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Ras Signaling is Involved in the Expression of Fas-L in Glioma

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SUMMARY: Fas-L expresses on a variety of tumors and is suspected to modify the dialog between tumor and the immune system. However, the cellular abnormality in tumor cells leading to an aberrant expression of Fas-L is unclear. In this study, we demonstrate the involvement of Ras signaling in the Fas-L expression in several ways. First, the activated *Ha-ras*^{Val12} gene enhanced the Fas-L expression of primary human glial cells. Second, blocking the Ras signal pathway in glioma cells by lovastatin or the *Ha-ras*^{Asn17} dominant-negative mutant gene resulted in reduced Fas-L expression. Transfection of the *Ha-ras*^{Asn17} into glioma cells also inhibited the activation of NFκB, which is a downstream component of Ras signaling. Accordingly, the membrane-permeable NFκB competitor suppressed the Fas-L expression. Furthermore, the Fas-L expression coincided with the Ras activity in the murine 212 cells, in which the Ras activity could be induced by isopropyl β-D-thiogalactoside. In summary, these results suggest that the enhanced Ras signaling with consequential NFκB activation, which is a frequent defect found in tumors, could mediate the Fas-L expression of tumors. (*Lab Invest 2000, 80:529–537*).

Fas-L (CD95L, APO-1L), a member of the tumor necrosis factor receptor superfamily, sparks the death signal into Fas-bearing cells by the engagement with Fas molecules (Itoh et al, 1991; Takahashi et al, 1994). Apoptosis triggered by the Fas/Fas-L signaling cascade is an important pathway involving not only homeostasis of the immune system (Chervonsky et al, 1997), but also embryo organogenesis (Seino et al, 1997; Yeh et al, 1998). In contrast to the tightly regulated expression in lymphocytes (Alderson et al, 1995; van Parijs and Abbas, 1996), Fas-L is constitutively expressed in several non-lymphoid tissues of immune privilege sites (Ferguson and Griffith, 1997). The recent findings that Fas-L-bearing melanoma and hepatoma cells induced apoptosis of T cells in vitro (Hahne et al, 1996; Strand et al, 1996) and that tumorigenesis of melanoma cells was delayed in Fas-deficient *lpr* mutant mice (Hahne et al, 1996) raise an interesting hypothesis that Fas-L renders tumor to escape from immune surveillance (O'Connell et al, 1999; Walker et al, 1998). Since then, ectopic expression of Fas-L has been found in many malignancies. Fas-L appears to modulate both immunosuppressive and proinflammatory immune responses in various tumor models (Walker et al, 1998). Despite the wealth of information, signaling in tumor cells for the Fas-L expression is not well elucidated.

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The NFκB binding site, a downstream component of the Ras signaling, has been identified in the promoter region of *Fas-L* gene and could affect the regulation of Fas-L in T cells (Faris et al, 1998; Latinis et al, 1997). Oncogenic mutations that alter the Ras activity have often been found in tumor cells (Kaba et al, 1990; Lowy and Willumsen, 1993). Therefore, we speculated that the trigger of the Ras signaling pathway that results in NFκB activation may mediate the Fas-L expression in tumor cells.

Glioma is one of the common tumors in the central nervous system and expresses a high level of Fas-L (Gratas et al, 1997; Saas et al, 1997). Moreover, glioma cells showed high Ras activity because of either enhanced transcription of the N-*ras* gene or reduced function of Ras suppressor RSU-1 (Tsuda et al, 1995; Tsurushima et al, 1996). Blocking the Ras activity by the *Ha-ras*^{Asn17} dominant-negative mutant gene decreased cell proliferation, indicating that Ras is active and mediates an important mitogenic pathway in glioma (Guha et al, 1997). In this study, the Ras signaling in glioma cells was blocked by lovastatin, by *Ha-ras* dominant-negative mutant gene, or by the NFκB competitive peptides. Reductions in Ras and NFκB activities in treated cells were confirmed by luciferase-based reporter assays. Transcripts and protein levels of Fas-L in cells were detected by reverse transcription-polymerase chain reaction and flow cytometric analysis, respectively. Activated *Ha-ras*^{Val12} gene was transiently expressed in primary human glial cells to evaluate its effect on the Fas-L expression. In addition, we utilized an isopropyl β-D-thiogalactoside (IPTG)-inducible Ras expression system constructed in murine NIH3T3 cells to explore the association of the Ras activity and the Fas-L expression.

Results

Activated Ha-ras^{Val12} *Stimulated Fas -L Expression in Glial Cell*

U-373MG and U118MG cells showed elevated expression of Fas-L at both transcriptional and translational levels as compared with normal glial cells (Fig. 1, a and b). At 36 hours posttransfection with activated *Ha-ras*^{Val12}, normal glial cells had increased amounts of Fas-L mRNA (Fig. 2a). Moreover, Fas-L-positive glial cells, being stained immunohistochemically with Fas-L-specific antibody, appeared in the EGFP-expressing cells, which would simultaneously receive *Ha-ras*^{Val12} in this experimental condition (Fig. 2b). Normal glial cells transfected with pEGFP-N1 alone were flat and polygonal with little overlapping, whereas *Ha-ras*^{Val12}-transfected glial cells became spindle form and overlapped.

