Tuberous Sclerosis-associated Neoplasms Express Activated p42/44 Mitogen-activated Protein (MAP) Kinase, and Inhibition of MAP Kinase Signaling Results in Decreased in Vivo Tumor Growth

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ABSTRACT

Purpose: Tuberous sclerosis (TS) is a common autosomal disorder attributable to inactivation of the tumor suppressor genes tuberin and hamartin. To determine whether mitogen-activated protein (MAP) kinase signaling plays a role in the pathogenesis of TS, we stained human TS-associated neoplasms with antibodies directed against activated MAP kinase, and observed high-level expression.

Experimental Design: To determine whether MAP kinase is functionally important for the development of neoplasia in TS, we established a murine model of TS-associated neoplasia (Tsc2Ang1 cells) from a tumor arising in a mouse heterozygous for tuberin. Tsc2Ang1 cells demonstrate tumorigenesis in vivo and high-level expression of activated MAP kinase in vitro. The functionality of MAP kinase signaling was assessed by inactivating MAP kinase using a dominant-negative MAP kinase kinase in tsc2Ang1 cells and assessing the effect of this intervention on in vivo tumorigenicity and production of the potent angiogenic factor vascular endothelial growth factor (VEGF).

Results: Human TS-related neoplasms demonstrate high-level expression of activated MAP kinase, as does a tumor arising in a mouse heterozygous for tuberin. The inhibition of MAP kinase signaling by the introduction of a dominant-negative MAP kinase kinase leads to the inhibition of tumor growth in vivo and decreased production of VEGF.

Conclusions: MAP kinase is activated in TS-related neoplasia in mice and humans. Inhibition of MAP kinase leads to decreased tumor growth in vivo. Pharmacological inhibition of MAP kinase may be a therapeutic target in the prevention and treatment of TS-related tumors.

INTRODUCTION

TS is a tumor susceptibility cancer syndrome characterized by a high incidence of benign and malignant neoplasms involving the kidney, brain, and skin. This disease is caused by mutations in one of two genes, tsc1 (hamartin) or tsc2 (tuberin; Refs. 1, 2). The precise function of these genes is not fully understood, but it is known that tuberin and hamartin bind to each other and localize to the Golgi (3). In addition, tuberin has rap1 and rab5 GTPase activities (4, 5).

Several potential mechanisms for tumorigenesis in inherited cancer susceptibility syndromes have been identified. The first and one of the best characterized occurs in accordance with the two-hit theory of neoplasia (6), in which tumors arise when the normal allele is lost, resulting in LOH in the tumor. Multiple examples of LOH have been described in syndromes such as Li-Fraumeni syndrome (LFS), TS, and NF1. However, many tumors, especially benign tumors in TS and NF1, do not necessarily exhibit LOH (7, 8). Thus, other mechanisms must account for tumor growth. Dominant-negative forms of tumor suppressor genes that confer an opposing phenotype to a tumor suppressor gene have been found and described. For example, mutations in the rap1 GTP-binding portions of the COOH terminus of tuberin cause oncogenesis in NIH3T3 cells (9).

Finally, selective up-regulation of tyrosine kinase receptor activity, in part through autocrine loops, has been described in autosomal dominant tumor susceptibility genes. In the progression of neurofibromas to MPNSTs, up-regulation of the EGFR signaling pathway has been observed (10). In von Hippel-Lindau syndrome (VHL), kidney tumorigenesis is promoted

3 The abbreviations used are: TS, tuberous sclerosis; LOH, loss of heterozygosity; NF1, neurofibromatosis type 1; MPNST, malignant peripheral nerve sheath tumor; EGF, epidermal growth factor; EGFR, EGF receptor; GFP, green fluorescent protein; MAP, mitogen-activated protein; MAPK, MAP kinase; MAPKK, MAP kinase; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor receptor β.
by activation of tumor-growth-factor-α-EGFR autocrine loops (11). These autocrine loops are often accompanied by the loss of other tumor suppressor genes, such as p16\(^{ink4a}\), and the loss of p16\(^{ink4a}\) is a common event in MPNST and renal cell carcinoma (12, 13).

Mice heterozygous for tuberin develop renal cell carcinomas, hepatic hemangiomas, and cutaneous sarcomas at an increased incidence compared with wild-type mice (14). We generated a cutaneous sarcoma cell line, tsc2ang1 from a representative sarcoma, and have analyzed signal transduction events and genetic mutations that contribute to tumorigenesis. Using our murine model, we demonstrate a functional role of MAPK signaling in TS-associated neoplasia.

