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H-Ras Increases Urokinase Expression and Cell Invasion in Genetically Modified Human Astrocytes Through Ras/Raf/MEK Signaling Pathway

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Abstract

Previous study reported that the activation of Ras pathway cooperated with E6/E7-mediated inactivation of p53/pRb to transform immortalized normal human astrocytes (NHA/hTERT) into intracranial tumors strongly resembling human astrocytomas. The mechanism of how H-Ras contributes to astrocytoma formation is unclear. Using genetically modified NHA cells (E6/E7/hTERT and E6/E7/hTERT/Ras cells) as models, we investigated the mechanism of Ras-induced tumorigenesis. The overexpression of constitutively active H-RasV12 in E6/E7/hTERT cells robustly increased the levels of urokinase plasminogen activator (uPA) mRNA, protein, activity and invasive capacity of the E6/E7/hTERT/Ras cells. However, the expressions of MMP-9 and MMP-2 did not significantly change in the E6/E7/hTERT and E6/E7/hTERT/Ras cells. Furthermore, E6/E7/hTERT/Ras cells also displayed higher level of uPA activity and were more invasive than E6/E7/hTERT cells in 3D culture, and formed an intracranial tumor mass in a NOD-SCID mouse model. uPA specific inhibitor (B428) and uPA neutralizing antibody decreased uPA activity and invasion in E6/E7/hTERT/Ras cells. uPA-deficient U-1242 glioblastoma cells were less invasive *in vitro* and exhibited reduced tumor growth and infiltration into normal brain in xenograft mouse model. Inhibitors of Ras (FTA), Raf (Bay 54–9085) and MEK (UO126), but not of phosphatidylinositol 3-kinase (PI3K) (LY294002) and of protein kinase C (BIM) pathways, inhibited uPA activity and cell invasion. Our results suggest that H-Ras increased uPA expression and activity via the Ras/Raf/MEK signaling pathway leading to enhanced cell invasion and this may contribute to increased invasive growth properties of astrocytomas.

Keywords

Ras; astrocyte; urokinase; invasion; SCID mouse model

INTRODUCTION

Astrocytomas are the most common human gliomas and divided into four grades by World Health Organization (WHO) standards, of which grade III anaplastic astrocytoma (AA) and

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grade IV glioblastoma multiforme (GBM) are considered to be malignant and believed to develop as a result of stepwise accumulations of genetic lesions (Cavenee et al., 2000). AA typically exhibit loss of a functional p53/pRb pathway, Ras pathway activation, and telomerase reactivation (Cavenee et al., 2000; Hiraga et al., 1999). Interestingly, human telomerase reverse transcriptase (hTERT) expression, in combination with inactivation of p53/pRb (E6/E7) did not transform normal astrocytes, while hTERT expression and inactivation of p53/pRb cooperated with H-Ras pathway activation to form intracranial tumors resembling human malignant gliomas (Sonoda et al., 2001). However, the Ras-dependent signaling pathway in E6/E7/hTERT/Ras cells that converts astrocyte to tumor still remains undefined.

Proteases are required for the degradation of the ECM for cell invasions and migrations. Increased protease expressions are correlated with the invasive properties of migrating cells. One of the best-characterized groups of extracellular proteolytic enzymes in human glioma is the urokinase plasminogen activator (uPA). Increased expression of uPA has been found in AAs and GBMs *in vivo* (Yamamoto et al., 1994). uPA activity in human gliomas correlates with poor prognosis (Hsu et al., 1995). In addition, a role for uPA in astrocytomas invasion and metastasis has been demonstrated (Axelrod et al., 1989; Gondi et al., 2003; Mohanam et al., 2001; Rao et al., 1994; Yu and Schultz, 1990). uPA promotes the degradation of ECM proteins either acting alone or through activation of plasminogen to plasmin (Mignatti and Rifkin, 1993). In this report, we have investigated the mechanism involved in H-Ras-mediated cell invasive growth using the genetically modified human astrocytes.