Lovastatin Suppressed the Expression of Fas-L in Glioma Cells

Treatment with 5 μg/ml lovastatin for 24 hours apparently did not affect the viability of U-373MG cells. U-118MG cells were relatively sensitive to lovastatin, that growth was reduced approximately 40% in 24 hours after treatment with 5 μg/ml lovastatin (data not shown). Treatment with lovastatin for 12, 24, or 36 hours, both U-118MG and U-373MG showed less Fas-L mRNA than DMSO-treated controls (Fig. 3a). The transcripts of Fas-L expression were significantly reduced by lovastatin at 24 hours posttreatment, then gradually recovered. Decrease in Fas-L protein level was further confirmed in 24 hours-lovastatin treated cells by flow cytometric analysis (Fig. 3b).

Transfection of Ha-ras^{Asn17} *Suppressed the Ras Activity, the NFκB Activity, and the Fas-L Expression in Glioma Cells*

The inhibitory effect of *Ha-ras*^{Asn17} dominant-negative mutant gene on Ras activity in glioma cells was determined directly with the Pzy-luc reporter plasmid, which requires the Ras activity to express luciferase. By transfection with Pzy-luc alone for 24 hours, U-118MG cells showed a higher level of luciferase activity than U-373MG cells. Cotransfection with Pzy-luc and *Ha-ras*^{Asn17} significantly reduced the luciferase units of reporter in both U-118MG and U-373MG cells, as compared with those of Pzy-luc-tranfected cells, indicating that *Ha-ras*^{Asn17} inhibited effectively the Ras activity (Fig. 4a). Similarly, cells cotransfected with *Ha-ras*^{Asn17} and NKκB-luc showed less luciferase units than cells transfected with NKκB-luc alone (Fig. 4b), indicating that *Ha-ras*^{Asn17} also effectively suppressed the NKκB activity.

After cotransfection with *Ha-ras*^{Asn17} combining excess amounts of pEGFP-N1 and growth in fresh medium for 36 hours, cells were subjected to measuring Fas-L protein by flow cytometric analysis gating on EGFP-positive population. Compared with transfection with an empty vector, expression of *Ha-ras*^{Asn17} in glioma cells suppressed the Fas-L expression in glioma cells in two independent experiments (Fig. 4c).

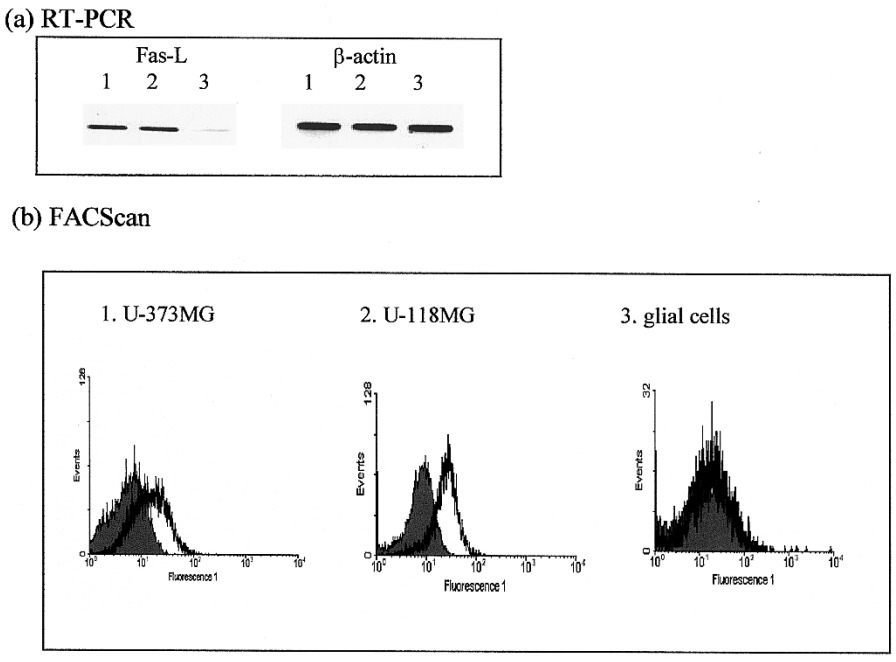

Figure 1.

Enhanced expression of Fas-L in glioma cells. a, After growth for 2 days in 20% FCS/DMEM, cells were harvested. Fas-L transcripts were analyzed by RT-PCR. Lanes 1 to 3: U-373MG, U-118MG, and normal glial cells, respectively. b, Level of Fas-L protein was detected by flow cytometry analysis with antibody recognizing human Fas-L. Staining with secondary antibody alone was the negative control.