**MATERIALS AND METHODS**

**Immunohistochemical Analysis of Human TS Neoplasms for Active p42/44 MAPK.** Paraffin-fixed sections of subependymal giant-cell astrocytomas and periungual fibromas were stained with an antibody specific for phosphorylated MAPK according to the procedure of Arbisier et al. (15, 16).

**Generation of Murine Model of TS.** Tumor tissue from a cutaneous sarcoma arising in a tsc2\(^{+/−}\) mouse was dissociated with sterile filtered collagenase type II (Worthington, Lakewood, NJ) in PBS after manual dissociation through repetitive pipetting with a plastic pipette. Collagenase was neutralized with serum-containing medium, and the cells were cultured in type II complete medium (50/50 mixture of DMEM/Ham F12), supplemented with 5\(^{−}\)M cholesterol, 1.6\(^{−}\)M sodium selenite, 25 \(\mu\)g/ml insulin, 2 \(\times\) \(10^{−7}\) m hydrocortisone, 10 \(\mu\)g/ml transferrin, 1 \(\times\) \(10^{−8}\) m sodium selenite, 25 \(\mu\)g/ml insulin, 2 \(\times\) \(10^{−7}\) m hydrocortisone, 10 \(\mu\)g/ml transferrin, 1 \(\times\) \(10^{−8}\) m cholesterol, 1.6 \(\times\) \(10^{−8}\) m ferrous sulfate, 10 ng/ml EGF, and 15% fetal bovine serum, (supplied by Elizabeth Henske, Fox Chase Cancer Center, Philadelphia, PA).

**Signal Transduction Analysis.** Three million cells were lysed in 1 ml of lysis buffer containing 20 m\(\mu\)l Tris HCl (pH 7.5), 150 m\(\mu\)l NaCl, 1% (v/v) Triton X-100, 10% glycerol, 1 m\(\mu\) EDTA, 10 \(\mu\)g/ml leupeptin, 10 \(\mu\)g/ml aprotinin, 1 m\(\mu\)l benzamidine, 1 m\(\mu\)l phenylmethylsulfonylfluoride, and 1 m\(\mu\)l Na\(_{2}\)VO\(_{4}\). The lysate was spun in a microfuge, and the pellet was discarded. Protein concentration of the supernatant was determined by the Bradford assay using BSA as a standard. Samples were treated with Laemmli sample buffer and heated to 90°C for 5 min before SDS-PAGE (National Diagnostics, Atlanta, GA) and was transferred to nitrocellulose membranes. The membranes were then blocked with 5% nonfat dry milk in 10 m\(\mu\)l Tris/0.1% Tween 20/100 \(\mu\)l NaCl and were subsequently incubated with the appropriate antibody for immunoblotting. Anti-phospho-MAPK polyclonal antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Western blotting was performed according to Arbisier et al. (17).

**Inhibition of MAPK Signaling.** Tsc2ang1 cells were infected with retroviruses encoding GFP (pDIVA-GFP; plasmid gift from A. Kowalczyk, Emory University) or a dominant-negative rabbit MAPKK mutant A221 (a gift from C. Marshall, Institute of Cancer Research, London, United Kingdom; Ref. 18). Both vectors encode puromycin resistance, and cells were selected in 2 \(\mu\)g/ml puromycin and pooled to avoid clonal variation. GFP expression was confirmed by microscopic examination, and expression of the dominant-negative MAPK gene was confirmed by performing Western blot analysis with an antibody specific to phosphorylated MAPK and an antibody specific to the rabbit allele of MAPKK (antibody 177; C. Marshall, Institute of Cancer Research, London, United Kingdom) encoded by the retrovirus (18). Protein extracts were prepared as described in Arbisier et al. (19). Tsc2ang1 expressing GFP termed Tsc2GFP, and Tsc2ang1 cells expressing dnMAPK were termed tsc2dnmapkk. Clones were pooled to avoid clonal artifact.

**In Vivo Tumorigenesis.** Tsc2GFP and tsc2dnmapkk (1 \(\times\) \(10^{6}\)) cells were injected into the flank of 6-week-old nude male mice obtained from Massachussetts General Hospital. Three weeks after tumors appeared, they were excised and fixed in formalin for H&E staining.