In our study, we provide evidence that overexpression of H-RasV12 in E6/E7/hTERT cells induced uPA up-expression at both protein and mRNA levels, increased proteolytic activity and cell invasion via Ras/Raf/MEK signaling pathway. In contrast, the inhibition of phosphatidylinositol 3-kinase (PI3K) or protein kinase C (PKC) signaling pathway did not significantly alter uPA level, activity or cell invasion. The E6/E7/hTERT/Ras cells have increased invasive phenotype *in vitro* and also formed intracranial tumors in NOD-SCID mouse model. A specific uPA inhibitor (B428) and a neutralizing antibody against the protease significantly attenuated transmembrane invasion in E6/E7/hTERT/Ras cells. Similarly, silencing uPA expression in U1242 GBM cells reduced cell invasion *in vitro* and tumor growth in a NOD-SCID mouse model. These findings suggest that H-Ras may contribute to glioma invasion by increasing uPA expression and activity via Ras/Raf/MEK-dependent pathway.

MATERIALS AND METHODS Cell Culture

Normal human astrocytes (NHA) were grown in Clonetics EBM (Endothelial Cell Basal Media, No.CC-2131) supplement with Hydrocortisone (1 µg/mL), hEGF (20 ng/mL), Insulin (25 µg/mL) Progesterone (25 ng/mL) Transferrin (50 µg/mL) and 5% FBS. The NHA clones (E6/E7/hTERT and E6/E7/hTERT/Ras) were grown in a α -MEM supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 µg/mL) in a humidified incubator containing 5% CO₂ at 37°C.

Northern Blot Assay

Total cellular RNA (20 µg) was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) and electrophoresed on 1.0% agarose gels followed by electrotransfer to Zeta probe nylon membranes. Prehybridization, hybridization with randomly primed ³²P-labeled probes were carried out as previously described. The probes for uPA and GAPDH have been reported previously (Hussaini et al., 1999).

Reverse Transcriptase-PCR Analyses

RT-PCR analysis was carried out as described (Zhao et al., 2003). The uPA forward primer was 5'-CGG AGA GAT GAA GTT TGA GGT GGA GCA GCT-3', the reverse primer was 5'-CAC TCT GGG TCA GCA GCA CAC AGC ATT TTA-3'; the β -actin forward primer was 5'-CAC CAT GGA TGA TGA TAT CG-3', the reverse primer was 5'-TGG ATA GCA ACG TAC ATG G-3'. All PCR reactions were performed using a Cyclor (Bio-Rad) with 35 thermal cycles of 45 s at 94°C denaturing, 45 s at 57°C annealing, and 1 min at 72°C elongation.

Western Blotting

The conditioned media of the cultured cells were collected. The cultured cells were lysed with Tris-buffered saline containing 1% (v/v) Triton X-100 and 1× proteinase inhibitor cocktails (Sigma). The protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL). Equal amounts of total protein (25 μ g) were loaded onto a 10% SDS-polyacrylamide mini-gel. After electrophoresis and transfer to a nitrocellulose, the immunoreactive H-Ras, uPA, MMP-2 and α -tubulin bands were visualized according to a previous report (Hussaini et al., 1999). The antibodies used were rabbit anti-H-Ras antibody (Santa Cruz Biotechnology), monoclonal mouse anti-human uPA antibody (American Diagnostica, Stamford, CT), rabbit anti-MMP-2 antibody (Cell signaling Technology, Beverly, MA), and mouse anti-human α -tubulin antibody (Sigma).

Fibrinogen and Gelatin Zymography

The conditioned media (treated with different inhibitors) were collected. Protein concentrations were determined with BCA Protein Assay. uPA activity was detected by fibrinogen zymography. Briefly, aliquots (15 μ g) of conditioned media were resolved under nonreducing condition on 10% SDS-PAGE gels containing fibrinogen (1 mg/mL), thrombin (0.2 unit/mL) and plasminogen (20 μ g/mL). Then the gels were processed as previously reported (Zhao et al., 2003) except that the incubation buffer was 0.1 M glycine (pH 8.0).

