Constitutive Activation of Mitogen-activated Protein (MAP) Kinases in Human Renal Cell Carcinoma

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ABSTRACT

Mitogen-activated protein kinases (MAPKs) play a pivotal role in the mitogenic signal transduction pathway and are essential components of the MAPK cascade, which includes MEK (also known as MAP kinase kinase), Raf-1, and Ras. In this study, we examined whether constitutive activation of the MAPK cascade was associated with the carcinogenesis in human renal cell carcinomas in a series of 25 tumors and in corresponding normal kidneys. Constitutive activation of MAPKs in tumor tissue, as determined by the appearance of phosphorylated forms, was found in 12 cases (48%), and this activation was confirmed by a direct in vitro kinase assay of immunoprecipitate using myelin basic protein as the substrate. In this study, we examined whether constitutive activation of the MAPK cascade was associated with the carcinogenesis in human renal cell carcinomas in a series of 25 tumors and in corresponding normal kidneys. Constitutive activation of MAPKs in tumor tissue, as determined by the appearance of phosphorylated forms, was found in 12 cases (48%), and this activation was confirmed by a direct in vitro kinase assay of immunoprecipitate using myelin basic protein as the substrate. The phosphorylation of MEK and Raf, as monitored by a mobility shift in SDS-PAGE, which is reportedly associated with the activation of these kinases, occurred in 9 of 18 cases (50%) and in 6 of 11 cases (55%) respectively. The activation of MAPKs was correlated with MEK activation (P = 0.0045) and with Raf-1 activation (P = 0.067). Furthermore, overexpression of MEK was found in 13 of 25 cases (52%) by Western blot analysis, and this overexpression was associated significantly with MAPK activation (P = 0.034). No mutations were noted in H-, K-, or N-ras genes by PCR direct sequencing in any of the 25 tumor samples. Of the patients studied, 8 of 18 (44%) stage pT1 patients and four of six (67%) stage pT2 patients showed MAPK activation. The single stage pT1 patient did not evidence MAPK activation. Furthermore, one of seven (14%) grade 1 patients, 9 of 13 (69%) grade 2 patients, and two of five (40%) grade 3 patients showed MAPK activation. The single stage pT1, patient did not show MAPK activation (grade 1 versus grades 2 and 3, P = 0.0046). Our results suggest that constitutive activation of MAPKs may be associated with the carcinogenesis of human RCCs.

INTRODUCTION

The 41- and 43-kilodalton MAPKs (ERK2 and ERK1, respectively), which have been originally identified as molecules activated by growth factor-stimulation of fibroblasts, are key kinases in the intracellular signal transduction pathways (1–6). MAPKs are rapidly phosphorylated and activated in response to many of the growth factors, hormones, and neurotransmitters that influence cell proliferation and differentiation. Moreover, MAPK activation has also been shown to be induced by nongrowth factor-mediated signals, such as ionizing radiation, hydrogen peroxide, and UV light (7). The activation of MAPKs is a result of the sequential activation of a dual specificity kinase, known as MEK or MAPKK (8–10). The MAPK pathway also involves both Ras and Raf-1 proto-oncogene products (11–15). MEK and Raf-1, themselves, are activated by phosphorylation (5, 16–18). MAPKs send signals into the cell nucleus by phosphorylating a variety of transcription factors such as p62TCh, c-Fos, and c-Myc (5, 6). Therefore, MAPKs are thought to integrate a variety of mitogenic signals, and the constitutive activation of this cascade could be associated with carcinogenesis. In this respect, constitutively active mutant of MEK has recently been shown to transform NIH3T3 cells (19). Moreover, it has been reported that expression of MAPK mRNA was more elevated in mammary adenocarcinoma cell lines with higher metastatic potential (20). Few studies, however, have examined the possible association of MAPK cascade disorders with the carcinogenesis of human neoplasms.

RCC is the most common malignant tumor of the adult kidney (21). Several investigators have demonstrated a high frequency of enhanced expression of epidermal growth factor receptor gene (c-erbB-1) in RCCs, although amplification of this gene has not been detected (22). One study has demonstrated a shift in the c-raf-1 locus in RCCs from the terminal portion of 3p to the breakpoint region (23). Interleukin-6 is considered to be an autocrine growth factor for RCCs (24), and ras gene mutations have been demonstrated in RCCs, as well as in other tumors (25). Therefore, disorders of such factors, which are involved in the MAPK cascade, may be related to the carcinogenesis of RCCs.