**RT-PCR Distinguishes Endogenous Murine MAPKK.** Total RNA was isolated using TRI Reagent (Sigma). RT-PCR was performed with Promega Accession RT-PCR kit. Primers used were: β actin (728 bp), forward AAG ATG ACC CAG ATC ATG TTT GAC AC; and reverse, CTG CTT GCT GAT CCA CAT CTG CTG G; mouse MAPKK(285 bp), forward, AGG AGA CGC AGC CGA AAC; and reverse, GAG CCA GCC TGC GAA GTC TAC; rabbit MAPKK (285 bp), forward, GGG GCA GCC TGC GAA GTC TAC; GAG CCA ACC AGC AAA ATC AAC. Reactions were performed in an Eppendorf master cycler. One PCR cycle at 45°C for 45 min and 94°C for 2 min followed by forty PCR cycles under standard conditions with an annealing temperature of 60°C were performed. β actin mRNA was used as a reference message to normalize the content of total RNA. MAPKK expression was calculated as the relative expression ratio to that of β actin. All of the reactions were carried out in triplicate.

**Real-Time RT-PCR for VEGF.** Two-step quantitative RT-PCR was performed on cDNA generated by using the SYBR Green PCR Master Mix (Perkin-Elmer). Primers used were: β actin (76 bp), forward, AAG ATG ACC GGT GAC ATC CGT AA; and reverse, TGC CTG GGT ACA TGG TGG TA, giving rise to a 76-bp product, and VEGF, forward, CAT CTT CAA GCC GTG CTG CTG; and reverse, CAC CCT AGG GCT TCA TCG T; giving rise to a 69-bp product. Reactions were performed in MicroAmp Optical 96-well Reaction Plate (Perkin-Elmer). Thirty-five PCR cycles were performed under standard conditions with an annealing temperature of 60°C. Quantification was determined by the cycle number at which exponential amplification began (threshold value), and values were averaged from the values obtained from the triplicate repeats. β actin mRNA was used as a reference message to normalize the initial content of total cDNA. VEGF expression was calculated as the relative expression ratio of either VEGF threshold cycle to that of β actin. All of the reactions were carried out in triplicate, and threshold cycles were averaged.

**PCR Amplification and Gene Sequence Analysis for Tumor Suppressor Genes and Oncogene.** DNA (1–2 \(\mu\)g) was amplified using the Perkin-Elmer GeneAmp PCR Reagent kit. All of the reactions were carried out in a 50-\(\mu\)l reaction volume, which consisted of buffer [10 m\(\mu\)l Tris-HCl (pH 8.0/50 mm KCl), 2.5 m\(\mu\)M MgCl\(_{2}\); concentration adjusted to 4.0 mm for exon 8 of the p53 gene], 200 \(\mu\)M dNTPs (dATP, dCTP, dGTP, dTTP), 0.2 \(\mu\)M of each primer, 2.5 \(\mu\)l of cDNA, and 2.5 U of AmpliTaq DNA Polymerase (Perkin-Elmer). The reaction mixture was incubated at 95°C for 5 min, followed by 40–45 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C. The PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide.
dTTTP), and 2 units of Ampli-Taq Gold (Perkin-Perkin-Elmer Corp., Norwalk, CT). Primers for the Ki-ras, Ha-ras, p53, p16\textsuperscript{ink4a}, and p19\textsuperscript{ARF} genes were added at a final concentration of 0.2 µM for each primer as described previously (20-22). Primer sequences for Ki-ras exon 2, Ha-ras exon 2, p53, p16\textsuperscript{ink4a}, and p19\textsuperscript{ARF} were described previously (18-20); primers for exon 1 of Ki-ras were forward, 5'TAGACATGT-TCTAATTTAG-3', and reverse, 5'TCCTTTACAAGGC-CACACAG-3', and for exon 1 of Ha-ras were forward, 5'GGTGCCACAGGTGATCAACTG-3', and reverse, 5'AAAGTGGTTCTGGATCAG-3'. Intronic primers were used for the p53 gene because of the presence of pseudogenes in the mouse genome (23). The samples were overlaid with 50 µl of mineral oil to prevent evaporation and cross-contamination of the samples. All of the PCR conditions started with an initial extended denaturation step for 4 min at 94°C and ended with a prolonged extension step for 5–7 min at 72°C. Gene-specific amplification for 30–40 cycles was as follows (20-22): for exon 1 of the Ki-ras gene, 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C; for exon 1 of the Ha-ras gene, 45 s at 94°C, 1 min at 60°C, and 90 s at 72°C; for exons 1α and 2 of p16\textsuperscript{ink4a} and exon 2 of the Ki-ras and Ha-ras genes, 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; for exon 1β of p19\textsuperscript{ARF}, 30 s at 94°C, 30 s at 67°C, and 30 s at 72°C; and for exon 5–8 of p53, 30 s at 94°C and 90 s at 65°C (22, 24). Each reaction included negative controls that lacked template DNA and served as negative buffer controls for the PCR amplification reactions. All of the samples were amplified in a MJ Research PTC 200 thermal cycler.