Immunocytochemistry

The immunocytochemistry was performed as previously reported (Zhao et al., 2003). Briefly, cells were fixed and blocked prior to overnight incubation with monoclonal anti-human uPA antibody (5 μ g/mL) at 4°C. Cells were incubated with alkaline phosphatase-conjugated secondary antibody (Sigma). The signals were detected by adding Fast-Red (Sigma). Purified normal mouse IgG was used as negative control.

Cell Invasion Assay

The cell invasion assay was performed as described previously (Zhao et al., 2003). Modified Boyden chambers (Becton Dickinson, Boston, MA) were coated with 0.25 mg/mL human type IV collagen (Sigma). 0.5×10^5 cells in serum-free media were added to each insert in the presence or absence of 4-iodo benzo[b]thiophene-2-carboxamide (B428, 7.5 and 15 μ M), uPA antibody (25 μ g/ml), FTA (10 μ M, Biomol, Plymouth Meeting, PA), UO126 (10 μ M, Calbiochem), and Bay 54-9085 (10 μ M, Bayer Pharmaceuticals). The control groups were treated with DMSO or normal mouse IgG. After incubation, invaded cells were stained with 0.1% crystal violet solution and photographed. The cells were then counted and the number of invaded cells was used for subsequent comparative analyses by one-way ANOVA with the least significant different correction.

Three-Dimensional Cell culture and 3D Invasion Assay

Gelfoam (Pfizer Inc) was cut into 0.9 mm diameter and 10 mm thickness to grow E6/E7 and Ras cells (1×10^6) for 9 days and media was changed every 2 days. The cell-containing gelfoam

was then washed with serum free media and moved into the upper chamber of the pre-coated Boyden insert and incubated for 24 h. Then the invaded cells were stained with 0.1% crystal violet solution and photographed. The cells were counted using Photoshop software. Media from each insert were collected for western blot and zymogram analyses.

Stable Expression of uPA Antisense RNA Constructs in the Invasive GBM U1242 Cells

uPA antisense RNA expression constructs were designed for stable integration and constitutive RNA synthesis. The uPA partial cDNA served as our starting template. A 637 bp fragment of uPA cDNA (bp 727–1364, Gene Bank Access No. K02284) was excised with EcoR I. This fragment was ligated, in reverse orientation, into the multiple cloning site of eukaryotic expression vector pBK-CMV to generate uPA antisense construct, in which, the SV40 3'-splice site and polyadenylation signal in pBK-CMV were left intact. We chose U1242 cells as a model because they express high level of uPA and exhibit highly invasive phenotype. U1242 cells were transfected with 5 µg/mL of pBK-CMV-uPA antisense (a-U1242) or with empty pBK-CMV vector (v-U1242) by incubation for 6 h with lipofectamine (4 µg/mL). The cells were then washed twice with serum-free α -MEM and cultured with 10% FBS-supplemented media containing amino-glycoside G418 (400 µg/mL). After 4 weeks, the mixed cultures were screened for uPA expression and single-cell cloning was initiated. Cells that did not express uPA were selected as positive clones.

Xenograft Tumor Mouse Model

Ras clones, v-U1242 and a-U1242 cells were grown in 3D-gelfoam for 7 days and stereotactically injected into the brain of adult male NOD SCID mice (Jackson Laboratories, Bar Harbor, Maine). The Animal Care Committee of the University of Virginia reviewed and approved all procedures and experiments performed in our studies. The mice were anesthetized with ketamine (17.4 mg/20 g), acepromazine and xylazine (2.6 mg/20 g) and placed in a Kopf stereotactic frame with a mouse adapter. The transformed Ras clones grown in gelfoam (1.3×10^6 cells) were implanted into the right striatum of the mouse at coordinates from the bregma 1 mm anterior, 2 mm lateral and 4.5 mm intraparenchymal. The rodent was removed from the stereotactic apparatus and maintained according to the University of Virginia ACUC protocols. The mice were sacrificed 5 weeks post-implantation of Ras clones and 7 weeks postimplantation of the v-U1242 and a-U1242 cells. The formalin-fixed and paraffin embedded specimens were counter-stained with hematoxylin and eosin.

