Effects of Raf Kinase Inhibitor Protein Expression on Suppression of Prostate Cancer Metastasis

Zheng Fu, Peter C. Smith, Lizhi Zhang, Mark A. Rubin, Rodney L. Dunn, Zhi Yao, Evan T. Keller

Background: Raf kinase inhibitor protein (RKIP), an inhibitor of Raf-mediated activation of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK), is expressed at lower levels in human C4-2B metastatic prostate cancer cells than in the parental non-metastatic LNCaP prostate cancer cells from which they were derived. We examined whether RKIP functions as a suppressor of metastasis. Methods: Immunohistochemistry was used to detect RKIP expression in clinical samples of primary prostate cancer and prostate cancer metastases. LNCaP and C4-2B cells were stably transfected with plasmids that constitutively expressed antisense and sense RKIP cDNA, respectively, or with empty vector. Assays of cell proliferation, soft-agar colony formation, and in vitro invasion were used to examine the malignant phenotypes of the transfected cells. An orthotopic murine model was used to examine the effect of expressing RKIP in C4-2B cells on the development of spontaneous metastasis. Results: Clinical samples of primary prostate cancer had detectable RKIP expression, whereas clinical samples of prostate cancer metastases did not. There were no differences in the in vitro proliferation rate or colony-forming ability between the control vector-transfected and sense RKIP vector-transfected C4-2B cells or between the control vector-transfected and the antisense RKIP vector-transfected LNCaP cells. Overexpression of RKIP in C4-2B cells was associated with decreased in vitro cell invasion, decreased development of lung metastases in vivo, and decreased vascular invasion in the primary tumor but did not affect primary tumor growth in mice. Conclusions: RKIP does not influence the tumorigenic properties of human prostate cancer cells. It appears to be a novel and clinically relevant suppressor of metastasis that may function by decreasing vascular invasion. [J Natl Cancer Inst 2003;95:878–89]
MEK-1 (a mitogen-activated protein [MAP] kinase kinase, which specifically phosphorylates the regulatory threonine and tyrosine residues present in MAP kinases) by Raf-1 (9). The purpose of the current study was to investigate whether RKIP functions as a suppressor of prostate cancer metastasis.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture**

Human prostate cancer LNCaP cells (American Type Culture Collection [ATCC], Manassas, VA) were grown in RPMI-1640 medium (Life Technologies, Gaithersburg, MD) with 10% fetal bovine serum (FBS), and C4-2B cells (UroCor, Oklahoma City, OK) were grown in T medium (80% Dulbecco’s modified Eagle medium [Life Technologies], 20% Ham’s F12 medium [Irving Scientific, Santa Ana, CA], NaCO₃ at 3 g/L, penicillin G at 100 U/L, streptomycin at 100 µg/mL, insulin at 5 µg/mL, triiodothyronine at 13.6 µg/mL, transferrin at 5 µg/mL, biotin at 0.25 µg/mL, and adenine at 25 µg/mL) supplemented with 5% FBS (10). Cells were grown in 150-mm culture dishes in a 37°C incubator equilibrated with 5% CO₂ in humidified air.

**Real-Time Reverse Transcription–Polymerase Chain Reaction**

Total RNA was extracted from LNCaP and C4-2B cells using TRIzol reagent (Life Technologies), as directed by the manufacturer. We subjected 200 ng of total RNA to real-time reverse transcription–polymerase chain reaction (RT–PCR) using a LightCycler SYBR Green I RNA amplification kit according to the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). RT–PCR reactions were subjected to 45 cycles of 94°C for 5 seconds, 55°C for 10 seconds, and 72°C for 1 minute. RT–PCR of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts was used as an internal control to ensure equal loading of samples. We used oligonucleotide primers purchased from Clontech (Palo Alto, CA) to amplify a 250-base pair (bp) fragment of RKIP RNA (sense primer: 5‘-CAATGCCAGATCCAGATGGCAGCAGTC-3‘; antisense primer: 5‘-CACAATCGATCCCCACTGCGCTG-3‘). We amplified a 960-bp fragment of GAPDH RNA (sense primer: 5‘-TGAGGTCGTTGTAACCGATTGTTTGCTTGA-3‘; antisense primer: 5‘-CATG TAGGCTAGGTTTCCACAC-3‘).

**RKIP Expression Vectors**

Human RKIP cDNA (ATCC) was subcloned into the BamHI and EcoRI (Life Technologies) sites of the pcDNA3.1(+) and pcDNA3.1(−) expression vectors (Invitrogen, San Diego, CA). We used DNA sequence analysis and restriction enzyme mapping to distinguish between the resulting plasmids, pcDNA3.1(+)ssRKIP (i.e., sense RKIP vector) and pcDNA3.1(−)ssRKIP (i.e., antisense RKIP vector), which constitutively expressed the RKIP cDNA in the sense and antisense orientations, respectively, from a cytomegalovirus promoter. Sequence analysis showed that the subcloned RKIP cDNA had 100% homology to the published sequence for RKIP cDNA (11). We used the pcDNA3.1(+) and pcDNA3.1(−) vectors without RKIP inserts as negative controls in subsequent experiments.

**Generation and Selection of Cells Stably Transfected With pcDNA3.1(+)ssRKIP and pcDNA3.1(−)ssRKIP**

LNCaP cells (5 × 10⁵) were transfected with 1 µg of pcDNA3.1(−)ssRKIP or pcDNA3.1(+)ssRKIP and pcDNA3.1(+) with the use of SuperFect reagent (Qiagen, Valencia, CA), according to the manufacturer’s instructions. Selection for the neomycin gene contained on each of the plasmid vectors was initiated 48 hours after transfection by adding 600 µg of G418 (Life Technologies)/mL to the culture medium. This selection medium was changed every 4 days for 5 weeks, at which point all untransfected (i.e., G418-sensitive) cells had died. Clones of G418-resistant cells were isolated and expanded for further characterization.