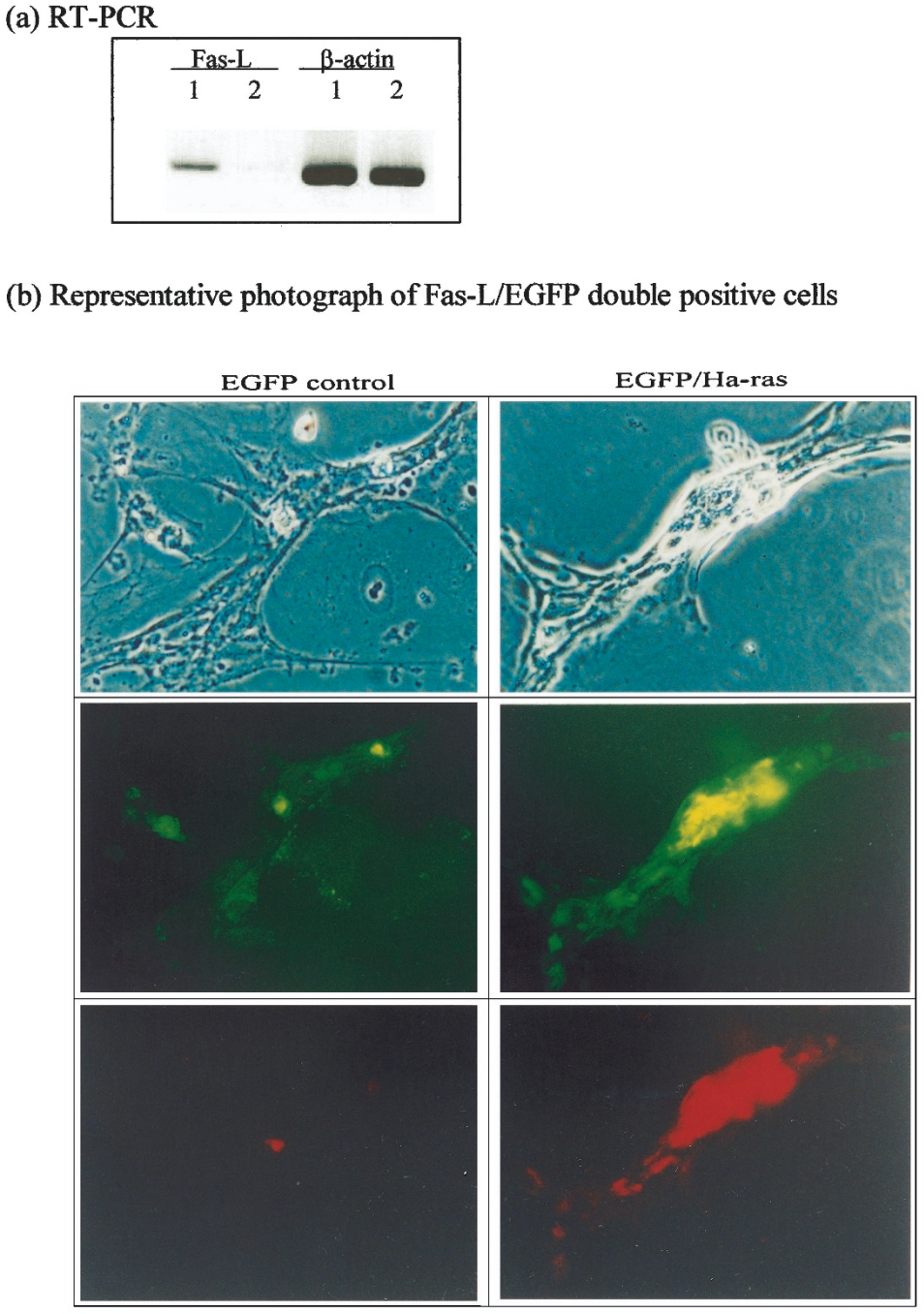

Figure 2.
Activated *Ha-ras*^{val12} gene stimulated the expression of Fas-L in normal glial cells. DNA transfection was done as described in "Material and Methods." Levels of Fas-L were compared in 36 hours posttransfected cells. a, The transcripts of Fas-L were analyzed by RT-PCR. Lane 1, *Ha-ras* ^{val12} plus pEGFP-N1; Lane 2, pEGFP-N1 alone. b, Fas-L protein was immunohistochemically stained using PE-labeled Fas-L specific antibody. Upper panel, light microscopy; middle panel, EGFP-fluorescence; lower panel, Fas-L staining.

