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 established a human TS-associated neoplasm 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 neoplasms with antibodies directed against activated MAP kinase, and observed high-level expression.

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-EGFR signaling pathway has been observed (10). In von Hippel-Lindau syndrome (VHL), kidney tumorigenesis is promoted by...

Received 10/21/02; revised 3/24/03; accepted 4/14/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by the Tuberous Sclerosis Alliance and NIH Grants AR02030, R01 AR47901, and National Institute of Arthritis, Musculoskeletal, and Skin Diseases (NIAMS) Emory Skin Disease Research Core Center P30 AR42687 (to J. L. A.), and Grants RO1 ES06501 and ES08252 (to M. S. M.) from the National Institute of Environmental Health Sciences and Cancer Center Support Grant P30 CA12197 from the National Cancer Institute, which provided support for the Wake Forest University Analytical Imaging Core Facility, the DNA Synthesis Core Laboratory, and the DNA Sequencing and Gene Analysis Facility.

2 To whom requests for reprints should be addressed, at Department of Dermatology, Emory University School of Medicine, WMB 5309, 1639 Pierce Drive, Atlanta, GA 30322. Phone: (404) 727-5063; Fax: (404) 727-0923; E-mail: jarbise@emory.edu.

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; PI3 kinase, phosphatidylinositol 3’-kinase; PDGR2X 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 p16INK4a, and the loss of p16INK4a 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. Paraaffin-fixed sections of subependymal giant-cell astrocytomas and periungual fibromas were stained with an antibody specific for phosphorylated MAPK according to the procedure of Arbiser 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), with serum-containing medium, and the cells were cultured in type II complete medium (50/50 mixture of DMEM/Ham F12), supplemented with 5 × 10−6 M sodium selenite, 25 μg/ml insulin, 2 × 10−7 M hydrocortisone, 10 μg/ml transferrin, 1 × 10−8 M T3 (triiodothyronine), 10 μU/ml vasopressin, 1 × 10−4 M cholesteroll, 1.6 × 10−6 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 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 10% glycerol, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM benzamidine, 1 mM phenylmethylsulfonylfluoride, and 1 mM Na3VO4. The lysate was spun in a microtube, and the pellet was discarded. Protein concentration of the supernatant was determined according to Arbiser et al. (17).

Inhibition of MAPK Signaling. Tsc2ang1 cells were infected with retroviruses encoding GFP (pDIVA-GFP; plasmid a 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 μg/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 Arbiser et al. (19). Tsc2ang1 expressing GFP termed Tsc2GFP, and Tsc2ang1 cells expressing dnMAPKK were termed tsc2dnmapkk. Clones were pooled to avoid clonal artifact.

In Vivo Tumorigenesis. Tsc2GFP and tsc2dnmapkk (1 × 106) were injected into the flank of 6-week-old nude male mice obtained from Massachusetts General Hospital. Three weeks after tumors appeared, they were excised and fixed in formalin for H&E staining.

RT-PCR Distinguishes Endogenous Murine MAPKK from Transduced Rabbit 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 TGT GAC AC, and reverse, CTG CTI 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 CGA TGC GGC CGA AAC, and reverse, GAG CCA ACC AGC AAA ATC CAC. 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 were used: β actin (76 bp), forward, AAG TGT GAC GTT GAC ATC CGT AA, and reverse, TGC CTG GGT ACA GTA CAG TA, giving rise to a 76-bp product, and VEGF, forward, CAT CTT CAA GCC GTG TGC TG T, and reverse, CAC TCC AGG GCT TCA TGG TT, 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 μg) was amplified using the Perkin-Elmer GeneAmp PCR Reagent kit. All of the reactions were carried out in a 50-μl reaction volume, which consisted of buffer [10 mM Tris-HCl (pH 8.0/50 mM KCl)], 2.5 mM MgCl2 (concentration adjusted to 4.0 mM for exon 8 of the p53 gene), 200 μM dNTPs (dATP, dCTP, dGTP,
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 in giant cells (Fig. 1) is notable, compared with negative staining in non-neoplastic brain (A).

