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Different Regulation of Vascular Endothelial Growth Factor Expression by the ERK and p38 Kinase Pathways in v-*ras,* v-*raf,* and v-*myc* Transformed Cells

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Here we show that vascular endothelial growth factor (VEGF) mRNA expression is up-regulated in oncogene transformed rat liver epithelial (RLE) cell lines and that the extracellular signal-regulated kinase (ERK) and p38 kinase differentially regulate the oncogene-mediated stimulation of VEGF. The highest level of VEGF mRNA expression was observed in the v-H-*ras* transformed RLE cell line, followed by the v-*raf* and v-*myc* transformed lines. The PD98059 MEK inhibitor was used to block the ERK pathway and SB203580 inhibitor to block the p38 pathway. The parent and the v-H-*ras* transformed RLE cell lines showed up-regulation of VEGF RNA expression through the ERK pathway and down-regulation of VEGF through the p38 pathway. VEGF was regulated in a comparable manner in a human breast carcinoma cell line. In the v-*raf* and v-*myc* transformed RLE lines, positive regulation of VEGF was transduced through the p38 pathway. These findings suggest that (1) oncogenic *ras* differs from *raf* and *myc* in the recruitment of the MAPK signaling pathways for VEGF regulation; (2) that VEGF is regulated in *ras* transformed and human cancer cell lines in a positive and negative manner by the ERK and p38 signaling pathways.

Formation of new blood vessels is required for supporting growth of solid tumors (1). VEGF is an important angiogenic factor, which has been shown to be essential in experimental tumorigenesis (2, 3) and is expressed in different types of human cancers (4). VEGF expression is induced by various cytokines and growth factors (4, 5). The relationship between oncogene activation and the induction of tumor angiogenesis has mainly been studied in *ras* transformed cells (6). Mutant *ras* has been shown to contribute to angiogenesis through up-regulation of VEGF expression (7, 8) and stabilization of VEGF mRNA (9). Further evidence that VEGF is important for *ras*-induced tumorigenesis came from a study on H-ras transformed embryonic fibroblasts with null mutations in both alleles of VEGF (10). However, the role of activated *ras* in tumor maintenance is believed to go beyond the regulation of VEGF expression (11).

The MAPK cascade is one of four signal transduction systems used by mammalian cells. It has been classified into the ERK, JNK, and p38 signaling pathways (12). Phosphorylated MAPK can activate a range of substrates, including transcription factors, which results in increased or decreased expression of target genes. It has been shown that *ras* induces VEGF mRNA expression through ERK kinase activation (13). Signaling pathways underlying induction of VEGF by the *raf* or *myc* oncogenes remain to be elucidated.

The objective of the present study was to examine if the ERK and p38 pathways are involved in VEGF induction in cloned cell lines, derived from v-H-*ras,* v-*raf,* and v-*myc* transformed RLE cell lines. These lines have been extensively studied with respect to various parameters associated with carcinogenesis (14–20). We show that stimulation of VEGF expression in v-H-*ras* transformed RLE cells is transduced through the ERK pathway, whereas the p38 pathway is used for VEGF stimulation in the v-*raf* and v-*myc* transformed cells.

MATERIALS AND METHODS

The origin and the characterization of the rat liver epithelial (RLE)-derived cell lines was described previously (14–19). Briefly, the RLE cells were originally derived from the liver of 10-day old Fischer 344 rats. They were infected with retroviral constructs containing the following oncogenes: v-H-*ras* (pRNR16), v-*raf* (3611-MSV), or v-*myc* (J-5). The cell lines were maintained in Ham's F12 medium (Biofluids, Rockville, MD), 2 mM L-glutamine, 50 mg/l,

Abbreviations used: VEGF, vascular endothelial growth factor; RLE, rat liver epithelial; ERK, extracellular signal-mediated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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gentamicin sulfate (Gibco, Grand Island, NY), and 10% fetal bovine serum (Biofluids, Rockville, MD). The MCF-7 human breast cancer cell line was maintained in Eagle's minimum essential medium with Earle's balanced salt solution supplemented with non-essential amino acids, 2 mM L-glutamine, and 10% fetal bovine serum (FBS). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