Suppression of Fas-L *Gene in Glioma Cells by NFκB Competitive Peptide*

Cells treated with membrane-permeable NFκB competitive peptide for 24 hours were harvested and subjected to detection on mRNA by RT-PCR and protein and by flow cytometric analysis. NFκB competitive peptide significantly reduced the amounts of Fas-L transcript (Fig. 5a), as well as protein (Fig. 5b) in glioma cells as compared with those of control.

Expression of Fas-L Coincided with the Ras Activity in 212 Cells

On exposure to 2.5 mM IPTG for 24 hours, 212 cells expressed elevated amounts of Ras protein (Fig. 6a).

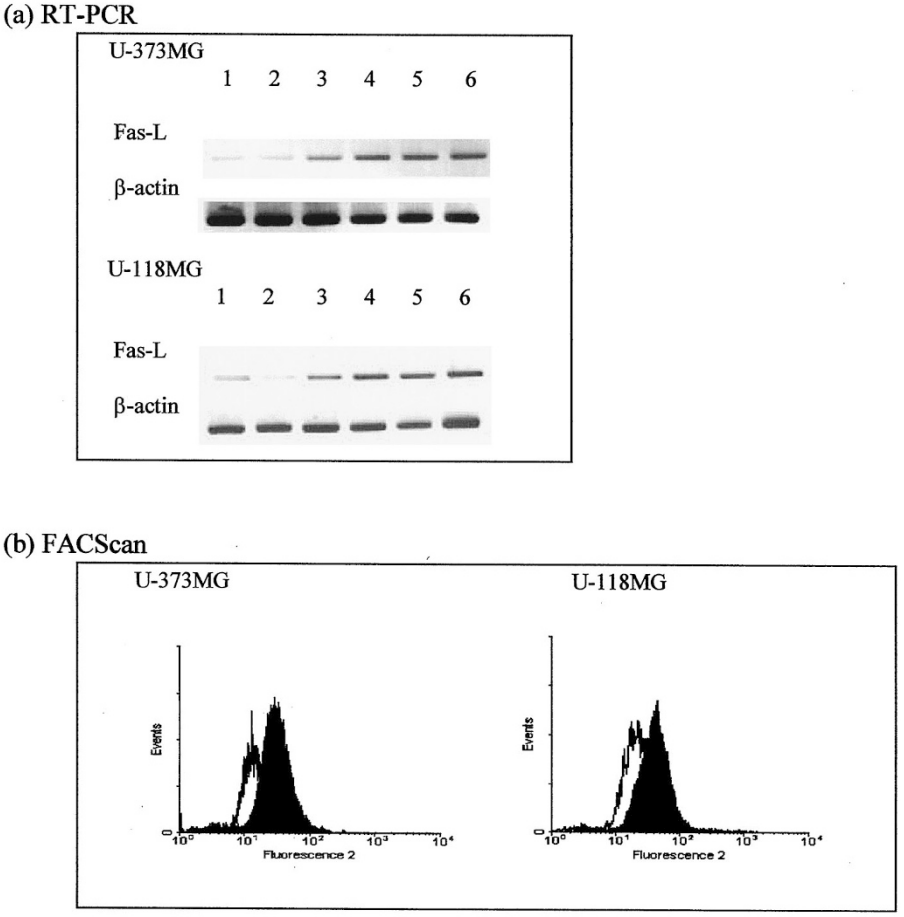

Figure 3.
Effect of lovastatin on Fas-L. After treated with either 5 μg/ml lovastatin (Lanes 1 to 3) or DMSO (Lanes 4 to 6), cells were harvested at intervals. a, The Fas-L transcripts were analyzed by RT-PCR. Lanes 1 and 4, 12 hours; Lanes 2 and 5, 24 hours; Lanes 3 and 6, 36 hours posttreatment. b, Fas-L protein in cells treated with lovastatin for 12 hours was detected by flow cytometry analysis. Lovastatin-treated cells, blank curve; DMSO-treated cells, filled curve.

The Ras activity in 212 cells reached maximal in 24 hours after induction with IPTG. Accordingly, the Ras activity in IPTG-treated 212 cells, revealed by the luciferase units in measurement with Pyz-luc reporter, was enhanced approximately to 3.5-fold over mock-treated cells. The expression of Fas-L, both at RNA and protein levels, agreed well with the Ras activity in 212 cell upon IPTG induction (Fig. 6b).