**In Vitro Cell Invasion Assay**

The invasiveness of LNCaP and C4-2B cells stably transfected with control, sense, or antisense plasmids was evaluated in 24-well transwell chambers (Costar, Cambridge, MA), as directed by the manufacturer. Briefly, the upper and lower culture compartments of each well are separated by polycarbonate membranes (8-µm pore size). The membranes in some wells were pre-coated with 100 µg/cm² of collagen matrix (Matrigel; Collaborative Biomedical Products, Bedford, MA), which was reconstituted by adding 0.5 mL of serum-free T medium to the well for 2 hours. To assess the ability of the cells to cross the polycarbonate membrane (i.e., baseline migration), 2.5 × 10⁴ cells in 0.5 mL of T medium containing 5% FBS was placed into the upper compartment of wells that did not contain collagen matrix, and 0.75 mL of T medium containing 10% FBS was placed into the lower compartment. In parallel, we assessed the ability of the same cells to penetrate a collagen matrix by placing 2.5 × 10⁴ cells in 0.5 mL of T medium containing 5% FBS in the upper compartment of wells that were coated with the reconstituted matrix and 0.75 mL of T medium containing 10% FBS in the lower compartment. The transwell chambers were incubated for 24 hours at 37°C in 95% air and 5% CO₂. Cell penetration through the membrane was detected by staining the cells on the porous membrane with a Diff-Quik stain kit (Dade Behring, Newark, DE) and quantified by counting the numbers of cells that penetrated the membrane in five microscopic fields (at ×200 magnification) per filter. Invasive ability was defined as the proportion of cells that penetrated the matrix-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). The results are reported as the mean of triplicate assays (12).

To test the effect of protein kinase inhibitors on the invasiveness of C4-2B cells, the in vitro invasion assay was performed using C4-2B cells in the presence and absence of various protein kinase inhibitors (Calbiochem, San Diego, CA). Briefly, C4-2B cells were seeded in upper compartments of 24-well transwell chambers in the presence of either the MEK-1 inhibitor PD098059 (20 µM), the p38 MAP kinase inhibitor SB203580 (20 µM), the phosphoinositide-3 kinase inhibitor LY294002 (10 µM), the protein kinase C inhibitor bisindolylmaleimide I (10 µM), or the protein kinase A inhibitor H89 (3 µM) for 24 hours. The experiment was repeated three times.

**Cell Proliferation Assay**

Cells were grown for further characterization.

**Cell Proliferation Assay**

Cell proliferation was measured with the use of a CellTiter 96 AQUous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Briefly, parent cells (LNCaP and C4-2B cells) and their transfectants were plated in 96-well plates at a density of 500 cells/well in 200 µL of T medium with 5% FBS. The cells were incubated at 37°C in a humidified 5% CO₂ atmo-
sphere for 2, 4, 6, or 8 days, at which time 20 µL of combined MTS/PMS solution (Promega) was added per well. After incubation for 2 hours at 37 °C, we measured the absorbance of each well at 490 nm by using an enzyme-linked immunosorbent assay (ELISA) plate reader. Data represent the average absorbance of six wells in one experiment. The experiment was repeated twice with similar results.

**Soft-Agar Colony Formation Assay**

Assays of colony formation in soft agar were performed using standard methods. Briefly, 1-mL underlayers consisting of 0.6% agar medium were prepared in 35-mm dishes by combining equal volumes of 1.2% Noble agar (Difco, Detroit, MI) with either 2x RPMI-1640 medium with 20% FBS or 2x T medium with 10% FBS. Parent cells (LNCaP and C4-2B cells) and their transfectants were trypsinized, centrifuged, and resuspended in 0.3% agar medium (equal volumes of 0.6% Noble agar and 2x RPMI-1640 medium with 20% FBS and 2x T medium with 10% FBS); 10³ cells were then plated onto the previously prepared underlayers. The cells were kept wet by adding a small amount of RPMI-1640 medium (Life Technologies) with 10% FBS for LNCaP cells and their transfectants or T medium with 5% FBS for C4-2B cells and their transfectants. The cells were incubated at 37 °C in a humidified 5% CO2 atmosphere for 5–6 weeks and were then stained with methylene blue. Stained colonies were photographed and counted.

**Western Blot Analysis**

Whole-cell lysates were prepared by incubating parent cells (LNCaP and C4-2B cells) or their transfectants grown to 85%–90% confluence in six-well plates with 80 µL of ice-cold lysis buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, leupeptin at 5 µg/mL, pepstatin at 5 µg/mL, and 0.5 mM phenylmethylsulfonyl fluoride for 2 minutes. Cells were sonicated for 8 seconds and then placed on ice for 15 minutes. Lysates were precleared by centrifugation at 20 000g for 15 minutes, and protein concentrations were determined using the bicinchoninic acid assay (Pierce Bioc hemicals, Rockford, IL). The protein samples were mixed with 4x sodium dodecyl sulfate (SDS) loading buffer (final concentrations were 62.5 mM Tris–HCl [pH 6.8], 2% SDS, 10% glycerol, 0.05% bromphenol blue, and 2% mercaptoethanol); 40 µg of protein was loaded per lane and separated on 12% SDS–polyacrylamide gels, and the proteins were transferred to nitrocellulose membranes. The membranes were incubated in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk to blocking nonspecific binding, probed with a goat polyclonal anti-RKIP primary antibody (SC-5423 or SC-5426; 1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated donkey anti-goat secondary antibody (1:5000 dilution; Santa Cruz Biotechnology), and visualized using an enhanced chemiluminescence (ECL) detection system (Amersham Life Science, Arlington Heights, IL). Those membranes were subsequently stripped by incubating with stripping buffer (final concentrations were 250 mM Tris–HCl [pH 6.7], 2% SDS, and 100 mM 2-mercaptoethanol) at 50 °C for 30 minutes and then reprobed using a rabbit polyclonal anti-actin antibody (1:1000 dilution; Sigma-Aldrich, St. Louis, MO) with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at a 1:5000 dilution (Amersham International, Buckinghamshire, U.K.) and visualized using an ECL detection system. For quantification, the bands for RKIP and β-actin protein were analyzed with ChemiImager version 3.3 software (Alpha Innotech, San Leandro, CA). The RKIP protein levels were normalized against β-actin to control for variance in sample loading and transfer.