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\(^{INK4a}\); therefore, we examined the status of this gene in our murine tumor model. Genetic analysis of tsc2ang1 cells reveals deletion of both exons 1\(\alpha\) and 1\(\beta\) at the Ink4a gene locus, resulting in inactivation of both the p16\(^{INK4a}\) and the p19\(^{ARF}\) 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\(^{INK4a}\) (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

---

**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 the functional role in the development of TS-associated neoplasia. The production of a dominant-negative MAPKK led to decreased MAPK in a murine model of TS, tsc2ang1 cells, which like the human lesion may predict the functional status of this pathway. Multiple pathways have been implicated but have not been demonstrated that these genes are authentic tumor suppressor genes in that they inhibit tumor growth when reintroduced into appropriate tumor cells. Mice heterozygous for tuberin and hamartin develop an increased incidence of tumors, notably renal cell carcinomas, sarcomas of the skin, and hemangiommas of the liver. However, the signal transduction pathways downstream of these genes are not well understood. Multiple pathways have been implicated but have not been functionally tested in an in vivo tumorigenesis assay.

Our previous studies have suggested that the MAPK activation in a human lesion may predict the functional status of this pathway. 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 tumors, 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, reveals a majority of tumors exhibiting inactivation of p16INK4a rather than of p53.

Therapeutic interventions resulting in decreased MAPK signaling in tumor cells may be feasible. We have recently demonstrated that PDGFRβ is activated in TS. 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 tyrosine kinase inhibitor STI 571 (gleevec), which inhibits MAPK signaling downstream of bcr-abl, c-kit, and PDGFRβ. Tsc2ang1 cells are not sensitive to STI 571, preventing us from evaluating it in this model, but human angiomylipoma cells are sensitive, suggesting a role for gleevec in TS. In addition, other drugs may down-regulate MAPK signaling through other mechanisms, including receptor recycling and may provide novel therapies for TS.

ACKNOWLEDGMENTS

We thank Elyse Jung for assistance with the automated sequencing.

REFERENCES


4 Peter Crino, University of Pennsylvania, personal communication.


FUTURE ANNUAL MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH
2004 March 27–31, Orlando, FL
2005 April 16–20, Anaheim, CA

SECOND ANNUAL INTERNATIONAL CONFERENCE ON FRONTIERS IN CANCER PREVENTION RESEARCH
October 26–30, 2003
JW Marriott Desert Ridge Resort, Phoenix, AZ
Chairperson
Raymond N. DuBois, Nashville, TN

SIXTH JOINT CONFERENCE OF THE AACR AND JCA, ADVANCES IN CANCER RESEARCH
January 25–29, 2004
Hilton Wai Koloa Village, Wai Koloa, Hawaii
Chairpersons
Waun Ki Hong, Houston, TX
Takahashi Tsuruo, Tokyo, Japan

AACR
The American Association for Cancer Research (AACR) was founded in 1907 to bring together active investigators of the cancer problem for the presentation and discussion of new findings and to foster advances in cancer research. Today the Association has over 19,000 members working in all of the subdisciplines of cancer research in the United States, Canada, and more than 60 other countries. Information on AACR programs and activities can be obtained from:

American Association for Cancer Research
615 Chestnut Street, 17th Floor
Philadelphia, PA 19106-4404
Phone: (215) 440-9300
Fax: (215) 440-9313
E-mail: aacr@aacr.org

The AACR welcomes applications for membership from the readership. Scientists engaged in all areas of cancer research are eligible for membership. There are three categories of membership: active membership, open to cancer researchers all over the world; associate membership, open to graduate and medical students, postdoctoral fellows, and physicians-in-training; and affiliate membership, open to qualified health professionals and educators working in cancer research and to survivors and advocates who are members of organizations that advance cancer research. Further information on the qualifications for each category as well as the benefits of membership can be found on the AACR website at www.aacr.org.

AACR SPECIAL CONFERENCES IN CANCER RESEARCH
A number of meetings are now being organized in the AACR’s series of smaller scientific meetings. Following are the topics, dates, locations, and program committees for these meetings. When full details of each meeting are available, AACR members will be the first to receive complete brochures and application forms for participation in these important conferences. Nonmembers may receive this information by sending their names and addresses to Meetings Mailing List, American Association for Cancer Research, 615 Chestnut Street, 17th Floor, Philadelphia, PA 19106-4404. Up-to-date program information is also available via the Internet at the AACR’s website (http://www.aacr.org).

AACR-NCI-EORTC INTERNATIONAL CONFERENCE ON MOLECULAR TARGETS AND CANCER THERAPEUTICS
November 17–21, 2003
Hynes Center, Boston, MA
Chairpersons
Charles I. Sawyers, Los Angeles, CA
Edward A. Sausville, Bethesda, MD
Jaap Verweij, Rotterdam, The Netherlands

Correction
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.
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

Baskaran Govindarajan, Melissa C. Mizesko, Mark Steven Miller, et al.


Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/9/9/3469

Cited articles  This article cites 53 articles, 26 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/9/9/3469.full#ref-list-1

Citing articles  This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/9/9/3469.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.