RESULTS

Overexpression of Constitutively Active H-Ras in Immortalized Human Astrocytes

A previous report (Sonoda et al., 2001) showed that four genetic alterations (hTERT expression, inactivation of p53/pRb and ras pathway activation) converted NHA into cells that formed intracranial tumors resembling human AA. Using the same cell models, we confirmed that a 21 kDa H-Ras was robustly expressed in E6/E7/hTERT/Ras astrocytes, but not in NHA and E6/E7/hTERT cells by western blot analysis (Fig. 1a). This data is in agreement with the finding of Sonoda et al. (2001). Additionally, morphological transformation in H-ras transfected E6/E7/hTERT/Ras cells was observed. E6/E7/hTERT/Ras cells were morphologically changed to round or oval shapes (Fig. 1b).

Overexpression of Constitutively Active H-RasV12 Induced uPA Expression and Cell Invasion in Immortalized NHA Cells

Ras induces uPA up-regulation in OVCAR-3 and NIH 3T3 cells (Aguirre-Ghiso et al., 1999; Lengyel et al., 1995; Li et al., 2005). To explore the possibility that constitutively active H-RasV12 contributed to AA formation partly by up-regulating uPA expression, uPA mRNA

was determined in immortalized NHA cells using Northern blot analysis. The result showed a strong 2.2 kb uPA mRNA band in Ras clones, but not in NHA cells and E6/E7 clone (Fig. 2a, top panel). In a similar pattern, western blot and fibrinogen zymography analyses revealed a robust increase of uPA protein/activity in the Ras clones as compared with the NHA cells and E6/E7 clone (Fig. 2a middle and bottom panels). Furthermore, immunocytochemical staining showed that uPA was localized in cytoplasm of Ras cells (Fig. 2b). The uPA signals were very faint in NHA and E6/E7/hTERT cells. There was also a very weak immunoreactivity in control cells using purified normal mouse IgG (Fig. 2b). These results indicate that H-Ras induced uPA expression at both protein/activity and mRNA levels. Cell invasion plays a pivotal role in tumor progression and metastasis. Invasive and metastatic cells require protease expression for migration through the extracellular matrix. We speculate that increased expression of uPA contributes to the increased invasive phenotype of Ras oncogene-transformed E6/E7 cells. To explore the functional role of H-RasV12-induced uPA in Ras clone, *in vitro* cell invasion assay was performed. Infection of H-RasV12 significantly enhanced cell invasion by 8-fold ($P < 0.001$) and 2-fold ($P < 0.01$) compared with NHA and E6/E7 cells, respectively (Fig. 2c).

H-RasV12 Promotes Cell Invasion Via Upregulation of uPA in Three-Dimensional Culture System

Cells grown in 2D culture can be different considerably in their morphology, cell–cell and cell–matrix interactions, and differentiation from those cultured in more pathophysiological 3D environments (Yamada and Cukierman, 2007). Next, we investigated the role of HRas-induced uPA upregulation in cell invasion using 3D-gelfoam culture system. As expected, cells with HRasV12 expression produced higher level of uPA compared with uninfected E6/E7 cells as determined by both fibrinogen zymography (Fig. 3a) and western blot (Fig. 3b). The invasion of Ras clone increased 10-fold compared with E6/E7 clone (Figs. 3c,d) in our 3D cell invasion system.

Overexpression of H-RasV12 Forms Tumor in Xenograft Mouse Model

To explore whether our *in vitro* results represent a true *in vivo* situation, we implanted Ras clones and assessed whether these cells would form tumors following implantation in brains of NOD-SCID mice. All five mice implanted with the Ras clones formed small tumor masses with minor infiltration into the surrounding normal brain tissues 5 weeks postimplantation (see Fig. 4).