To examine the phosphorylation status of endogenous MEK and ERK in LNCaP and C4-2B cells and their stable transfectants, the cells were lysed and subjected to western blot analysis as described above, except that the membranes were probed with an anti-p-MEK antibody (Cell Signaling Technology, Beverly, MA) to detect phosphorylated MEK or an anti-p-ERK antibody (Santa Cruz Biotechnology) to detect phosphorylated ERK, visualized by ECL reagents (Amersham Life Science, Arlington Heights, IL). Those membranes were subsequently stripped as described above and then reprobed with anti-MEK or anti-ERK antibodies (Santa Cruz Biotechnology) to detect both the phosphorylated and unphosphorylated forms of MEK and ERK.

**Immunohistochemistry of Human Tissue**

Radical prostatectomy specimens were obtained at the time of surgery from prostate cancer patients treated at the University of Michigan and frozen in liquid nitrogen within 30 minutes after surgical excision, as previously described (13). Histologic confirmation of both tumor and normal regions of each prostate gland and tumor grading was performed using a previously described protocol (14). Metastases found in a variety of tissues, including lymph node, bone, liver, lung, dura, adrenal gland, bladder, thyroid, and stomach, were obtained from patients that died of metastatic prostate cancer and were frozen in liquid nitrogen within 4 hours of the patient’s death using a rapid autopsy method, as previously described (15). (All samples were grossly trimmed to ensure that 95% of the sample represented the desired lesion.) A genitourinary pathologist (M. A. Rubin) confirmed that the metastases were of prostate cancer origin. Written informed consent was obtained from all patients or their families (for autopsy specimens), and all tissue procurement was approved by the University of Michigan Institutional Review Board.

Frozen tissue blocks were sectioned on a cryostat, and frozen sections were placed on slides, which were immersed in acetone at 4 °C for 15 minutes, air dried for 30 seconds, and then stored in phosphate-buffered saline (PBS) until immunostaining was performed. RKIP protein expression was detected using a rabbit polyclonal anti-RKIP antibody (1:400 dilution; Upstate Biotechnology, Lake Placid, NY) detected by the streptavidin–biotin method, which included horseradish peroxidase and diaminobenzidine chromogen (Histostain Kit; Zymed, San Francisco, CA). Immunostaining intensity was scored by a genitourinary pathologist (M. A. Rubin) blinded to Gleason score, tumor size, and clinical outcome. Staining was scored as negative (0), weak (1), moderate (2), or strong (3) on the basis of the amount of stain detected, as previously described (16). Briefly, the pathologist evaluated the spectrum of staining intensity in all the samples and then arbitrarily categorized the staining intensity into the various scores on the basis of overall staining.

**Immunofluorescence Double Staining for RKIP and Prostate-Specific Antigen**

Human tissue sections were incubated overnight at 4 °C with a mixture of goat anti-RKIP antibody (1:50 dilution; Santa Cruz Biotechnology) and rabbit anti-human prostate-specific antigen
(PSA) antibody (1:2000 dilution; Dako, Carpinteria, CA). The sections were then washed with PBS and incubated for 2 hours at room temperature with a mixture of fluorescein isothiocyanate-labeled anti-rabbit immunoglobulin G (IgG) and Texas red-labeled anti-goat IgG (Vector Laboratories, Burlingame, CA). Slides were rinsed with PBS, mounted in fluorescence mounting medium (Vector Laboratories), and viewed with the use of a fluorescence microscope (Carl Zeiss, New York, NY). We obtained no fluorescent signals when we omitted the primary antibodies. Additionally, to ensure that the secondary antibody to RKIP did not cross-react with the primary antibody for PSA and also that the secondary antibody to PSA did not cross-react with the primary antibody for RKIP, we incubated primary tumor tissue sections with one of the primary antibodies followed by incubation with both secondary antibodies. We obtained no fluorescent signals when the secondary antibody to RKIP was used to stain the primary antibody for PSA, nor were fluorescent signals obtained when the secondary antibody to PSA was used to stain the primary antibody for RKIP.

**Mice**

Male CB17 severe combined immunodeficient mice (Charles River, Wilmington, MA) were housed under pathogen-free conditions in accordance with National Institutes of Health guidelines. Our animal protocol was approved by the University of Michigan Animal Care and Use Committee. The mice used in this study were 5–6 weeks old.

**Spontaneous Metastasis Assay**

Mice were anesthetized with an intraperitoneal injection of xylazine (9 mg/kg) and ketamine (100 mg/kg) and then injected with C4-2B cells that were stably transfected with either pcDNA3.1(+) (i.e., control vector) or pcDNA3.1(+)-ssRKIP (i.e., sense RKIP vector) (1 × 10^6 cells per injection) into the dorsolateral lobe of the prostate gland with the use of a 30-gauge needle (BD, San Diego, CA). To ensure that our results were not specific to any individual subclone of transfected cells, we injected pools of clones of each type of transfected. Specifically, for injections of C4-2B cells stably transfected with control vector, we pooled clones C4-2B-(+)#3 and C4-2B-(+)#4; for injections of C4-2B cells stably transfected with sense RKIP vector, we pooled clones C4-2B-ssRKIP#2, C4-2B-ssRKIP#5, and C4-2B-ssRKIP#9 (each clone was represented in equal parts).

