%</td>
</tr>
<tr>
<td>L-NAME</td>
<td>NOS</td>
<td>121% 120%</td>
</tr>
<tr>
<td>NDOA</td>
<td>LOX</td>
<td>NT 122%</td>
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<tr>
<td>Olidac acid</td>
<td>Protein phosphatases</td>
<td>50% 124%</td>
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<td>Genistein</td>
<td>Tyrosine kinases</td>
<td>659% 173%</td>
</tr>
<tr>
<td>Calphostin C</td>
<td>Protein kinase C</td>
<td>155% 186%</td>
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<tr>
<td>GF10920X</td>
<td>Protein kinase C</td>
<td>168% 106%</td>
</tr>
<tr>
<td>BAY 11-7085</td>
<td>Nuclear factor κB</td>
<td>NT 124%</td>
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<tr>
<td>PP1</td>
<td>src tyrosine kinases</td>
<td>103% 101%</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>p70/S6K</td>
<td>116% 106%</td>
</tr>
<tr>
<td>NAC</td>
<td>ROIs</td>
<td>97% 77%</td>
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*Cells were treated for 9 h with the respective inhibitors or vehicle in DMEM containing 1% FBS. Expression of VEGF transcript in total RNA preparation was quantitated by PhosphorImager scanner, normalized to the abundance of 28S RNA, and expressed as a percentage of normalized VEGF expression in cultures incubated with DMSO (in all cases except NAC and L-NAME, which was dissolved in medium directly).

COX-2, cyclooxygenase-2; NOS, nitric oxide synthase; LOX, lipooxygenase; NT, not tested.
In Epithelial Cells, ROIs Do Not Mediate Up-Regulation of VEGF Protein. Mutant ras is known to exert its transforming activity in part through activation of the multimolecular complexes containing rac-1 and NADPH-oxidase (16), which in turn stimulate the production of ROIs. It has been postulated that PI3K may participate in the activation of rac-1, triggering this signaling cascade (57). Interestingly, ROIs have been directly implicated in up-regulation of VEGF via increased mRNA stability (58). Taken together, these observations suggest that PI3K-dependent up-regulation of VEGF by mutant ras may be mediated by ROIs. However, comparative analysis of VEGF mRNA half-life in RAS-3 and IEC-18 cells did not reveal any significant differences (Fig. 5). In addition, treatment of RAS-3 cells with the antioxidant NAC did not obliterate expression of VEGF mRNA (Fig. 6C), nor could we detect such an inhibitory effect in

Fig. 3. Up-regulation of VEGF in H-ras-transformed epithelial cells is dependent on PI3K activity and dissociable from regulation of MEK/MAPK and mitogenesis. A, absence of VEGF up-regulation in IEC-18 cells expressing constitutively activated, mutant MEK-1 (MEK-1 exog.) at levels comparable to endogenous enzyme (MEK-1 end.). Similar low levels of VEGF were detectable in conditioned medium of all clonal sublines of IEC-18 cells expressing mutant MEK-1 (H1A-4, H1A-2, H1A-2.3, H1A-2.2), wild-type MEK-1 (wt-4,4), or parental IEC-18 cells. Mutant ras-dependent up-regulation of VEGF was not abrogated by treatment of RAS-3 cells with the MEK-1 inhibitor (PD98059), even at the maximal concentration (50 μM). B, suppression of MAPK activity (MAPK-dependent MBP phosphorylation) during treatment of RAS-3 cells with PD98059 (50 μM). C, inhibition of DNA synthesis and mitogenesis by PD98059 treatment of RAS-3 cells. D, down-regulation of VEGF mRNA expression in RAS-3 cells treated with inhibitor of PI3K (LY294002, 20 μM, LY). PD98059 (PD) was unable to cause a significant change in VEGF mRNA unless combined with LY294002 (PD + LY).

Fig. 4. Dose-dependent down-regulation of VEGF protein production in ras-transformed epithelial cells treated with PI3K inhibitor (LY294002). Culturing the RAS-3 cells under high density conditions (dense) blunted the effect of LY294002 as compared to the cells in the log phase of growth (sparse). Addition of 10% FBS modified both cell density-dependent and -independent responses of RAS-3 cells to LY294002.