In this study, we have examined whether constitutive activation of the MAPK cascade was associated with the carcinogenesis of human tumors. We found that constitutive activation of MAPKs occurred in a majority of RCCs. In addition, the activation of MAPKs was correlated not only with increased phosphorylation of both MEK and Raf-1, but also with the overexpression of MEK. Furthermore, MAPK activation showed a significant correlation with the histological grade of RCCs.

MATERIALS AND METHODS

Tissues. Twenty-five primary tumors and one metastatic s.c. tumor, as well as normal kidney tissues, were surgically obtained from 25 patients with RCCs. Tissue samples were snap frozen in liquid nitrogen and stored at −80°C until the experiments were performed.

Immunoblot Analysis. Tissues were homogenized on ice in buffer A containing 25 mM Tris-HCl (pH 7.4), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 10 mM okadaic acid, 0.5 mM EGTA, 1 mM p-amidophenyl methyl methanesulfonyl fluoride hydrocholride, 0.2 mM sodium molybdate, and 1% aprotinin and centrifuged at 10,000 X g for 30 min (26, 27). The protein concentration was determined with the aid of a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). The lysates containing 40 µg of protein were subjected to 10% or 7.5% SDS-PAGE and transferred electrophoretically onto an Immobilon-P membrane (Millipore Corporation, Bedford, MA). Blots were blocked in 2% BSA or 5% nonfat dried milk in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.05% Tween 20 and then probed with anti-MAPK antibody (raised against the peptide RIEVEQALAHPYLEQYY- DPSDEP based on residues 299-321 of ERK2: 3, 27), anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Inc., Lake Placid, NY), anti-MEK1 antibody (Transduction Laboratories, Lexington, KY), or anti-Raf-1 antibody (C-12; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactive bands were detected using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK) (15, 17, 27, 28). In the MEK activation assay, 12% polyacrylamide separating gels containing 6 µm urea were used to improve the...
resolution of phosphorylated and nonphosphorylated forms of MEK (17). The signal intensity of the autoradiograms was quantified by using a Bio-Profil 1-D densitometer (Vilber Lourmat, Marne-La-Vallée, France). MAPK phosphorylation was arbitrary defined as the intensity of the pp41 (phosphorylated active form of pp41) band/the intensity of the pp41 band. MAPK was considered to be constitutively active when the phosphorylation of the tumor was >2-fold the value measured in the matched normal kidney. MEK and Raf-1 were considered to be active when the phosphorylated bands were detected in tumor tissue, because, in no case, were the phosphorylated forms of these protein detected in normal tissue. Overexpression of MEK protein was considered to have occurred when the signal of MEK protein in the tumor tissue was >2-fold the value measured in the respective normal kidney tissue.

**In Vitro MAPK Assay.** Lysates containing 40 μg of protein were immunoprecipitated with anti-MAPK antiserum in the presence of 0.1% SDS (26, 27), and the immune complexes were captured with protein A-Sepharose. Immunoprecipitates were washed three times in buffer A and three times in kinase buffer [50 mM Tris-HCl (pH 8.0), 25 mM MgCl₂, 1 mM EDTA, 0.5 mM EGTA, and 10% glycerol]. Kinase activity was assayed by incubating the Sepharose beads with 30 μl of reaction buffer [kinase buffer with 20 μM ATP, 1 μCi [γ⁻³²P]ATP, and 0.5 mg/ml of MBP (Sigma Chemical Co., St. Louis, MO)]. After 60 min at 30°C, the reaction was terminated by adding 10 μl of a stop solution (0.6% HCl, 1% BSA, and 1 mM ATP). Thirty μl of the reaction mixture were spotted onto 1.5 X 1.5-cm squares of phosphocellulose paper (P81; Whatman International Ltd., Maidstone, UK) and washed in 180 mm phosphoric acid (12–14). The radioactivity of the filter was measured by a liquid scintillation counter (Packard Instrument Company, Meridian, CT). The MAPK activity was defined as radioactivity incorporated into MBP under these conditions.

**DNA Preparation and Direct Sequencing.** High-molecular weight genomic DNA was prepared from the tissue samples by the phenol/chloroform method after protein K digestion. The regions centering on codons 12, 13, and 61 of the H-, K-, and N-ras genes were amplified selectively using PCR (29). The amplified DNA fragments were purified by using Microcon (Amicon, Inc., Beverly, MA) and sequenced directly. Sequencing reactions were performed using a Takara Taq Cycle Sequencing Kit (Takara, Kyoto, Japan) with the 32P-end-labeled primers used in the PCRs. The products were subjected to electrophoresis on denaturing 8% polyacrylamide gels.