The mice were killed with carbon dioxide asphyxiation 10 weeks after being inoculated with tumor cells. After they were killed, the primary (i.e., prostate) tumors were measured and their volumes were calculated using the formula for hemi-ellipsoids: \( V = \frac{length \times width \times height}{2} \times 0.5236 \) (17). Lungs were excised and fixed in 10% formalin, and lung metastases were quantified as previously described (17), with minor modifications. Briefly, formalin-fixed lungs were serially cut from the apex to the base into 20-μm-thick sections, and adjacent sections were immunostained for PSA (rabbit anti-human PSA antibody (1:2000 dilution; Dako) and stained with hematoxylin and eosin, as previously described (17). We examined 24 sections, which represented the entire length of each lung, for the presence of metastases.

**Assessment of Angiogenesis and Vascular Invasion**

To quantify vascular endothelial growth factor (VEGF) expression in C4-2B vector and sense-transfected cells, 6.4 × 10^4 cells were seeded in 24-well plates containing T medium with 5% FBS, incubated at 37 °C overnight, and then switched to T medium with 1% FBS. After incubation for an additional 24, 48, or 72 hours, the supernatants were collected and stored at −80 °C. VEGF levels in the supernatants were measured using a human ELISA kit (Human VEGF Quantikine kit; R&D Systems, Minneapolis, MN), as recommended by the manufacturer.

To quantify vascularity in the primary tumors in mice, we performed immunohistochemistry for blood vessels. Briefly, the primary tumors were fixed in 10% formalin, embedded in paraffin, and sectioned. Unstained sections were deparaffinized, rehydrated, and then stained for CD31 (an adhesion molecule present on blood vessels) with a rat polyclonal anti-CD31 antibody (1:250 dilution; Pharmingen, San Diego, CA) using standard immunohistochemistry techniques, as described above. The total number of blood vessels (indicated as CD31-positive) in a representative section from each tumor was quantified. To determine the proportion of blood vessels that were invaded by tumor cells, a pathologist (M. A. Rubin) who was blinded to the transfusion status of the tumor cells enumerated blood vessels that contained tumor cells in a representative section (stained for CD31) from each tumor. The proportion of blood vessels invaded by tumor cells in the primary orthotopic tumors was calculated by dividing the number of vessels invaded by tumor cells by the total number of vessels for each tumor section.

**Statistical Analysis**

We used the Mantel–Haenszel chi-square test to test the significance of the association between RKIP expression level and the degree of prostate cancer progression. Differences in tumor size, in the number of lung metastases per mouse (among mice that developed metastases), and in the percentage of vessels that were invaded (among mice that displayed any invasion) between mice injected with cells transfected with sense RKIP vector and mice injected with cells transfected with control vector were tested using a nonparametric Wilcoxon rank sum test. Incidences of primary tumor formation and metastases were reported along with their 95% exact binomial confidence intervals (CIs). Incidences of lung metastases development and vessel invasion were compared between the two groups using Fisher’s exact test. For the in vitro studies, single comparisons were performed using Student’s t test, and multiple comparisons were performed using one-way analysis of variance (ANOVA) with Fisher’s protected least significant difference method for post hoc analysis. All statistical tests were two-sided. For all tests, the level of significance was set at \( P < 0.05 \). Statistical calculations were performed with the use of SAS System software (SAS Institute, Cary, NC).

**RESULTS**

**RKIP Expression in Metastatic Prostate Cancer**

To confirm our previous gene array findings, that RKIP expression is decreased in the human C4-2B metastatic prostate cancer cell line compared to its non-metastatic parental LNCaP cell line (8), we measured the levels of RKIP mRNA by realtime RT–PCR and protein by western blot analysis in these cell lines. We found that C4-2B cells have four- to fivefold less RKIP mRNA and approximately threefold less RKIP protein than LNCaP cells (Fig. 1). To examine the potential relevance of these findings to clinical prostate cancer, we used an immunohistochemical approach
Fig. 1. Differential expression of Raf kinase inhibitor protein (RKIP) in human prostate cancer cells. A) Analysis of RKIP mRNA expression in LNCaP and C4-2B cells. Total RNA was collected from LNCaP and C4-2B cells, and 200 ng was subjected to real-time reverse transcription–polymerase chain reaction for RKIP mRNA. Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA was used as an internal control for variance in loading samples. A representative graph that demonstrates fluorescence is presented in the left panel. The summary results from three separate experiments are presented in the right panel. Bars represent the mean values of RKIP mRNA levels normalized to GAPDH levels from three individual experiments; error bars represent the upper 95% confidence intervals. *P = .0012 compared with LNCaP cells; Student’s t test. B) Analysis of RKIP protein expression in LNCaP and C4-2B cells. Total cellular protein from LNCaP or C4-2B cells (40 μg) was subjected to western blot analysis for RKIP protein. β-actin was used as an internal control for loading. RKIP band intensity was measured using densitometry and was normalized to β-actin levels. A representative stained membrane is shown in the left panel. The summary results from three separate experiments are presented in the right panel. Bars represent the mean RKIP protein expression after normalization with β-actin; error bars represent the upper 95% confidence intervals. *P = .0088 compared with LNCaP cells; Student’s t test.

to detect RKIP in human normal and cancerous prostate tissue samples. RKIP was detectable in all noncancerous prostate tissue (n = 10) and primary prostate cancers (n = 12) examined but was undetectable in all prostate cancer metastases examined (n = 22) (Fig. 2 and Table 1). Specifically, RKIP expression level was highest for benign tissue, lower for cancerous tissue (declining with increasing Gleason score), and absent in metastases (P < .001, Mantel–Haenszel chi-square test). To ensure that our inability to detect RKIP in the metastatic tissue samples was not due to the quality of the sections, the samples were also stained for PSA and all were positive (data not shown). Our ability to detect PSA in these metastatic samples, as well as our observation that we could not detect RKIP in the cancer cells of a liver metastases but could detect RKIP in the surrounding normal liver (Fig. 2; liver metastasis), demonstrates that RKIP protein was detectable using immunohistochemistry in these samples.