Discussion

We provide evidence to show an active participation of Ras signaling in the Fas-L expression. Transfection with the activated *Ha-Ras*^{Val12} gene enhanced the Fas-L expression in normal glial cells. Conversely, suppressing Ras signal pathway in glioma cells by lovastatin, *Ha-Ras*^{Asn17} dominant-negative mutant gene or NFkB competitive peptide could down-regulate the Fas-L expression. The suppression of NFκB activity that resulted in reduced Fas-L in glioma cells is consistent with the findings that NFκB binding to the Fas-L promoter region is required for the Fas-L expression in T cells (Chen et al, 1997; Kasibhatla et al, 1998). NFκB has been known to be a downstream mediator of Ras signaling pathway, and may regulate the transcription of genes for cell survival and immune responses (Ljungdahl et al, 1997). As expected, *Ha-Ras*^{Asn17} dominant-negative mutant gene inhibited not only the Ras activity but also the NF-κB activity in glioma cells. Our results indicate that the Ras signaling in glioma is transmitted down through NFκB to activate Fas-L. Furthermore, the stimulatory effect of Ras on the Fas-L expression was demonstrable in murine fibroblast-like cells with an IPTD-inducible Ras system, suggesting that Ras signal-regulated expression of Fas-L might be common in malignant cells of different origins.

Ras protein has been known to transduce multiple signals regulating cell growth (Campbell et al, 1998; White et al, 1995). As mentioned earlier, Ras dominant-negative mutants decrease the proliferation of astrocytoma cell lines (Guha et al, 1997). We failed to establish a stable cell clone carrying *Ha-Ras*^{Asn17} dominant-negative mutant gene (data not shown), which further confirms the essential role of Ras mitogenic pathway for the survival of glioma cells. Recently, accumulated information has shown that Ras could also mediate immune down-regulation through several growth-unrelated mechanisms (Weijzen et al,

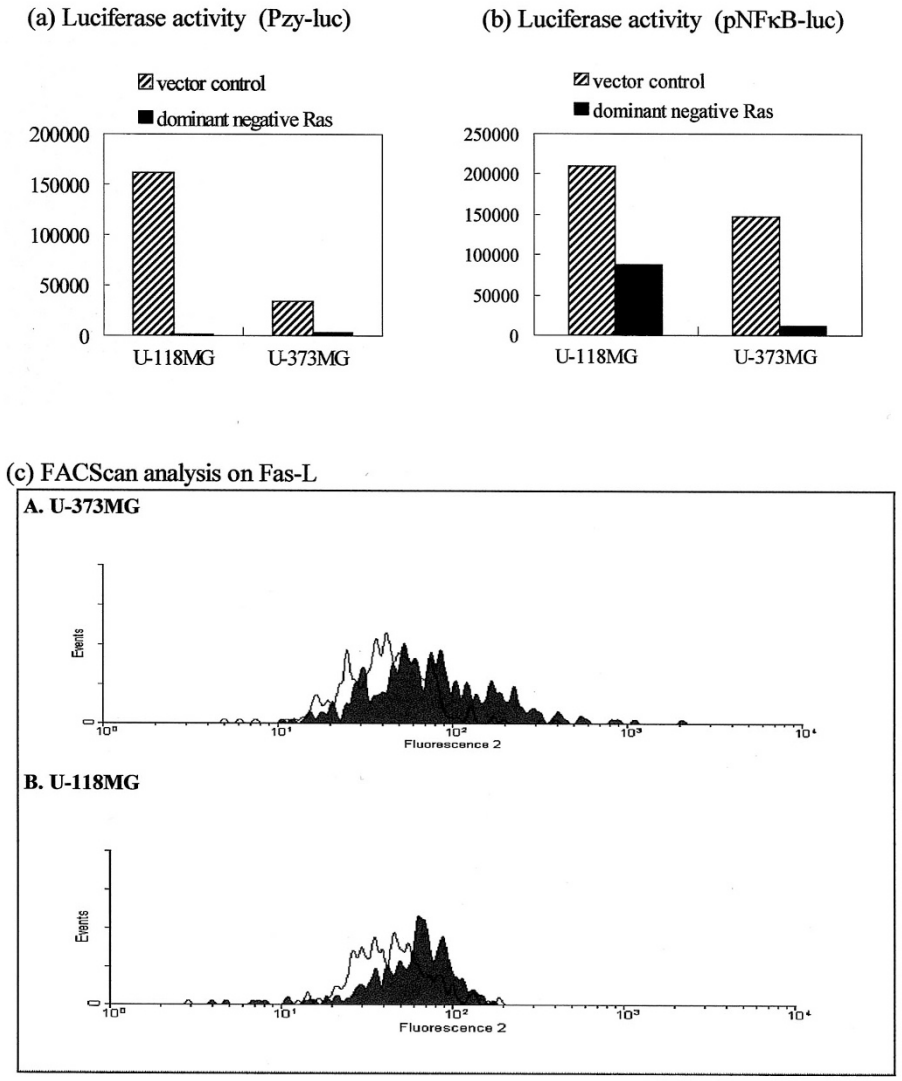

Figure 4.