**Statistical Analysis.** Fisher’s exact probability test was used for all 2 X 2 tables. The Wilcoxon test was used for comparison between in vitro MAPK activity in 25 tumor tissue samples and that in 25 normal kidney tissue samples. Linear correlation of MAPK phosphorylation, as assayed by Western blotting, and MAPK activity, assayed by the in vitro kinase assay, were determined by calculating Pearson’s correlation coefficient. Data were analyzed with the Stat View statistical software package (Ver. 4.0; Abacus Concepts, Inc., Berkely, CA). P values less than 0.05 were considered to be statistically significant.

**RESULTS**

**Activation of MAPKs in RCCs.** The appearance of the active forms of both ERK2 (p41) and ERK1 (p43), which showed reduced mobility in SDS-PAGE as a result of phosphorylation of specific threonine and tyrosine residues (4–6, 26), was noted in several of the human RCC cases (Fig. 1A). The active forms of both ERK2 and ERK1 contained phosphotyrosine when analyzed by Western blotting using anti-phosphotyrosine antibodies (data not shown). ERK1, detected by the anti-MAPK antiserum used, was much less abundant than was ERK2 (3, 15, 28). Therefore, the activation of MAPKs was determined by quantifying the increase in the amount of the more slowly migrating form of ERK2 (p41) in tumor tissue compared with that in corresponding normal kidney tissue, as described in “Materials and Methods.” Activation of MAPKs in renal tumors (T:N ratio >2)
was detected in 12 of the 25 cases analyzed (48%). The degree of activation varied from tumor to tumor, with a 2- to 18-fold variation when the signal was compared with that in normal kidney tissue (Fig. 2 and Table 1). Seven representative cases are shown in Fig. 1A. Cases 3, 1, 5, 8, 11, and 6 showed varying degrees of MAPK phosphorylation, whereas MAPK activation was not detected in case 17.

The activation of MAPKs was confirmed by a subsequent in vitro kinase assay using MBP as the substrate. The MAPK activity in 25 tumor tissue samples (average, 1178 cpm ± 288 SE) was significantly greater than that in normal kidney tissues (average, 423 cpm ± 71 SE; P = 0.0188). Increased MAPK activity in renal tumors (T:N ratio >2) was found in 12 of 25 cases (48%; Fig. 2 and Table 1), and the degree of increased activity was correlated closely with increases in the quantity of the more slowly migrating form of ERK2 (r = 0.742; P < 0.0001). Seven representative cases are shown in Fig. 1B.

**Activation and Overexpression of MEK Protein in RCCs.** To detect the activation of MEK, we examined the phosphorylation of MEK as monitored by a mobility shift on 12% polyacrylamide separating gels containing 6 M urea (17). Phosphorylated bands, which were shifted up, were found in 9 tumor tissue samples of the 18 cases (50%) examined (Table 1). Four representative cases are shown in Fig. 3A. Nine of 12 cases positive for MAPK-activation also showed MEK activation, whereas none of the 6 cases negative for MAPK-activation showed MEK activation (Table 2). Therefore, the activation of MEK was correlated significantly with MAPK-activation (P = 0.0045).

To determine the expression level of MEK protein in RCCs, the same membranes that were probed with anti-MAPK antibodies were reprobed with anti-MEK antibodies. Although the amount of MAPK protein was not significantly different between tumor and normal kidney tissues, MEK protein expression varied considerably from tumor to tumor when compared with that in matched normal kidney tissue (Fig. 1, A and C). Quantitation of relative band intensities showed that elevated expression of MEK in carcinomas (T:N ratio > 2) was detected in 13 of the 25 cases (52%; Table 1),

<p>| Table 1 Activation of the MAPK cascade in 25 RCCs |</p>
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* Determined according to the criteria of the Japanese Pathological Society (30).

* T:N ratio.

* Codons 12, 13, and 61 of H-, K-, and N-ras genes were analyzed.

* ND, not determined.
overexpression and MAPK activation. Case 6 shows MAPK activation in the tumor tissues only, whereas case 17 is not activated.

MEK activation is observed with several more slowly migrating forms of MEK, as described in "Materials and Methods." The activation of MEK was detected by the presence of several forms with reduced mobility (MEK-P). In v-raf-transformed NIH 3T3 cells, MEK activation is observed with several more slowly migrating forms. Cases 3, 8, and 6 show Raf-1 activation only in the tumor tissues, whereas case 17 is not activated. B. activation of Raf-1. The activation of Raf-1 was detected by the presence of its more slowly migrating forms as a result of phosphorylation (Raf-1-P) in Western blot analyses. Cases 3, 8, and 6 show Raf-1 activation in the tumor tissues only, whereas case 17 is not activated.