RKIP Expression Level and In Vitro Invasiveness of Prostate Cancer Cells

To examine the function of RKIP during prostate cancer progression, we modulated RKIP expression in prostate cancer cells by stably transfecting C4-2B cells with sense RKIP vector (or empty expression vector for control) and LNCaP cells with antisense RKIP vector (or empty expression vector for control). The sense RKIP vector-transfected C4-2B cells demonstrated increased RKIP expression, and the antisense RKIP vector-transfected LNCaP cells demonstrated decreased RKIP expression compared with the corresponding control vector-transfected cells (Fig. 3, A).

To examine whether modulation of RKIP expression influenced the tumorigenic properties of the prostate cancer cells, we measured the in vitro proliferation rates and the ability of LNCaP and C4-2B cells stably transfected with the various plasmids to form colonies in soft agar. There were no statistically significant differences in in vitro proliferation rates or colony-forming abilities between the control vector-transfected and sense RKIP vector-transfected C4-2B cells or between the control vector-transfected and the antisense RKIP vector-transfected LNCaP cells (data not shown; one-way ANOVA). These data suggest that modulation of RKIP expression has no effect on these two primary tumorigenic properties of human prostate cancer cells.

Invasion is one of the key components of the metastatic cascade. Accordingly, to examine whether RKIP expression is associated with cancer cell invasiveness, we measured the invasive ability of the parental (i.e., untransfected) LNCaP and C4-2B cells and their stable transfectants using an in vitro invasion assay. Increasing RKIP expression in C4-2B cells by stable transfection with the sense RKIP vector was associated with an average 48.5% decrease (95% CI = 29.8% to 67.0%) in in vitro invasive ability among the different RKIP sense-transfected clones compared with control vector-transfected C4-2B cells.
Conversely, decreasing RKIP expression in LNCaP cells by stable transfection with the antisense RKIP vector was associated with an average 102.3% increase (95% CI 7.1% to 198.5%) in in vitro invasive ability among the RKIP antisense-transfected clones compared with control vector-transfected LNCaP cells (Fig. 3, C). These results suggest that RKIP expression is inversely associated with the invasiveness of prostate cancer cells in vitro.

Effect of RKIP Expression on Tumor Growth and Metastasis

To examine the effect of RKIP expression on the metastatic phenotype of C4-2B cells, we implanted pooled clones of C4-2B cells stably transfected with either control vector or sense RKIP vector into the dorsal lobe of the prostate in mice and monitored prostate tumorigenesis after 10 weeks. The individual and pooled results of two independent experiments (five mice in each experiment) are reported in Table 2. Ten weeks after the orthotopic implantation of tumor cells into the mice, there was no statistically significant difference in the mean size of the primary (i.e., prostate) tumors of mice injected with sense RKIP vector-transfected C4-2B cells and those of mice injected with control vector-transfected C4-2B cells (Table 2). However, fewer mice injected with sense RKIP vector-transfected C4-2B cells had lung metastases than did mice injected with control vector-transfected C4-2B cells (Table 2 and Fig. 4). Furthermore, the average number of lung metastases among the three mice injected with sense RKIP vector-transfected C4-2B cells that developed lung metastases was 85.0% (95% CI 37.9% to 96.4%) lower than that among mice injected with control vector-transfected C4-2B cells (Table 2).

We used immunohistochemistry to examine RKIP expression levels in the mouse primary tumors and metastases. Primary tumors derived from sense RKIP vector-transfected C4-2B cells displayed more RKIP expression than did primary tumors derived from control vector-transfected C4-2B cells (Fig. 4, A). To detect RKIP expression in prostate cancer metastases, we stained primary tissues and metastases for both PSA (green fluorescence), which identifies human prostate cancer cells, and RKIP (red fluorescence). The lung normally expresses RKIP; thus, we could identify RKIP expression in the lung sections. Yellow fluorescence indicates co-localization of RKIP and PSA staining, which indicates RKIP expression in prostate cancer. In addition, green fluorescence indicates prostate cancer tissue with undetectable RKIP expression. RKIP was not detectable in the lung metastases of mice bearing primary tumors derived from control vector-transfected C4-2B cells. Furthermore, although RKIP expression was readily detectable in the primary tumors derived from sense RKIP vector-transfected C4-2B cells, RKIP was not detectable in the few metastases that developed from those tumors (Fig. 4), which suggests that RKIP expression was decreased in the metastases.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gleason score</th>
<th>No. evaluated</th>
<th>RKIP expression†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncancerous prostate</td>
<td>—</td>
<td>10</td>
<td>3.0 (3.0–3.0)</td>
</tr>
<tr>
<td>Primary prostate cancer</td>
<td>6</td>
<td>2</td>
<td>2.0 (2.0–2.0)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>6</td>
<td>1.75 (1.0–2.0)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4</td>
<td>0.5 (0.0–1.0)</td>
</tr>
<tr>
<td>Prostate cancer metastases‡</td>
<td>—</td>
<td>22</td>
<td>0.0 (0.0–0.0)</td>
</tr>
</tbody>
</table>

*The staining intensity was scored as follows: negative, 0; weak, 1; moderate, 2; or strong, 3. †Reported as median (range). ‡The metastases were obtained from lymph nodes (three cases), bone (three cases), liver (seven cases), lung (two cases), adrenal (two cases), and other (five cases).