PD98059, a selective inhibitor of ERK activator, MEK1 (New England BioLabs, Beverly, MA) and SB203580, a p38 kinase specific inhibitor (Sigma Chemical Company, St. Louis, MO) were dissolved in DMSO to make stock solution of 50 mM and 10 mM. The cell lines were trypsinized and plated at the density of 2×10^6 cells per 100 mm dish in 10 ml of culture medium described above with 0.5% FBS, instead of 10%. After overnight incubation, confluent cell cultures were changed to 10 ml of medium containing 10 μl of DMSO (control), 10 μl of 50 mM PD98059 (final conc. 50 μM), or 10 μl of 10 mM SB203580 (final conc. 10 μM). After 6 h of treatment, extraction of total RNA and preparation of whole cell lysates were carried out as described below. All experiments were repeated 2–4 times.

Total RNA was extracted using RNA Zol B (Tel Test Inc.) according to the manufacturer's instruction. For Northern blot analysis, 10 μg aliquots of total RNA were run on 1% agarose/2.2.M formaldehyde gel, immobilized onto a nylon membrane and hybridized with 1×10^6 cpm/ml of ^{32}P-labeled rat or human VEGF cDNA probes. Quantitation of VEGF mRNA expression was carried out using PhosporImager (Molecular Dynamics; software: Image Quant). The 18S RNA stained with ethidium bromide or GAPDH expression levels were used for control of RNA loading.

Western blot hybridization was carried out on RLE cell lines with and without PD98059 and SB203580. Confluent cells in 100 mm dishes were disrupted in 500 μl of lysis buffer which consisted of 10 mM Tris (pH 7.5), 10 mM EDTA, and 0.5% Triton X-100. The mixture was then sonicated ($2 \times$ 10-s pulses), and the supernatant was stored at −20°C after centrifugation at 15000 g for 15 min. Protein concentrations were determined using the BCA method (Pierce, Rockford IL), and 5 μg aliquots of total protein were boiled for 3 min together with 2 X loading buffer (Novex, San Diego, CA). SDS-PAGE was carried out using 12% tris-glycine polyacrylamide gels (Novex, San Diego, CA) at a constant 120 V for 1 h and 30 min in a Novex XCell II minicell (San Diego, CA). Transfer of protein from gels to PVDF (polyvinylidene difluoride) membranes (Novex, San Diego, CA) was accomplished in 2 h using a Novex XCell II Blot Module (Novex, San Diego, CA) at a constant 30 V. After incubating the membrane in a blocking buffer (Tris Buffered Saline with 0.2% Tween 20 and 7.5% non-fat dry milk) for 1 h, PhosphorPlus p44/p42 MAP kinase (Thr202/Tyr204) Antibody kit and p38 kinase sampler kit (New England BioLabs, Beverly, MA) were used to detect phosphorylated and non-phosphorylated MAP kinases, respectively, according to the manufacturer's instructions. The signal was visualized by exposing the blot to X-ray film (Amersham-Pharmacia, Piscataway, NJ) for 10 to 60 s.

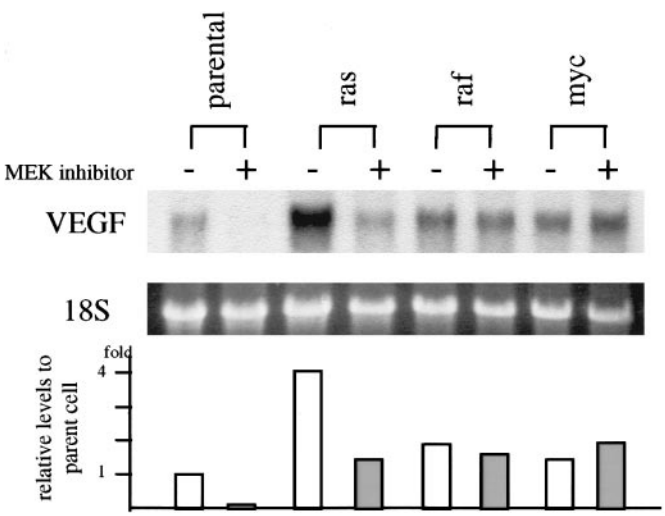

FIG. 1. Northern blot analysis of VEGF mRNA expression in parent, v-H-*ras*, v-*raf*, and v-*myc* transformed RLE cell lines in the presence and absence of an ERK pathway specific inhibitor, PD98059. Ethidium bromide staining of 18S RNA shows loading of total RNA. Bar graphs indicate relative VEGF mRNA levels of the oncogene transformed cells, compared to the basal expression by the parent RLE cell line.