Inhibition of uPA Expression Reduced U1242 Glioblastoma Invasion *In Vitro* and Tumor Growth *In Vivo*

To identify the role of active H-Ras-induced uPA in E6/E7/hTERT/Ras cell invasion, a selective uPA inhibitor (B428) (Towle et al., 1993) and functional uPA-blocking antibody were employed. As shown in Figure 5a, B428 blocked uPA activity at concentration of 7.5 and 15 μ M compared with the same volume of DMSO (Fig. 5a top panel). B428 (15 μ M) also significantly inhibited the invasion of Ras cells (40%, $P < 0.05$) as compared with a DMSO control (Fig. 5a, bottom panel). In addition, functional neutralizing antibody specifically targeting uPA significantly reduced the invasive potential of Ras cells by 60% ($P < 0.001$) when compared with normal mouse IgG treatment (Fig. 5a, bottom panel). These results suggest that uPA is involved in H-Ras-mediated invasion.

To extend our finding to glioblastoma cells, we determined the role of uPA in U-1242 invasion *in vitro* and in our xenograft mouse model. We knocked down endogenous uPA expression by introducing uPA antisense cDNA constructs into U1242 GBM cells. The results showed that uPA mRNA (Fig. 5b), protein (Fig. 5c) and activity (Fig. 5d) were decreased by 90–95% in uPA anti-sense transfected U1242 cells (a-U1242), when compared with an empty vector transfected U1242 (v-U1242) and parental U1242 cells. However, MMP-2 activity and proteins

were not affected (Figs. 5c,d). There was also a significant reduction (66.4%, $P < 0.001$) in invasion of a-U1242 cells when compared with empty vector transfected U1242 cells and parental U1242 cells (Fig. 5e). To confirm the role of uPA in GBM tumor growth *in vivo*, we implanted v-U1242 and a-U1242 cells in brains of NOD-SCID mice. The results demonstrate that all 5 v-U1242 cell implants formed large tumor masses and infiltrated into the normal brain 7 weeks postimplantation (Fig. 5f, left panel). Four out of five of the uPA-silenced clones (a-U1242 cells) formed much smaller tumor masses with well circumscribed tumor-normal tissue borders (Fig. 5f, right panel).

H-Ras stimulated uPA activity and cell invasion via Ras-Raf-MEK signaling pathway, but not Ras-PI3K or PKC signaling pathway

To determine which Ras-mediated signal transduction pathway is involved in increased uPA expression and cell invasion, Ras specific inhibitor farnesylthiosalicylic acid (FTA), Raf inhibitor (Bay 54-9085), MEK inhibitor (UO126), PI3K inhibitor (LY294002), or a broad spectrum PKC inhibitor bisindolylmaleimide (BIM) was used. UO126 (10 μ M), but not LY294002 (10 μ M) or BIM (1 μ M) inhibited uPA activity in E6/E7/hTERT/Ras cells. However, MMP-2 expression was not affected by treatment of these inhibitors (Fig. 6a). FTA, Bay 54-9085, and UO126 inhibited uPA activity in a concentration-dependent manner (1–10 μ M) (Fig. 6b). These results further suggest that Ras/Raf/MEK signaling pathway, but not PI3K and PKC signaling pathways, is involved in the up-regulation of uPA expression in the genetically modified NHA cells. To determine whether Ras/Raf/MEK-mediated uPA upregulation plays a role in H-Ras-induced cell invasion, transmembrane invasion assay was performed. FTA (10 μ M), Bay 54-9085 (10 μ M) and UO126 (10 μ M) significantly decreased Ras cell invasion by 32, 63, and 65% respectively ($P < 0.05$ or $P < 0.01$) (Fig. 6c). These results suggest that Ras/Raf/MEK signaling pathway is involved in H-Ras-induced uPA upregulation and cell invasion.