Fig. 2. Immunohistochemical staining for Raf kinase inhibitor protein (RKIP) in human clinical tissue samples. Frozen samples of benign prostatic hyperplasia (BPH), primary prostate tumors (Gleason score indicated in parentheses), and prostate cancer metastases from the adrenal glands, dura, liver, and lungs were stained with a goat polyclonal antibody against RKIP (brown) and counterstained with hematoxylin (blue). Note in the liver metastasis sample that the tumor (upper right) was negative for RKIP expression, whereas the normal surrounding liver tissue (lower left) was positive for RKIP expression. The scale bar indicates 50 μm.
Fig. 3. In vitro invasion assay. LNCaP cells were stably transfected with empty vector (−) or with a vector that constitutively expressed antisense (as) RKIP, and C4-2B cells were stably transfected with empty vector (+) or with a vector that constitutively expressed sense (ss) RKIP. Several stable clones of each transfectant were isolated for further studies. A) Representative immunoblot demonstrating levels of RKIP expression in LNCaP (left panel) and C4-2B (right panel) cells and their transfectants (40 μg of total cellular protein loaded in each lane). β-actin was used as an internal control for loading. Band intensity was quantified using densitometry and was normalized to β-actin band intensity. Results are reported as band intensity relative to untransfected parental LNCaP and C4-2B cells, which were set at an expression level of 1. B and C) In vitro invasiveness was measured by determining the percentage of cells that penetrated through Matrigel-coated Transwell chambers (8-μm pore size). These experiments were repeated three times. Bars represent the mean values; error bars represent the upper 95% confidence intervals. *Spontaneous metastatic ability was determined by orthotopic injection of 1 × 10⁶ C4-2B cells stably transfected with either empty vector [C4-2B-(+)] or a vector that constitutively expressed RKIP (C4-2B-ssRKIP) into 10 mice (five mice in each of two separate experiments) and quantification of the number of lung metastases that developed 10 weeks after injection. (Lung sections were stained for prostate-specific antigen expression to identify lung metastases derived from the orthotopic prostate tumors.)

Table 2. Metastatic ability of C4-2B cells after stable transfection with human Raf kinase inhibitor protein (RKIP) cDNA*

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>No. of mice with primary tumor formation/total No. of mice</th>
<th>Tumor size, cm³</th>
<th>No. of mice with lung metastases/total No. of mice</th>
<th>No. of lung metastases/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>C4-2B-(+) 5/5</td>
<td>2.25, 1.69, 1.87, 1.61, 1.43</td>
<td>5/5</td>
<td>15, 79, 17, 12, 18</td>
</tr>
<tr>
<td>C4-2B-ssRKIP</td>
<td>5/5</td>
<td>2.14, 2.06, 2.36, 2.35, 1.59</td>
<td>2/5</td>
<td>0, 4, 0, 3, 0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>C4-2B-(+) 5/5</td>
<td>1.71, 2.81, 2.17, 2.63, 1.80</td>
<td>5/5</td>
<td>12, 54, 18, 21</td>
</tr>
<tr>
<td>C4-2B-ssRKIP</td>
<td>5/5</td>
<td>2.59, 2.20, 1.44, 2.49, 1.70</td>
<td>1/5</td>
<td>0, 5, 0, 0, 0</td>
</tr>
<tr>
<td>Total (95% CI)</td>
<td>C4-2B-(+) 10/10 (6.92/10 to 10/10)</td>
<td>2.20 (1.89 to 2.51)†</td>
<td>10/10 (6.92/10 to 10/10)</td>
<td>26.30 (10.45 to 42.15)‡</td>
</tr>
<tr>
<td>C4-2B-ssRKIP</td>
<td>10/10 (6.92/10 to 10/10)</td>
<td>2.09 (1.81 to 2.37)§</td>
<td>3/10 (0.67/10 to 6.53/10)¶</td>
<td>4.00 (1.52 to 6.48)¶</td>
</tr>
</tbody>
</table>

*Spontaneous metastatic ability was determined by orthotopic injections of 1 × 10⁶ C4-2B cells stably transfected with either empty vector [C4-2B-(+)] or a vector that constitutively expressed RKIP (C4-2B-ssRKIP) into 10 mice (five mice in each of two separate experiments) and quantification of the number of lung metastases that developed 10 weeks after injection. (Lung sections were stained for prostate-specific antigen expression to identify lung metastases derived from the orthotopic prostate tumors.)

†Mean tumor size (95% confidence interval).
‡Mean number of lung metastases per mouse among those that developed lung metastases (95% confidence interval).
§P = .4727 compared with that for C4-2B-(+) cells (Wilcoxon rank sum test).
¶P = .0031 compared with that for C4-2B-(+) cells (Fisher’s exact test).
¶P = .0139 compared with that for C4-2B-(+) cells (Wilcoxon rank sum test).

Effect of RKIP Expression on Vascular Invasion by Human Prostate Cancer Cells

We evaluated the degree of vascular invasion associated with the primary orthotopic tumors in mice injected with C4-2B cells stably transfected with control vector or sense RKIP vector. We observed vascular invasion (Fig. 5) in 10 of 10 (100%, 95% CI = 69.2% to 100%) mice bearing tumors derived from control vector-transfected C4-2B cells and in only 4 of 10 (40.0%, 95% CI = 12.2% to 73.8%) mice bearing tumors derived from sense RKIP vector-transfected C4-2B cells (P = .01, Fisher’s exact test). We also performed histologic analysis, as described in the “Materials and Methods” section, to examine the proportion of blood vessels that were invaded by tumor cells in the primary
orthotopic tumors. We found that the mean percentage of vessels showing tumor cell invasion was 22.0% (95% CI = 17.3% to 26.6%) for the 10 mice that bore tumors derived from control vector-transfected C4-2B cells. By contrast, the mean percentage of vessels showing tumor cell invasion was 3.0% (95% CI = 0.9% to 5.0%) among the four mice that bore tumors derived from sense RKIP vector-transfected C4-2B cells. Thus, in mice with tumors that demonstrated vascular invasion, RKIP expression was associated with an 86.3% decrease (95% CI = 83.0% to 89.6%) in the number of vessels that had vascular invasion by the C4-2B cells ($P = .006$, Wilcoxon rank sum test).