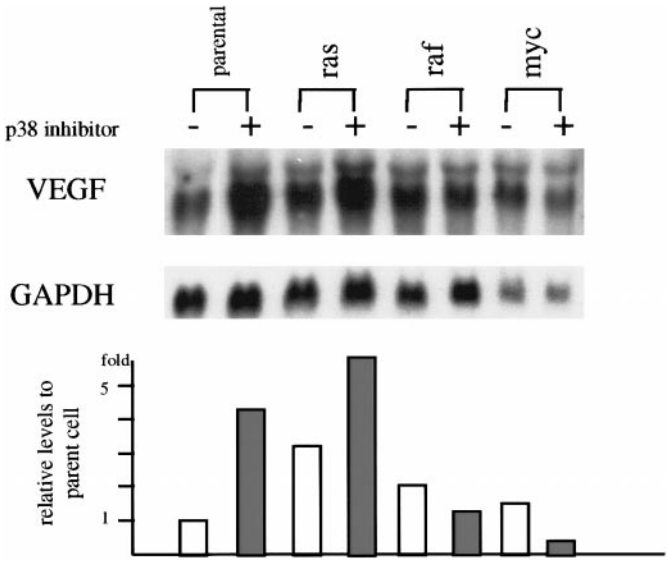

FIG. 2. Northern blot analysis of VEGF mRNA expression in parent, v-H-*ras*, v-*raf*, and v-*myc* transformed RLE cell lines in the presence and absence of a p38 kinase pathway inhibitor, SB203580. GAPDH is used to control loading of RNA. Bar graphs indicate relative VEGF mRNA levels of the oncogene transformed cells, compared to basal expression by the parent RLE cell line.

RESULTS

Regulation of VEGF by the ERK and p38 Signaling Pathways

Northern blot analysis was carried out to determine the relative contribution of the ERK and p38 pathways to regulation of VEGF mRNA expression in the parent and transformed RLE cell lines. Of the three transformed cell lines the v-H-*ras* transformant displayed the highest VEGF mRNA level, followed by the v-*raf* and the v-*myc* transformant (Figs. 1 and 2).

To find out if VEGF expression was dependent upon signaling through the ERK pathway, it was blocked by the MEK1 specific inhibitor, PD98059. The ERK pathway was critical for stimulation of VEGF in the parent RLE cell line, since the PD98059 treatment resulted in a complete loss of the VEGF signal (Fig. 1). Blocking of the ERK pathway with PD98059 resulted in a 2.7-fold decrease in the level of VEGF expression in the v-H-*ras* transformed line, but had minimal effect on VEGF

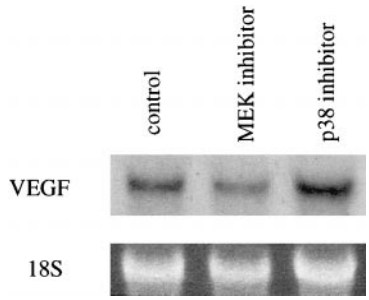

FIG. 3. Northern blot analysis of VEGF mRNA expression in the human MCF-7 breast cancer cell line in the presence and absence of the PD98059 MEK inhibitor and the SB203580 p38 kinase inhibitor. Ethidium bromide staining of 18S RNA shows loading of total RNA.

expression in the v-*raf* and v-*myc* transformed cell lines (Fig. 1). Western blot analysis of MAPK phosphorylation in the untreated parent and oncogene transformed cell lines showed comparable levels of phospholylated ERK1/2 which were completely abolished in all lines following PD98059 treatment (data not shown).