DISCUSSION

The Ras oncoprotein is overexpressed or mutated in 30–95% of advanced solid tumors (Bos, 1989). Oncogenic H-Ras is involved in the conversion of normal human fibroblasts and epithelial cells into tumors (Elenbaas et al., 2001; Hahn et al., 1999). In both *in vitro* and *in vivo* experimental models, constitutively active forms of Ras have been shown to upregulate uPA expression in ovarian OVCAR-3 and SK-OV-3 cells (Lengyel et al., 1995; Li et al., 2005), NIH 3T3 fibroblast cells (Aguirre-Ghiso et al., 1999; Zhang and Schultz, 1992) and human adrenocortical cells (Chen et al., 2005), and contributing to invasive and metastatic properties of NIT 3T3 cells (Aguirre-Ghiso et al., 1999; Zhang and Schultz, 1992). Ras is rarely mutated in gliomas; however, its activity is increased in high-grade gliomas (Carpentier, 2005; Kondo et al., 2004; Lassman, 2004). Increased Ras activity is believed to contribute to the growth of high-grade gliomas (Carpentier, 2005; Guha et al., 1997; Holland et al., 2000; Kondo et al., 2004; Lassman, 2004). It has been well documented that K-ras signal pathway contributes to gliomagenesis (Dai et al., 2005; Fomchenko and Holland, 2006; Momota et al., 2005; Parsa and Holland, 2004; Uhrbom et al., 2005). Moreover, human hTERT expression and inactivation of p53/pRb cooperated with H-Ras pathway activation to transform NHA into intracranial tumors strongly resembling human malignant gliomas (Sonoda et al., 2001).

In this investigation, we have demonstrated that HRasV12 overexpression dramatically enhanced uPA expression and activity, which is consistent with the observation in other cell-types (Aguirre-Ghiso et al., 1999; Chen et al., 2005; Lengyel et al., 1995; Li et al., 2005; Zhang and Schultz, 1992). In addition, we have demonstrated that H-RasV12 overexpression in E6/E7/hTERT cells increased invasion in 2D (Fig. 2c) and 3D (Fig. 3) cell culture conditions and tumor formation in a NOD-SCID mouse model (Fig. 4). In contrast, E6/E7/hTERT cells

exhibited low level of uPA (Fig. 2) and low invasive capacity (Figs. 2 and 3). uPA selective inhibitor (B428) and neutralizing antibody significantly inhibited the invasion of Ras clone (Fig. 5). These results suggest that transfection of active H-Ras in E6/E7/hTERT cells up-regulates uPA expression, which promotes *in vitro* invasion and may contribute to tumor formation *in vivo*. Moreover, the invasiveness of uPA-deficient U1242 clone was greatly reduced *in vitro* and tumor growth *in vivo* was significantly less compared with the parent U1242 cells (Fig. 5), indicating that uPA is necessary for GBM cell invasion and tumor growth *in vivo*. In contrast, Ras increased the expression of MMP-2 and -9 in NIH3T3 cells (Aguirre-Ghiso et al., 1999) and OVCAR-3 cells (Gum et al., 1996), but we have not observed these changes in transformed normal human astrocyte infected with H-RasV12.