**Effect of RKIP Expression on Tumor Angiogenesis**

Angiogenesis is a critical factor in the establishment of primary and metastatic tumors. Accordingly, we examined the total number of vessels contained within the primary tumors that developed in mice injected with human prostate cancer cells. Tumors that developed from the control vector-transfected C4-2B cells had a mean of 118.1 (95% CI = 97.2 to 139.0) vessels per tumor, whereas tumors that developed from sense RKIP vector-transfected C4-2B cells had a mean of 50.1 (95% CI = 25.2 to 75.0) vessels per tumor (difference = 68.0 vessels per tumor, 95% CI = 42.0 to 94.0 vessels per tumor; $P < .001$, Student’s $t$ test). A variety of factors can modify angiogenesis. For example, VEGF is a pro-angiogenic molecule that promotes angiogenesis in many tumors. Thus, it is possible that the decrease of vascularity observed with increased expression of RKIP may be due to a modulation of VEGF expression. To examine whether differences in VEGF expression were associated with the differing vascularity of the primary tumors derived from the
control vector-transfected cells and the RKIP sense-transfected cells, we used ELISA to measure VEGF production by these two cell lines. However, we found no statistically significant difference in the level of VEGF expression between the control-transfected and RKIP sense-transfected cells (data not shown; Student’s t test).

Effect of RKIP Expression on the Modulation of Raf/MEK/ERK Signaling in Human Prostate Cancer Cells

Protein kinases achieve cell signaling through phosphorylation and thereby activation of specific sites on target molecules. Because RKIP is an inhibitor of Raf-mediated signaling and RKIP expression is decreased in prostate cancer metastases, it follows that Raf-mediated signaling may be increased in metastatic prostate cancer cells. To test this hypothesis, we examined the phosphorylation status of two Raf target proteins, MEK and ERK, in untransfected non-metastatic (LNCaP) and metastatic (C4-2B) prostate cancer cells and in clones of those cells that stably expressed empty vector, sense or antisense RKIP. Although parental LNCaP and C4-2B cells had similar levels of total (i.e., combined phosphorylated and unphosphorylated) MEK and ERK, C4-2B cells had higher basal levels of the phosphorylated forms of MEK and ERK than did LNCaP cells (Fig. 6, A). C4-2B cells stably transfected with the sense RKIP vector had less phosphorylated MEK and ERK than did empty vector-transfected C4-2B cells (Fig. 6, B). LNCaP cells stably transfected with the antisense RKIP vector had more phosphorylated MEK and ERK than did empty vector-transfected LNCaP cells (Fig. 6, C). The level of RKIP expression in C4-2B or LNCaP cells was not associated with the levels of total MEK or ERK expression (Fig. 6, B and C). These data suggest that the decrease in RKIP expression that accompanies the transition of non-metastatic LNCaP prostate cancer cells to metastatic C4-2B prostate cancer cells promotes MEK and ERK phosphorylation.

The observation that diminished RKIP expression, which was associated with an increased in vitro invasive ability, was also associated with MEK activation (i.e., phosphorylation) is consistent with the possibility that decreased RKIP expression promotes invasiveness through activation of MEK. To test this possibility, we evaluated the effect of inhibiting MEK activity, with the use of the MEK kinase inhibitor PD098059, on the in vitro invasion ability of C4-2B cells. To examine whether our results were specific to MEK or whether other signaling pathways were involved, we used kinase inhibitors that blocked several signaling pathways that do not involve MEK (see Fig. 7 for description). Compared with untreated cells, C4-2B cells treated with PD098059 had a decreased invasive ability, whereas C4-2B cells treated with the other kinase inhibitors did not (Fig. 7). These results suggest that MEK contributes to in vitro invasion, which is consistent with the possibility that RKIP regulates tumor invasion through MEK activity.

DISCUSSION

In the current study, we have identified a novel anti-metastatic function for RKIP, a protein that regulates a signal transduction cascade. Specifically, we have demonstrated that RKIP expression is associated with the suppression of prostate cancer metastasis in a mouse model and that loss of RKIP expression is associated with clinical metastasis. Furthermore, we
have demonstrated that decreased RKIP expression is associated with the increased invasive capability of prostate cancer cells, presumably through the activation of MEK and ERK by phosphorylation. Relatively few metastasis suppressor genes have been identified; for most of those identified to date, the mechanism through which they suppress metastasis is not known. Thus, identification of a protein (i.e., RKIP) whose expression is associated with the inhibition of signal transduction and the suppression of metastasis as well as delineation of the cellular mechanism through which it promotes metastasis could substantially contribute to our understanding of the metastatic process.

Protein kinases are potentially important mediators of metastasis [reviewed in (18,19)]. For example, constitutively activated MEK induces cellular transformation (20,21). Furthermore, constitutive activation of Raf or MEK induces mouse NIH 3T3 fibroblasts to form lung metastases in rodent tumor models (22,23). Activation of Raf (a small GTPase)-specific guanine nucleotide exchange factor that mediates Ras-dependent c-Jun phosphorylation induces aggressive metastasis in rodent tumor models (23). The expression of MEK and ERK is increased in a steroid hormone-induced rodent model of prostate dysplasia, suggesting that this growth factor signaling pathway may be involved in prostate cancer initiation and progression (24–26).

Our study is the first to demonstrate that decreased expression of a metastasis suppressor gene is associated with increased MEK/ERK activation in vitro, results that are supported by clinical findings.