In Epithelial Cells, ROIs Do Not Mediate Up-Regulation of VEGF Transcript by Mutant ras but Are Required for Secretion of VEGF Protein. Mutant ras is known to exert its transforming activity in part through activation of the multimolecular complexes containing rac-1 and NADPH-oxidase (16), which in turn stimulate the production of ROIs. It has been postulated that PI3K may participate in the activation of rac-1, triggering this signaling cascade (57). Interestingly, ROIs have been directly implicated in up-regulation of VEGF via increased mRNA stability (58). Taken together, these observations suggest that PI3K-dependent up-regulation of VEGF by mutant ras may be mediated by ROIs. However, comparative analysis of VEGF mRNA half-life in RAS-3 and IEC-18 cells did not reveal any significant differences (Fig. 5). In addition, treatment of RAS-3 cells with the antioxidant NAC did not obliterate expression of VEGF mRNA (Fig. 6C), nor could we detect such an inhibitory effect in
found that constitutive MAPK activity detected in NIH3T3 fibroblasts is due to species specificity of the ELISA detection system. VEGF and v-ras analysis of discrepancy by testing D). We decided to study this apparent different from the results shown above, which were obtained using fibroblast result in strong up-regulation of VEGF (59). This is clearly of oncogenic mutants of ras, raf (2), and MEK-1 (59) in rodent phenotype (14, 15). Furthermore, it is well established that expression central and dominant role in mediating the transformed (tumorigenic) led to the notion that the raf/MEK/MAPK signaling pathway plays a transformation of mouse fibroblasts expressing a mutant conclusion (rat VEGF) vis-à-vis expression of exogenous growth factor suppression increased steadily with increasing NAC concentration (2.5-20 mM). VEGF levels remain unaffected (right panel). Absence of VEGF signal from H-ras or v-src-transformed IEC-18 cells in this panel (human VEGF) is due to species specificity of the ELISA detection system. B, VEGF mRNA expression in Ras-3 cells is unaffected by NAC treatment (20 mM), despite changes at the protein level.

various other tumor cell lines harboring a mutant ras oncogene (data not shown). Because the effect of H-ras on VEGF expression in IEC-18 cells is primarily exerted at the mRNA (transcriptional) level, we interpret these results as an indication that signals transmitted by ROS are not directly involved in this regulation. However, analysis of conditioned media obtained from IEC-18 variants transfected with mutant ras (RAS-3) or v-src (SRC-3) revealed that NAC treatment can almost completely block the expression of VEGF protein (Fig. 6A). A somewhat similar blockade was observed when human VEGF, was expressed in IEC-18 cells (clone 18V9) from a heterologous, constitutively active, viral (cytomegalovirus) promoter, further suggesting the involvement of a posttranscriptional mechanism. There were puzzling differences in the profiles of VEGF inhibitory activity of NAC in the context of oncogene-driven, endogenous production (rat VEGF) vis-à-vis expression of exogenous growth factor in 18V9 cells (human VEGF). In the former case, the degree of suppression increased steadily with increasing NAC concentration and was nearly complete at 20 mM, whereas in 18V9 cells, the effect was incomplete and biphasic, with a plateau beginning as low as at 2.5 mM of the antioxidant. The source of this discrepancy and the overall mechanism of VEGF inhibition by antioxidants remain to be elucidated.

Up-Regulation of VEGF in H-ras-transformed Fibroblasts Is Mediated by the MEK/MAPK Pathway. Studies on malignant transformation of mouse fibroblasts expressing a mutant ras oncogene led to the notion that the ras/MEK/MAPK signaling pathway plays a central and dominant role in mediating the transformed (tumorigenic) phenotype (14, 15). Furthermore, it is well established that expression of oncogenic mutants of ras, ras (2), and MEK-1 (59) in rodent fibroblast result in strong up-regulation of VEGF (59). This is clearly different from the results shown above, which were obtained using epithelial cells (see Fig. 3D). We decided to study this apparent discrepancy by testing ras-transformed NIH3T3 cells under identical conditions and using approaches similar to those employed in our analysis of ras-transformed epithelial (IEC-18) cells. Indeed, we found that constitutive MAPK activity detected in NIH3T3 fibroblasts harboring mutant H-ras (3T3RAS) could be blocked in a profound and lasting manner by treatment with the MEK-1 inhibitor (Fig. 7A). Again, as in the case of Ras-3 epithelial cells, this effect was accompanied by a gradual inhibition of cellular mitogenesis, as measured by the rate of [3H]thymidine incorporation into the DNA of asynchronously growing cells (Fig. 7B). However, in 3T3RAS fibroblasts, unlike their epithelial (RAS-3) counterparts, the antiproliferative effect of the MEK-1 inhibition was also accompanied by down-regulation of VEGF mRNA expression (Fig. 7C). Furthermore, in the case of 3T3RAS fibroblasts, inhibition of PI3K by treatment with LY294002 did not lead to abrogation of VEGF expression (Fig. 7C) as it so obviously did in Ras-3 cells (Fig. 3D). Combination of LY294002 and PD98059 treatments were only slightly more effective than treatment with the MEK-1 inhibitor alone (Fig. 7C). Finally, expression of VEGF protein was elevated by 2-3-fold in transformed and tumorigenic NIH3T3 fibroblasts (14) expressing a constitutively activated mutant of MEK-1 (ΔN3/S222D) as compared to cells transfected with the wild-type enzyme (MEK-1/wt). As expected, this up-regulation was completely abrogated by treatment with the MEK-1 inhibitor PD98059 (Fig. 7D).