It has been reported that Raf, MEK, ERK1/ERK2, JNK and c-Jun/AP-1 or Ral A signals mediate Ras-induced uPA up-regulation in OVCAR-3, SK-OV-3 cells, and NIH 3T3 fibroblast cells (Aguirre-Ghiso et al., 1999; Lengyel et al., 1995; Li et al., 2005; Zhang and Schultz, 1992). However, ERK activity is required for invasion in Ras-transformed cells, whereas JNK and c-Jun/AP-1 are not required (Janulis et al., 1999; Silberman et al., 1997). Consistent with this finding, we have observed that inhibition of Ras, Raf, and MEK activities by specific inhibitors decreased uPA expression and significantly reduced Ras-transformed cell invasion (see Fig. 6). In contrast, PI3K and PKC inhibitors did inhibit uPA activity in Ras clone (Fig. 6). These results suggest that Ras/Raf/MEK signaling pathway is central in the control of the uPA upregulation and cell invasion in astrocyte transformed Ras clone. Our results are consistent with the reports that Raf-1 is a stronger inducer of uPA expression than PI3K in SK-OV-3 cells (Li et al., 2005) and that the induction of uPA promoter by Ras is completely blocked by expression of a dominant negative CRaf or ERK1/2 inactivating phosphatase CL100 (Lengyel et al., 1995). In NIH3T3 cells, v-Ras-evoked increase in uPA expression and tumor formation are mediated by GTPase RalA-dependent activation of c-Src/phospholipase D/protein kinase C (PKC) signal pathways (Aguirre-Ghiso et al., 1999), which is different from our observation in immortalized astrocytes infected with constitutively active H-Ras in which inhibition of PKC with a broad-spectrum inhibitor (BIM) had no effect on uPA activation.

Ras proteins favor malignant transformation and survival by activating cellular proliferation, inducing cell migration, and inhibiting apoptosis. Their functional effects make them ideal targets for inhibition by potential therapeutic agents, many of which are in preclinical or clinical phases of development (Carpentier, 2005; Holland et al., 2000; Kondo et al., 2004). Ras/Raf/MEK/ MAPK, Ras/PI3K/Akt, and PKC pathways have been reported to contribute to cell transformation or tumor formation (Adjei, 2001; Lin et al., 1998). Our results clearly demonstrate that H-Ras-evoked increase in uPA and invasion in E6/E7/hTERH/Ras cells via the Ras/Raf/MEK pathway, but not PI3K and PKC pathways, suggesting that the Ras/Raf/MEK pathway may be an attractive target in combination with other therapeutic agents to prevent the growth and invasion of malignant astrocytomas which exhibit activated Ras-signaling pathway.

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Fig. 1.

Overexpression of constitutively active H-Ras in normal human astrocytes (NHA). **(a)** Western blot analysis of H-Ras expression in NHA, E6/E7 and Ras clones. Robust level of H-Ras was detected in Ras clones, but not in NHA and E6/E7 clones. α -tubulin was probed as a control. Equal amount of protein (25 μ g/lane) was loaded. **(b)** Cell morphology after H-Ras and E6/E7 infections. Photographs were taken under a microscope with $\times 100$ magnification. E6/E7: NHA infected with E6/E7; Ras: NHA infected with E6/E7 and constitutively active H-Ras V12.

Fig. 2.

Constitutively active H-Ras induced expression of uPA and cell invasion in Ras clones. **(a)** Northern blot of uPA mRNA expression in NHA, E6/E7 and Ras clones (top). A GAPDH probe was hybridized as a control. Western blot of uPA protein expression in NHA, E6/E7 and Ras clones (middle). α -tubulin was probed as control. Equal amount of protein (25 μ g/lane) was loaded. Fibrinogen zymography analysis of uPA activity in the conditioned media of NHA, E6/E7, and Ras clones (bottom). Equal amount of proteins (15 μ g/lane) was loaded. **(b)** Immunocytochemical staining of uPA expression in NHA, E6/E7 and Ras clones. Red indicates uPA immunoreactive signals. The normal mouse IgG was used as control. Photographs were taken with $\times 200$ magnification. **(c)** Invaded cells through type IV collagen for 6 h. The cells

were photographed with $\times 50$ magnification. The number of invaded cell in NHA cells was designated as 100%. Data shown are the mean $\pm$ s.e.m. values from three separate experiments for each group. $*P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 3. uPA activity and cell invasion of Ras clones in a three-dimensional (3D) gel foam environment. **(a)** uPA activity detected in 3D culture media by fibrinogen zymography. Equal amount of protein (15 μ g) was loaded in each well. **(b)** Western blot analysis of uPA expression in E6/E7 and Ras clones under 3D culture conditions. Equal amount of protein (25 μ g) was loaded in each well. **(c)** Invaded cells were significantly increased in Ras cells compared with E6/E7 cells in 3D culture for 24 h. The pictures were taken with $\times 50$ magnification. **(d)** The bar graph of the invaded E6/E7 and Ras cells. Data shown are the mean $\pm$ s.e.m. values from three separate experiments for each group. * $P < 0.001$.