Results of gene expression comparisons between the non-metastatic LNCaP cell line and its metastatic derivative cell line C4-2B initially suggested that RKIP expression was decreased in metastatic prostate cancers. In this study, we used immunohistochemistry to confirm the validity of those findings with respect to clinical prostate cancer. All of the prostate cancer lung metastases examined lacked RKIP expression, whereas all normal prostate tissue and primary prostate tumors expressed RKIP, suggesting an association between diminished RKIP expression and the occurrence of metastasis. A potential challenge to detecting RKIP expression in clinical samples of metastases is that fresh tissue is difficult to obtain. However, in our studies, we used a rapid autopsy method that allowed us to obtain tissue shortly after the patient’s death. Our ability to detect PSA expression in the metastases and RKIP expression in the normal liver surrounding the tumor metastases using immunohistochemistry indicates that these samples are adequate for detection of RKIP. Thus, our findings suggest that decreased RKIP expression promotes metastasis in the true clinical situation. Results of several studies suggest that activation of the MAP kinase signal transduction pathway is increased as prostate cancer progresses to a more advanced and androgen-independent disease (27,28). Results from these previous reports are also consistent with the association between decreased RKIP expression and the phosphorylation status of MEK and ERK in prostate cancer cells that we observed in this study. Taken together, these data suggest that the decrease in RKIP expression that occurs in prostate cancer is associated with the enhanced activation of MEK and ERK.

Metastasis suppressor genes encode proteins that suppress the formation of overt metastases without affecting the growth rate of the primary tumor (4). These genes differ from tumor suppressor genes, which suppress the growth of primary tumors. In our studies, the results of two independent experiments demonstrated that increased RKIP expression is associated with the decreased development of metastases but not with any changes in the growth of the primary tumors. These key data suggest that RKIP functions as a suppressor of metastasis. This finding is further supported by the immunohistochemical verification that all metastases that formed in the murine models had diminished RKIP expression compared with the primary tumor, even when the primary tumor overexpressed RKIP. Furthermore, these results are consistent with the discovery of an unidentified prostate cancer metastasis suppressor gene on chromosome 12q24 (29), because RKIP is located at 12q24.22 (International Radiation Mapping Consortium) (30).

For the majority of metastasis suppressor genes that have been identified to date, the mechanism through which they prevent metastasis is not well defined. Our data suggest that decreased expression of RKIP promotes metastasis through several different complementary mechanisms that altogether confer the ability of vascular invasion on prostate cancer cells. These mechanisms include both increased invasive potential and promotion of angiogenesis in the tumor tissue, resulting in an increased number of blood vessels available for invasion.

Among the factors known to influence the invasive ability of cancer cells, matrix metalloproteinases (MMPs) have been clearly implicated as pro-invasive factors through their ability to degrade the extracellular matrix (31–33). Moreover, the MMP inhibitor bastimastat has been shown to inhibit development of prostate cancer growth in human bone tissue implanted in mice (34). Tissue inhibitor of metalloproteinases (TIMP) also inhibits MMP activity; thus, when TIMP levels decrease, the cells gain an overall invasive phenotype (31–33,35). Ward et al. (23) reported that inhibition of ERK by conditional overexpression of an ERK phosphatase was associated with a decrease in the matrix-degrading activity of MMPs in vitro and in the tissue invasiveness of tumors in vivo. It is currently unknown if modulation
of MMP activity plays a role in loss of RKIP-mediated vascular invasion.

Angiogenesis is a requirement for the growth of primary tumors as well as for the growth of metastatic lesions at secondary sites (36). Although reduced angiogenesis was associated with increased RKIP expression, a decrease in angiogenesis was apparently not a limiting factor for primary tumor growth in this model because mice injected with cells expressing sense RKIP developed primary tumors similar in size to those that developed in mice injected with cells expressing empty vector. This finding suggests that transformation to a neoplastic phenotype was sufficient to allow the cancer cells to promote the degree of angiogenesis required for tumor growth; however, because decreased expression of RKIP promotes angiogenesis above and beyond the degree needed for primary tumor growth, this mechanism may preferentially enhance metastasis. Specifically, the increased number of blood vessels in the tumor offers a greater opportunity for vascular invasion. This is not a trivial point, because metastasis is inefficient, and many tumor cells that complete intravasation do not form successful metastases. Thus, the increased angiogenesis promoted by decreased RKIP expression increases the number of blood vessels available for cancer cells to invade, which results in a greater opportunity for the establishment of metastases. In addition, our observation that the proportion of vessels that were invaded by tumor cells was greater in mice injected with the control vector-transfected C4-2B cells than in mice injected with the sense RKIP vector-transfected C4-2B cells suggests that diminished RKIP expression promotes vascular invasion as well as increased angiogenesis. The mechanism through which altered RKIP expression modulates angiogenesis is unclear. However, our observation that VEGF levels were not modified by alterations in RKIP expression suggest that RKIP modulates angiogenesis independently of VEGF.

In summary, our results suggest that RKIP functions as a suppressor of metastasis. Furthermore, our data demonstrate that decreased RKIP expression is associated with increased invasive ability, vascular invasion, and angiogenesis. This is the first study, to our knowledge, to document the association between a cancer progression-associated decreased expression of a molecule that inhibits signal transduction and increased metastasis. These results suggest that inhibition of the MEK/ERK pathway may prevent metastasis.

REFERENCES

(8) Fu Z, Dozmorov I, Keller E. Osteoblasts produce soluble factors that induce a gene expression pattern in non-metastatic prostate cancer cells, similar to that found in bone metastatic prostate cancer cells. Prostate 2002;51:10–20.


NOTES

Supported in part by awards from the Stuart and Barbara Padnos Endowed Research Fund of the University of Michigan Comprehensive Cancer Center (to E. T. Keller), the Association for the Cure of Cancer of the Prostate (to E. T. Keller), grant PC020102 from the U.S. Department of Defense (to E. T. Keller), Public Health Service (PHS) grant RR07008 from the National Center for Research Resources, National Institutes of Health (NIH), Department of Health and Human Services (DHHS) (to E. T. Keller and P. C. Smith), and PHS Special Programs of Research Excellence (SPORE) grant P50 CA069568 from the National Cancer Institute, NIH, DHHS (to Z. Fu, E. T. Keller, and M. A. Rubin).

Manuscript received September 16, 2002; revised April 3, 2003; accepted April 15, 2003.