Cellular transformation under the influence of oncogenic ras is associated with a multitude of pleiotropic changes (15), each of which can have phenotypic consequences and potentially (directly or indirectly) impact VEGF expression (38). To explore some of these possibilities, we tested the effect of various signal transduction inhibitors on expression of VEGF mRNA by Ras-3 epithelial and 3T3RAS fibroblast (Table 1). With the exception of the Ras FTI (L-739,749) tested previously (1), MEK-1 inhibitor (PD98059), PI3K inhibitor (LY294002), and dexamethasone (60), no remarkable VEGF inhibition was observed on targeting such cellular activities as cyclooxygenase-2 (sulindac), lipooxygenase (NDGA), nitric oxide synthase (L-NAME), protein kinase C (calphostin C; GFX), S6 kinase (rapamycin), src-like kinases (PP1) or nuclear factor eB (BAY 11-7085). This suggests that these activities are nonessential for oncogenic ras-dependent VEGF up-regulation under standard testing conditions (1% FBS) but obviously does not rule out participation of the
respective target proteins in the control of VEGF expression under conditions in which there is a combined influence of ras and external physiological/environmental stimuli such as growth factors, cell-cell contacts, or hypoxia.

**DISCUSSION**

Our results should help resolve some conflicting findings regarding the mechanisms of ras oncogene-induced up-regulation of VEGF because they show that significant differences can be obtained, depending on whether transformed epithelial or fibroblastic cells are used for experimental analysis. Thus, just as the raf/MAPK pathway seems to be dominant in oncogenic (morphological) transformation of fibroblasts but not epithelial cells (19), the same appears to be the case for ras-mediated up-regulation of VEGF. Moreover, the PI3K pathway, which has been implicated in ras-mediated oncogenic transformation of epithelial cells in vitro or in vivo, was found in the present study to contribute to VEGF up-regulation in transformed intestinal epithelial cells, but in a much less conspicuous way, if at all, in fibroblasts.

The linkage between expression of mutant ras oncogenes and up-regulation of VEGF has now been demonstrated in a number of systems with rather remarkable consistency (1, 2, 29, 49, 61–63); however, as stated above, there has been no widely accepted molecular mechanism to account for this effect. For example, Grugel et al. (2) pointed out that VEGF up-regulation occurs in fibroblasts transformed with activated ras and v-raf, suggesting that by virtue of epistatic hierarchy, the entire raf/MAPK pathway is involved (2). This is consistent with the observation described recently by Milanini et al. (59), who reported that VEGF transcript is up-regulated in hamster fibroblasts transfected with activated MEK-1. However, there have been at least two reasons for doubts being raised regarding a universal and dominant role of the raf/MAPK pathway in the up-regulation of VEGF by oncogenic ras. First, as discussed above, there is mounting evidence that although this pathway is critical for transformation of rodent fibroblasts, the same is not the case for epithelial cells (19), which are the cellular progenitors of the vast majority of cancers in man. Thus, a similar difference might be extrapolated with respect to the proangiogenic mechanisms of ras oncogene-induced transformation in general, including up-regulation of VEGF. Second, experiments with immortalized human endothelial cells transfected with an activated H-ras oncogene suggest that elevated expression of VEGF mRNA, at least in this case, can be mitigated by the presence of the PI3K inhibitor wortmannin (64). Interestingly, wortmannin was more effective in restricting VEGF up-regulation under the influence of hypoxia than by mutant H-ras itself (64). This is consistent with earlier studies by Mazure et al., who reported that mutant ras amplifies the stimulatory effects of hypoxia on VEGF gene transcription in a manner that is dependent on the respective activities of PI3K, its downstream target-protein kinase B (PKB/Akt), and hypoxia inducible factor I (HIF-1) (29, 65). In this study and in others, the effect of hypoxia was clearly separable from the activity of the raf/MAPK cascade (29, 59), although some recent reports seem to indicate otherwise (45, 66).