After amplification, the samples were electrophoresed on a 3% Nu-Sieve gel (FMC Bioproducts, Rockland, ME) to visualize the PCR products. The PCR products were then either gel purified or taken directly from the PCR mix, purified with the QiAQuick PCR Purification kit (Qiagen Inc., Chatsworth, CA), and the sequences determined by direct automated DNA sequencing using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA) according to the manufacturer’s instructions. Analysis of the DNA sequences was performed using the DNASIS software (Hitachi Software Engineering America, Ltd., San Bruno, CA).

RESULTS

To determine whether MAPK plays an important role in the development of neoplasia in the autosomal disorder TS, we stained neoplasms from TS patients. High-level expression was noted in subependymal giant cell astrocytomas (Fig. 1), and we have previously shown high-level expression of MAPK signaling in a human angiomyolipoma cell line used as a model for TS (25, 26). A periungual fibroma from a patient with TS was also positive for MAPK expression (data not shown). Nonneoplastic brain showed faint immunoreactivity with antibodies directed against active MAPK. Western blot analysis revealed high levels of phosphorylation of MAPK, thus showing increased function of MAPK (Fig. 2). We demonstrated expression of dominant-negative MAPKK in the tsc2dnmapkk cells using sequence differences between endogenous murine MAPKK and retroviral rabbit MAPKK (Fig. 2B). Because MAPK is elevated in tsc2ang1 cells, we inhibited MAPK signaling through the introduction of a dominant-negative MAPKK. The inhibition of MAPK signaling leads to greatly decreased in vivo tumorigenesis (Fig. 2C). Consistent with the inhibition of tumorigenesis observed as a result of MAPK inhibition, we observed a down-regulation of VEGF mRNA synthesis (Fig. 3).

We have previously predicted that tumors that are MAPK dependent for in vivo growth are likely to have loss of p16\textsuperscript{ink4a}; therefore, we examined the status of this gene in our murine tumor model. Genetic analysis of tsc2ang1 cells reveals deletions of both exons 1α and 1β at the p16\textsuperscript{ink4a} gene locus, resulting in inactivation of both the p16\textsuperscript{ink4a} and the p19\textsuperscript{ARF} tumor suppressor genes. No mutations were detected in either the Ki- or Ha-ras oncogenes or the p53 tumor suppressor gene (Fig. 4).

DISCUSSION

Multiple signal transduction pathways have been implicated in the regulation of angiogenesis and tumorigenesis, including MAPK, reactive oxygen, and PI3 kinase (27-30). The choice of signal transduction pathway may be dependent on other factors, such as tissue of origin, carcinogenic insult, and status of other tumor suppressor genes, e.g., loss of p53 or p16\textsuperscript{ink4a} (31). Recent studies from our laboratory and others have implicated predictable patterns associating the loss of
specific tumor suppressor genes with the activation of particular signal transduction pathway (27). For example, we have found that in angiosarcoma, a tumor that is predominantly deficient in p53, activation of PI3 kinase is required for angiogenesis and tumorigenesis (19). Introduction of a dominant MAPK into angiosarcoma cells does not inhibit tumorigenesis (32). The analysis of human tumor samples for expression of MAPK by immunohistochemistry reveals a loss of MAPK expression with increasing grade of malignancy in angiosarcoma (15). Indeed, in mice homozygous for p53 deficiency, angiosarcoma is the second most common neoplasm (33, 34). Conversely, MAPK is elevated in a number of neoplasms, including prostate cancer, glioblastoma, and melanoma, tumors in which p16 Ink4a is commonly inactivated, and the introduction of dominant-negative MAPKK causes a loss of tumorigenesis and decreased angiogenesis in tumor cell lines deficient in p16 Ink4a (32, 35–39). On the basis of these findings, we hypothesized that a link exists between the inactivation of p16 Ink4a and the activation of PI3 kinase (27). In addition, our previous immunohistochemical studies of angiosarcoma and melanoma have shown that the presence of activated MAPK in human lesions may predict a functional role (15, 32, 38).