Inhibition of the p38 kinase pathway with a specific inhibitor, SB203580 resulted in 4.0-fold and 1.8-fold increase in VEGF mRNA expression in the parent and v-H-*ras* transformed RLE cell lines (Fig. 2). In the v-*raf* and v-*myc* transformed lines however, inhibition of the p38 pathway resulted in 0.3-fold decrease in VEGF expression in the v-*raf* transformed cell line and 0.6-fold decrease in the v-*myc* transformed line (Fig. 2).

Similar Effect of ERK and p38 Kinase Pathways on VEGF mRNA Expression in a Human Breast Cancer Cell Line

We examined one human cancer cell line to see if VEGF mRNA expression was affected in a similar manner as was observed in the oncogene transformed RLE cell lines. As shown by Northern blot analysis of RNA from the human MCF-7 breast carcinoma cell line in Fig. 3, VEGF expression was inhibited by PD98059 and stimulated by SB203580, indicating that regulation of VEGF signaling was similar to that observed in the parent and v-H-*ras* transformed RLE cell lines.

DISCUSSION

Oncogene activation results in up-regulation of a number of genes that contribute to tumorigenesis, including VEGF. Previous studies have suggested that H-*ras* and K-*ras* are involved in up-regulation of VEGF (7, 8) and v-*raf* was shown to stimulate VEGF expression in NIH/3T3 cells (21). Here, we studied VEGF RNA expression in v-H-*ras*, v-*raf*, and v-*myc* transformed RLE cell lines and examined if VEGF was regulated by the ERK and p38 pathways of the MAPK cascade.

The highest level of VEGF RNA expression was observed in the v-H-*ras* transformed RLE cell line. VEGF transcripts were also detected in the parent RLE line which is rare in epithelial cells without oncogenic changes. The RLE cells however, have many biochemical and ultrastructural characteristics of oval cells, which are considered to be derived from hepatic stem cells (20). It is therefore not surprising that the primitive RLE cell line expresses VEGF.

In the present study, the PD98059 MEK inhibitor was used to examine if the ERK pathway plays a role in VEGF signaling and SB205380 was used to assess the involvement of the p38 pathway. VEGF expression was almost completely abolished by the PD98059 in the parent RLE cells, which suggests that the ERK pathway is the principal positive regulator of VEGF in this primitive epithelial cell line. In the v-H-*ras* transformed RLE cells however, VEGF expression was partially suppressed by inhibition of the ERK pathway. This may conceivably suggest that additional pathway(s) are recruited in stimulating VEGF after transformation with oncogenic *ras*.

In the v-*raf* and v-*myc* transformed RLE cell lines, positive regulation of VEGF was transduced through the p38 signaling pathway and was minimally affected by the ERK pathway. This was unexpected in the v-*raf* transformed RLE cells however, blocking of the ERK pathway by the MEK inhibitor is upstream from *myc* and would therefore not be expected to affect VEGF expression. Suppression of VEGF by the p38 inhibitor in the v-*raf* and v-*myc* transformed cell lines was incomplete, which implies that VEGF may be regulated through additional pathways analogous to that observed in the v-H-*ras* transformed RLE cells. Others have suggested MAPK independent pathways are involved in gene induction by oncogenic *raf* (22). It must also be considered that oncogenic *raf* and *myc*, as well as *ras*, may exert negative function on suppressors of angiogenesis in non-transformed epithelial cells (23).

To find out if the ERK and p38 pathways are involved in regulation of VEGF expression in a malignant human tumor cell line, we chose the MCF-7 breast carcinoma line. Interestingly, the effects of the ERK and p38 inhibitors on VEGF RNA expression in the MCF-7 cells were comparable to that observed in the v-H-*ras* transfected RLE cells. It will be important to further characterize the role of the signaling pathways used in *ras*-mediated stimulation of VEGF since *ras* is the most commonly mutated oncogene in solid human malignant tumors. The possibility that the p38 pathway serves as a suppressor of VEGF in cancer cells may have significant therapeutic implications.

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