The results we have obtained with ras-transformed epithelial cells differ fundamentally from those that we and others have obtained using fibroblasts. Thus, even under normoxic conditions, the effect of

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**Fig. 7.** MEK/MAPK dependence of VEGF expression and mitogenesis in ras-transformed fibroblasts. A, suppression of MAPK activity in mutant ras-transformed NIH3T3 cells (3T3RAS) in the presence of MEK-1 inhibitor (PD98059, 50 μM); B, time-dependent inhibition of DNA synthesis in 3T3RAS cells in the presence of PD98059; C, inhibition of VEGF mRNA expression in 3T3RAS cells treated with PD98059 but not with LY294002; D, up-regulation of VEGF protein expression in NIH3T3 cells transformed with constitutively active mutant of MEK-1 [MEK-1/mut (ΔN3/S222D)] as compared to the cells expressing wild-type MEK-1 (MEK-1/wt). This up-regulation was abrogated by the addition of PD98059.

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mutant ras on VEGF expression in transformed epithelial cells was markedly reduced on inhibition of PI3K activity and was resistant to inhibition of MEK-1. The antagonistic responses to the latter treatment were not due to the differential potency of PD98059 in fibroblasts and epithelial cells because MAPK activity and mitogenesis were markedly inhibited in both cell types by the treatment with the drug. Our transfection experiments also indicate that whereas mutant MEK-1, which is known to readily transform rodent fibroblasts (14, 54), can also induce an increase in VEGF mRNA levels (59) and protein production in these cells (compare Fig. 7), it clearly fails to do so in IEC-18 epithelial cells. However, although our experiments suggest a lack of autonomous VEGF stimulating function of MEK-1 in IEC-18 cells, a degree of cooperation between this enzyme and PI3K signaling can be inferred from the cumulative effect of combined LY294002 and PD98059 treatment.

It is intriguing that the expression of VEGF mRNA did not always correspond to the VEGF protein levels in the conditioned medium of various transformed and nontransformed cell lines. Notably, treatment with NAC, an antioxidant and inhibitor of ras signaling via ROIs (16), had only a slight effect on expression VEGF mRNA in 3T3RAS cells and had no effect on their epithelial counterparts (RAS-3), whereas it profoundly inhibited VEGF protein secretion. This result suggests a requirement for ROIs at posttranscriptional levels of VEGF regulation that may or may not be influenced by oncogenic transformation. There are several possible examples of how such regulation might be exerted, e.g., by alteration of VEGF gene translation through function of internal ribosomal entry sites (36) or translational activity of the eIF-4E protein (34). Whatever the mechanism of its activity, NAC can serve as a prototype of a specific signal transduction inhibitor that has been used extensively in the clinic (67) and deserves further exploration in terms of its potential as a possible angiogenesis inhibitor.

Finally, it is important to keep in mind that the proangiogenic phenotype induced by oncogenic ras is clearly not restricted to up-regulation of VEGF. Whereas the latter factor is at the center of angiogenesis regulation in many systems (5–8, 68), there are growing numbers of reports suggesting the involvement of other oncogene-(and tumor suppressor gene)-dependent changes in expression of angiogenesis stimulators, inhibitors, and modulators (12, 42, 60). However, with few exceptions (49, 64, 69), these studies are focused on a single growth factor and a single tumor-associated genetic alteration (1, 48, 70). In this regard, we simultaneously examined the expression of VEGF and TSP-1 in the context of a single epithelial model system and under influence of two different transforming oncogenes, H-ras and v-src. This led us to the realization that the profile of proangiogenic changes expressed by tumor cells may, to some extent, be oncogene specific. An example of this is the lack of TSP-1 down-regulation in v-src but not in H-ras-transformed IEC-18 cells. Again, this pattern and its angiogenic consequences in vivo may vary between different cell types harboring similar oncogenic changes (49, 71).

In summary, an important practical implication of our results is that up-regulation of VEGF, which is a crucial and common element of the ras-dependent angiogenic phenotype (1, 5, 7, 8), is executed in a tissue/cell type-specific manner. Although in this context, targeting mutant ras itself (or a ras-related signaling modules) can, in principle, be viewed as a form of de facto antiangiogenic therapy, development of such similar approaches using signal transduction inhibitors should not rest on the mere presence of mutant ras (or other analogous genetic changes) in the target tumor cell population. For example, whereas an inhibitor of ras may block VEGF production and hence possibly, tumor angiogenesis in ras-transformed epithelial cells or fibroblasts, the same cannot be said of signal transduction inhibitors that act further downstream of ras. Thus, inhibitors of MAPK and PI3K may have quite different effects with respect to blocking ras-induced VEGF expression, depending on the cellular origin of the tumor. Hence, improved therapeutic opportunities may lie in a more detailed understanding of the molecular linkage between specific oncogenic changes and the resultant angiogenic phenotypes in specific types of cancer cells.

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