Effect of *Ha-ras*^{Asn17} dominant-negative mutant gene on Ras, NFκB, and Fas-L. Cells were cotransfected with *Ha-ras*^{Asn17} and pEGFP-N1 and grown further for 36 hours. Relative luciferase units detected by a, Pzy-luc reporter and b, pNFκB-luc reporter reflect the activities of Ras and NFκB, respectively. Values shown are means of two independent experiments. c, Fas-L protein stained with Fas-L-specific antibody was detected by flow cytometry analysis gating on EGFP-positive cell. Blank curve, *Ha-ras*^{Asn17}/pEGFP-N1-transfected; filled curve, pEGFP-N1-transfected.

1999). For instances, high Ras expression is correlated with an increased level of invasiveness of breast cancer, in which Ras has been linked to MHC down-regulation (Solana et al, 1992). In addition, Ras-expressing tumors are shown to have a marked decrease in Fas or TNF-α receptor, which renders tumors resistant to cytotoxic T cell-mediated killing (Fenton et al, 1998; Fernandez et al, 1992). These immune-associated mechanisms can explain the observations that the Ras confers progression and metastasis of some cell lines in vivo, but does not alter significantly the growth rate of those cells in vitro (Muschel et al, 1985; Wang et al, 1997). Taken together, our finding supports the idea that oncogenic potential of Ras may reside in the capacity not only to promote cell growth, but also to defeat immune surveillance by increasing the expression of Fas-L.

Materials and Methods

Cell Culture

Human glioma cell lines U-373MG and U-118MG were purchased from the American Type Culture Collection (Rockville, MD). Brain tissues were obtained from patients who underwent operations at National Cheng Kung university Hospital for brain damage that was unrelated to malignancy. Primary explant cultures of normal glial cells were then established according to a method described elsewhere (Yong et al, 1991). Cells were cultured in Dulbecco modified Eagle's medium (DMEM; Gibco BRL, New York) supplemented with 20% fetal calf serum (FCS), 1% penicillin, and 1% fungizone at 37° C/5% CO_2. The 212 cells, derived from NIH3T3 cells and mounted with an isopropyl β-D-thiogalactoside (IPTG; 2.5 mM; Sigma Chemical,

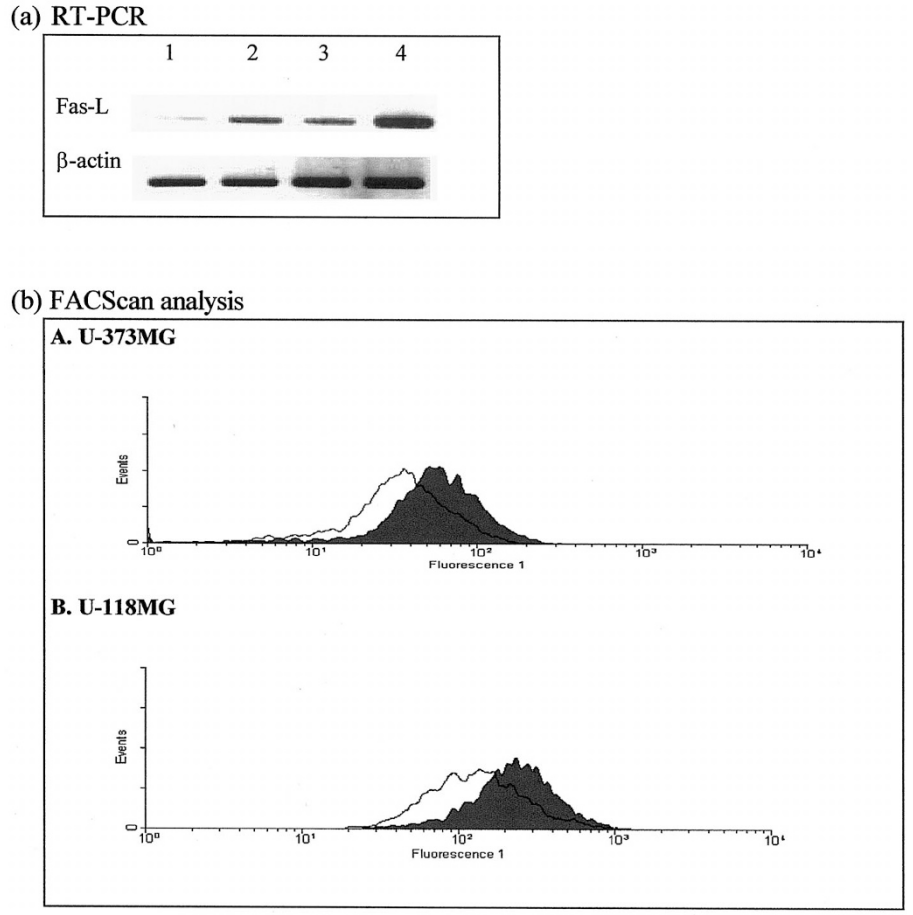

Figure 5.