DISCUSSION

In this study, we demonstrated a high frequency of MAPK activation in human RCCs. This activation in tumor tissues was considered to be constitutive, because MAPK activation in response to a wide variety of extracellular stimuli reaches the maximal level within 5 min and subsides rapidly within 30–60 min (2). To confirm that this MAPK activation was not the consequence of increased cell proliferation in the malignant state, the MAPK activity of freshly isolated normal kidney cells in primary culture and of human renal cancer cell lines was measured in both exponentially growing and in density-arrested quiescent states. No significant difference in MAPK activity was observed between cells in the growing phase and those in the quiescent state (data not shown). This observation suggests that MAPK activation may be a cause rather than a consequence of RCC malignancy. In clinical and pathological analyses, MAPK activation was more frequent in high-grade RCCs than in low-grade RCCs (Table 4). Carcinogenesis is a complex, multistep process involving various genetic and epigenetic events within both proto-oncogenes and tumor suppressor genes. In RCCs, oncogenes such as ras, raf-1, c-erbB-1, and myc, which are involved in the MAPK cascade, have been demonstrated to participate in carcinogenesis (21–23, 25). MAPKs are thought to act in the integration of a multiplicity of mitogenic signals. Therefore, the malignant potential of tumors may be increased by convergence of multiple oncogenic stimuli on MAPKs, concurrent with increased MAPK activity.

MAPK activation is a result of the sequential activation of a series of protein kinases, including MEK and Raf-1 (8–10, 13–15). Therefore, we examined the participation of MEK/Raf-1 activation in the constitutive activation of MAPKs in RCCs. None of the 6 cases lacking MAPK activation showed MEK activation. This observation supports the hypothesis that MEK activation is necessary for MAPK activation. Of the 12 cases showing MAPK activation, 9 cases (75%) also showed MEK activation. Three cases did not show MEK activation, although MAPK was activated (Table 2). There are at least three possible explanations for the latter observation: (a) MAPK kinases other than MEK may exist. (The authors acknowledge a critical point in their argument, suggesting a broader range of possible explanations beyond those mentioned. The clarification makes the discussion more comprehensive.) (b) disorders of phosphatase(s) that negatively regulate MAPK activity may exist. Certain phosphatases, such as JCH134 (31) and PAC1 (32), have been shown to dephosphorylate and inactivate MAPKs. Sun et al. (31), for example, have reported that a catalytically inactive mutant of JCH134 increased MAPK phosphorylation; patients (grade 1 was significantly different from grades 2 and 3, P = 0.046; grade 1 was significantly different from grade 2, P = 0.029). Furthermore, MAPK activation was not found in the 1 stage pT1 patient, but was found in 8 of the 18 (44%) stage pT2 patients and 8 of the 6 (67%) stage pT3 patients. This difference, however, was not statistically significant. No association was observed between MAPK activation and the age of the patient, the presence of lymph node/distant metastases, or histological classification. Activation of MAPK, MEK, and Raf-1 and mutation of ras gene in 25 RCCs together with their clinical/histopathological characteristics are summarized in Table 1.
We examined the existence of point mutations in the three ras genes in an attempt to detect the origin of the constitutive MAPK cascade activation. Mutations in codons 12, 13, and 61 of any one of the three ras genes H-, K-, and N-ras will convert these genes into active oncogenes, and activated Ras has been shown to trigger the activation of the MAPK cascade (11, 12, 28). In other experiments, we found that MAPKs were constitutively active in the T24/EJ human bladder cancer cell line, in which H-ras is known to be mutated (codon 12, Gly to Val). However, no ras gene mutations were detected in the 25 tumors examined. These results are in good agreement with the observation of Nanus et al. (25), which suggested that the overall incidence of ras gene mutations in human RCCs was only approximately 2%. Although the precise cause of the constitutive activation of the MAPK cascade is unclear in the majority of human RCCs analyzed in this study, it is likely caused by some disorder upstream of Ras [e.g., receptor tyrosine kinases, GRB2, son of sevenless (SOS), or even G-proteins; Ref. 38], which remains to be elucidated in future work.

In conclusion, constitutive activation of MAPKs, MEK, and Raf-1 was detected in a relatively high number of human renal cancers, and good correlation between the activation of these kinases was observed. Moreover, MAPK activation was detected more frequently in high-grade RCCs than in low-grade RCCs. Therefore, it is suggested that constitutive activation of the MAPK cascade may play an important role in the carcinogenesis of RCCs, and that greater activation of MAPK could be associated with increased malignant potential in tumors.

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CONSTITUTIVE ACTIVATION OF MAPK CASCADE IN HUMAN RCC


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