Fig. 4.

Overexpression of H-Ras V12 forms tumor in xenograft mouse model. Cultured Ras cells in 3D gelfoam were implanted in the brains of NOD-SCID mice. The tumor bearing mice were sacrificed 5 weeks postimplantation. The formalin-fixed and paraffin embedded specimens were counterstained with hematoxylin and eosin. The Ras cells formed intracranial tumors with minor infiltration into the surrounding normal brain (indicated by arrows). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 5.

Inhibition of uPA expression reduced cell invasion *in vitro* and tumor growth *in vivo*. (a) Cell invasion of Ras clones was associated with the levels of uPA expression. uPA specific inhibitor B428 (7.5 and 15 μM) treatment for 24 h decreased uPA activity in the conditioned media of Ras clones (top panel). Equal amount of proteins (15 μg/lane) was loaded. The invasion of Ras cells through type IV collagen was decreased by adding B428 (15 μM), or uPA neutralizing antibody (25 μg/ml) (bottom panel) for 24 h. DMSO and normal mouse IgG were used as controls. The number of invaded cell in the control was designated as 100%. Data shown are the mean ± s.e.m. values from three separate experiments. * $P < 0.05$, ** $P < 0.01$. (b) RT-PCR analysis of uPA mRNA expression in parental U1242 (U1242) cells, vector (pBKCMV) control U1242 cells (v-U1242) and uPA antisense transfected U1242 cells (a-U1242). β-actin used as control. (c) uPA protein was detected by western blot in U1242, v-U1242 and a-U1242 cells. MMP-2 was probed as a control. (d) Fibrinogen zymography analysis of uPA levels in U1242, v-U1242, and a-U1242 cells. Equal amount of protein (15 μg/lane) was loaded. MMP-2 was detected as loading control. (e) uPA antisense introduction decreased invasion of U1242 cells *in vitro*. The cells were counted and analyzed as described under Materials and methods section. Data shown are the mean ± s.e.m. $n = 3$, **, $P < 0.001$. (f) Xenograft GBM mouse model. Cultured v-U1242 and a-U1242 cells in 3D gelfoam were implanted in the brains of NOD-SCID mice. The tumor-bearing mice were sacrificed 7 weeks postimplantation. The formalin-

fixed and paraffin embedded specimens were counterstained with hematoxylin and eosin. v-U1242 cells grew and infiltrated the surrounding tissues of brain ($n = 5$, top panel). a-U1242 cells grew but were not infiltrative ($n = 5$, bottom panel). Arrows indicate the border of tumors. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 6.

H-Ras-mediated uPA expression and cell invasion via Ras/Raf/MEK signaling pathway. **(a)** uPA activity was blocked by MEK1/2 inhibitor UO126 (10 μM), but not by PI3K inhibitor (LY294002, 10 μM) and protein kinase C inhibitor (BIM, 1 μM) as detected by fibrinogen zymography. MMP-2 was not altered by treatment of these inhibitors as detected by gelatin zymography. **(b)** Ras inhibitor (FTA), Raf inhibitor (Bay 54–9085) and MEK1/2 inhibitor UO126 reduced uPA activity in a concentration-dependent manner (1, 5, and 10 μM) after treatment for 24 h. Equal amount of proteins was loaded in each group. **(c)** Cell invasion was significantly decreased by adding FTA (10 μM), Bay 54–9085 (10 μM) and UO126 (10 μM) in Ras clones for 24 h compared with DMSO. The number of invaded cell in the DMSO was designated as 100%. Data shown are the mean ± s.e.m. values from three separate experiments. * $P < 0.05$.