Effect of NFκB competitive peptide on Fas-L. Treated with 50 μg/ml NFκB competitive peptide for 24 hours, cells were harvested for analysis on Fas-L. a, The Fas-L transcript was analyzed by RT-PCR. Lanes 1 and 2, U-373MG; Lanes 3 and 4, U-118MG. b, Fas-L protein was detected by flow cytometry analysis using Fas-L-specific antibody. Blank curve, NFκB competitive peptide-treated; filled curve, mock treated.

St. Louis, Missouri)-inducible Ras system (Liu et al, 1992, 1998), were grown in 10% FCS/DMEM. Standard Chem. and Pharm. Co., Taiwan, Republic of China, kindly provided us with lovastatin, which is a competitive inhibitor of 3-hydroxyl-3-methylglutaryl coenzyme A reductase and inhibits the translocation-associated isoprenylation of several Ras family proteins, resulting in loss of their signal transducing ability (Cuthbert and Lipsky, 1997; Girgert et al, 1994). NFκB activity was inhibited by the membrane-permeable NFκB competitive peptide (50 μg/ml; BIOMOL Research Laboratories, Plymouth Meeting, Pennsylvania) according to the manufacturer's instructions.

DNA and Transfection

Plasmid DNA was delivered into cells by lipofection with a ratio of 1 μg DNA/20 μ1 lipofectamine (SuperFect Transfection Reagent; Qiagen, Hilden, Germany). After transfection, cells were grown in fresh 20% FCS/DMEM for 24–36 hours then harvested for detections on Fas-L, Ras, or NFκB. pEGFP-N1 plasmid (Clontech, Palo Alto, California) was used as the control to evaluate transfection efficiency and to label transfection-positive cells. EGFP-expressing cells were green under fluorescent microscopy. *Ha-ras*^{Val12} is an activated mutant cloned from the human T24 bladder carcinoma cell line (Capon et al, 1983). Dominant-negative mutant *Ha-ras*^{Asn17} (pZipAsn17; Feig and Cooper, 1988) was kindly provided by Dr. S.F. Yang (Academia Sinica, Taiwan, Republic of China). The transfection efficiency was approximately 10% in this experimental condition. By cotransfection experiments, plasmids coding *Ha-ras*^{Val12} activated mutant or *Ha-ras*^{Asn17} dominant-negative mutant were mixed with the pEGFP-N1, Pzy-luc, or the NKκB-luc at a ratio of 9:1. The excess amounts of reporter plasmids over Ras plasmids ensured that those pEGFP-N1-, Pzy-luc-, or NKκB-luc-transfected cells might simultaneously receive the *Ha-ras*^{Val12} or *Ha-ras*^{Asn17}.

Luciferase Reporter Systems for Ras and NFκB Activities

The luciferase-base reporter plasmids for Ras and NFκB activities were Pyz-luc (Galang et al, 1994,