TS is an autosomal dominant disorder characterized by the development of aberrant neurons that become calcified (tubers) and that cause seizures and by an increased incidence of benign

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**Fig. 2** Inhibition of MAPK signaling using a dominant-negative MAPKK in vitro and in vivo. A, overexpression of MAPKK is indicated in the tsc2ang1 cells that overexpress dominant-negative MAPKK, and decreased levels of phosphorylation of p42/44 MAPK (P-MAPK), the primary downstream target of MAPKK. β-actin serves as a loading control. B, the expression of dominant-negative (rabbit) MAPKK by RT-PCR in tsc2dnMAPKK cells is confirmed, compared with vector controls, using primers that can distinguish rabbit MAPKK from endogenous murine MAPKK. Lane labeled −, RNA from GFP vector control cells; Lane labeled +, RNA from cells expressing dominant-negative MAPKK (rabbit). Top band, rabbit MAPKK; middle band, mouse MAPKK; bottom band, β-actin as a loading control. C, the effect of MAPKK inhibition in vivo, resulting in a significant decrease in in vivo growth (P < 0.05).

**Fig. 3** Effect of MAPK inhibition on VEGF mRNA levels using quantitative RT-PCR. Lane 1, mRNA from tsc2ang1 cells expressing GFP; Lane 2, RNA from tsc2ang1 cells expressing dnMAPKK. Levels of VEGF mRNA were normalized for β-actin. The experiments were performed in triplicate, and the median value of the VEGF:actin ratios is expressed, with vector control tsc2gfp normalized to a value of 1.0.
which cause seizures. In addition, we have predicted that comes from the finding of MAPK activation in brain tubers, independent confirmation of a role of MAPK signaling in TS functional role in the development of TS-associated neoplasia. Production of a dominant-negative MAPKK led to decreased tu-
pberin and hamartin develop an increased incidence of tumors, notably renal cell carcinomas, sarcomas of the skin, and hemangio-
morigenesis assay. Multiple pathways have been implicated but have not been functionally tested in an in vivo tumorigenesis assay (45–49).

Our previous studies have suggested that the MAPK activation in a human lesion may predict the functional status of this pathway (15, 32, 38). We stained neoplastic lesions from patients with TS for active MAPK, and found that MAPK is activated in these lesions. We then functionally tested the role of MAPK in a murine model of TS, tsc2ang1 cells, which like the human lesions, express high levels of MAPK signaling. Introduction of a dominant-negative MAPKK led to decreased tumorigenesis in vivo, suggesting that MAPK plays an important functional role in the development of TS-associated neoplasia. Independent confirmation of a role of MAPK signaling in TS comes from the finding of MAPK activation in brain tubers, which cause seizures. In addition, we have predicted that tumors that exhibit MAPK activation are likely to have inactivation of p16ink4a rather than of p53; the tsc2ang1 cell line demonstrates inactivation of p16ink4a but not of p53, confirming our prediction. Interestingly, analysis of renal tumors arising in Eker rats, a strain of rat containing an insertion in the tuberin gene (50), reveals a majority of tumors exhibiting inactivation of p16ink4a rather than of p53 (51).

Therapeutic interventions resulting in decreased MAPK signaling in tumor cells may be feasible. We have recently demonstrated that PDGFRβ is activated in TS (26). Targeting of this receptor by selective tyrosine kinase inhibitors may be of therapeutic benefit in the treatment of TS. Indeed, this has already been accomplished using the clinically available tyro-
sine kinase inhibitor STI 571 (gleevec), which inhibits MAPK signaling downstream of bcr-abl, c-kit, and PDGFRβ (52, 53). Tsc2ang1 cells are not sensitive to STI 571, preventing us from evaluating it in this model, but human angiomylolipoma cells are sensitive, suggesting a role for gleevec in TS (26). In addition, other drugs may down-regulate MAPK signaling through other mechanisms, including receptor recycling (54) and may provide novel therapies for TS.

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In the August 15, 2003, issue of Clinical Cancer Research [9 (9): pp. 3469–3475], in the article titled “Tuberous Sclerosis-Associated Neoplasms Express Activated p42/44 Mitogen-Activated Protein (MAP) Kinase, and Inhibition of MAP Kinase Signaling Results in Decreased in Vivo Tumor Growth,” by B. Govindarajan, M. C. Mizesko, M. S. Miller, H. Onda, M. Nunnelley, K. Casper, D. Brat, C. Cohen, and J. L. Arbiser, the name of author Matthew Nunnelley was spelled incorrectly.
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