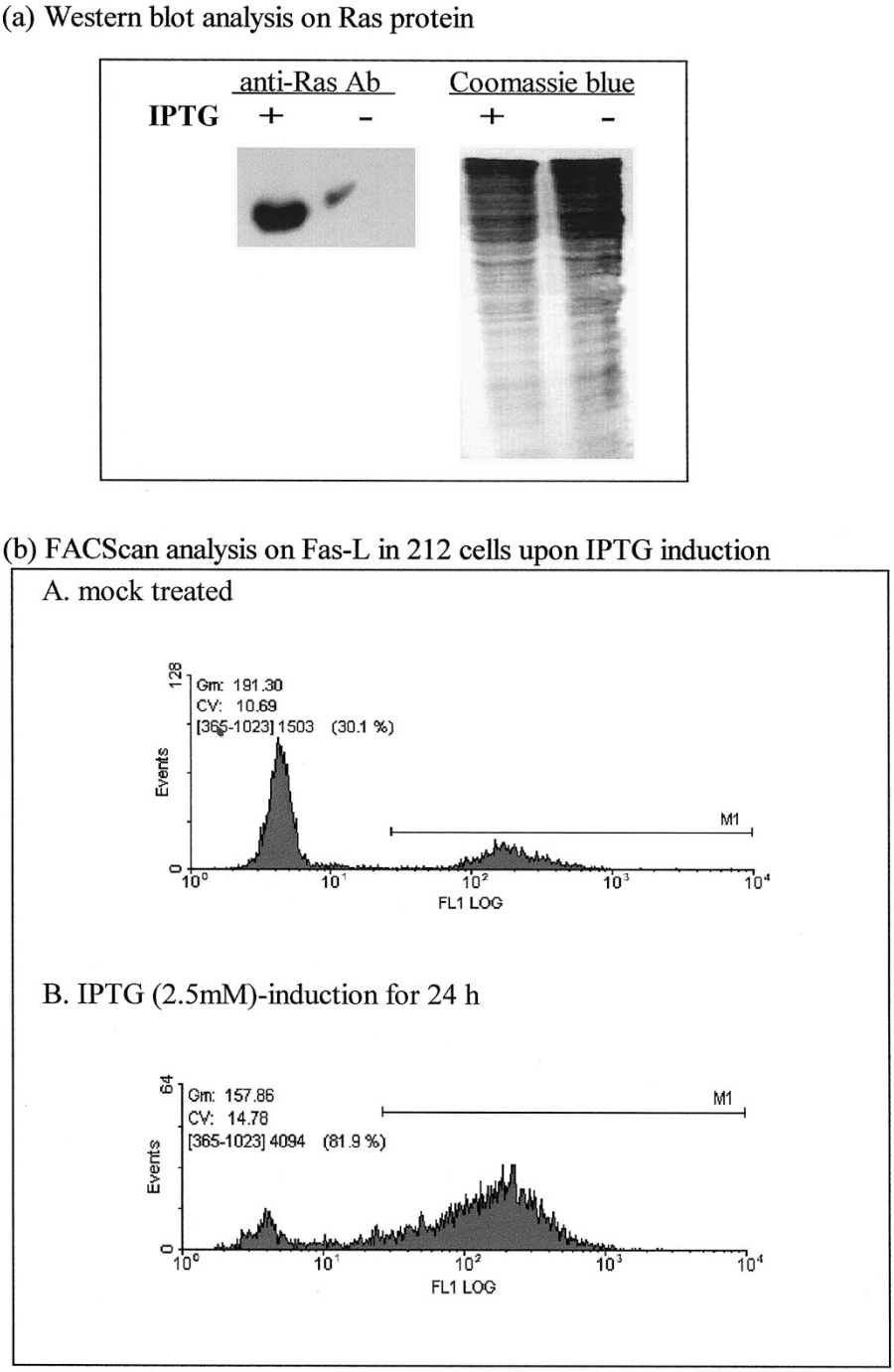

Figure 6.
Induction of Fas-L expression in murine 212 cells. The 212 cells were stimulated with 2.5 mM IPTG for 24 hours. Protein extract was subjected to Western blot analysis on Ras protein using Ras-recognizing antibody and detected with ECL system. a, Identical gel was stained with Coomassie blue and served as protein loading control. b, Fas-L protein was measured by flow cytometric analysis using Fas-L-specific antibody.

kindly provided by Dr. Hauser, The Burnham Institute, La Jolla, California and pNFκB-Luc (Stratagene, La Jolla, California), respectively. The activities of luciferase and β-galactosidase were determined by the Dual-light luciferase and β-galactosidase reporter gene assay system (Tropix, Bedford, Massachusetts).

Reversed Transcription-Polymerase Chain Reaction

Total RNA was prepared by the RNeasy Kit followed the manufacturer's instruction (Qiagen) and converted to cDNA by StrataScrip-H-Reverse transcriptase in the presence of RNAsin (Stratagene) with oligo-dT as

a primer. RT-PCR on the Fas-L transcripts was performed under conditions described previously (Yang and Yang, 1998). The generated cDNA was subjected to PCR amplification on a DNA thermal Cycler (Hybaid Omnigene, Middlesex, UK). β-actin served as a semi-quantitative control. PCR products were fractionated by agarose electrophoresis, stained with ethidium bromide, and visualized under UV light.

Flow Cytometric Analysis and Immunohistochemical Staining

Cells were harvested with a cell scraper and suspended in phosphate balanced buffer containing 1% BSA. They were fixed with ice-cold 4% formaldehyde for 15 minutes and then permeablized with 0.1% Tween 20. Fas-L protein was stained with a rabbit anti-Fas-L antibody (N-20; Santa Cruz, California), followed by PE-conjugated rat anti-rabbit Ig antibody (Gibco). Subsequently, cells were subjected to flow cytometry analysis with a gate set for examining a total of 10^4 cells. Fas-L of *Ha-ras*^{Val12}-transfected normal glial cells was detected by immunohistochemical staining. Glial cells were transfected with *Ha-ras*^{Val12} and pEGFP-N1 at a ratio of 9:1, then grown and treated on poly-L-lysine-coated slides. They were stained in situ with the rabbit anti-Fas-L antibody followed by the PE-conjugated rat anti-rabbit Ig. Fas-L-positive cells were visible under a fluorescent microscope.

Western Blot Analysis

On exposure to IPTG for 24 hours, 212 cells were extracted with buffer containing 1% triton X-100, 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1 U/ml aprotinin, and 50 μg/ml PMSF. Proteins were separated in a 12.5% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Ras protein was probed at first with a Ras-specific monoclonal mouse antibody (Ab-2; Oncogene, Cambridge, Massachusetts) followed by peroxidase-conjugated goat anti-mouse IgG antibody (DAKO, Carpinteria, California). The immune complexes were made visible by fluorography with an enhanced chemiluminescence detection kit (Amersham International PLC, Buckingham, UK).

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