Fas/Fas-L signalling system plays an important role in immune homeostasis (Nagata, 1997). Fas-L (also termed as CD95L, APO-1L) is a member of the tumour necrosis factor receptor superfamily and may trigger a death signal into Fas-bearing cells after engagement with Fas molecule (Itoh et al., 1991; Takahashi et al., 1994). Contrast to a well-regulated manner in lymphocytes (Alderson et al., 1995; Van Parijs and Abbas, 1996), Fas-L is constitutively expressed in several non-lymphoid tissues as well as in tumours of various origins. The expression of functional Fas-L on some tumour cells may confer them the ability to kill T cells in vitro as compared with vector controls. In nude mice, Fas-Lribozyme-carrying cells grew faster with lesser apoptosis, formed bigger tumour with significantly fewer infiltrating cells in the tumour area, and triggered relatively milder tumour-associated liver damage than vector controls did. Thus, down-regulation of Fas-L not only improved viability of glioma cells but also reduces local immune responses that may consequently affect tumour formation. Taken together, our findings imply that endogenous expression of Fas-L in malignant cells is not always growth promoting.

MATERIALS AND METHODS

Cells and cell culture condition

Human glioma cell lines U-373MG and U-118MG were purchased from the American Type Culture Collection (Rockville, MD). Transfectants carrying plasmid pEGFP-N1 (vector controls) or
Fas-L<sub>ribozyme</sub> were established in this study. Cells were cultured in Dulbecco modified Eagles’ medium (DMEM; Life Technologies, Grand Island, NY) supplemented with 20% fetal calf serum (FCS), 1% penicillin and 1% fungizone at 5%CO₂ /37°C in a humidified atmosphere. Growth rate was measured by counting viable cell, which was determined by trypan blue exclusion. MTT assay was applied to estimate cellular viability using a commercially available kit (CellTitre 96™ Aquous, Promega, Madison, WI). In brief, cells were seeded in 96-well plates (5 x 10<sup>3</sup> cells well<sup>-1</sup>), grew for 72 h to confluence, and the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolummon) was added the last 2 h before the end of culture. The cell-bound dye was measured by optical absorption at 490 nm using a microplate reader.

**Construction of Fas-L<sub>ribozyme</sub>**

The sequences of the oligonucleotides used to construct Fas-L<sub>ribozyme</sub> were as follow: sense sequence: 5′-ATG/AAT/TCC/CGG/AGTC/ATC/GTA/GTC/GTC/ATA/CAGA/GAA/AC/TTT/GGA/TCC/CCA/CGA-3′; antisense sequence: 5′-TCG/GGA/TCC/AAA/GTT/TGG/CTG/TAT/CAG/GAC/TCA/TCA/GTA/TTT/CCG/GGA/ATT/CA-3′. This sequence is corresponding to the coding region in the extracellular domain of Fas-L and does not share homology to other genes according to database search on GenBank, EMBL, PDB and DDBJ. After annealing of these oligonucleotide pairs, the generated fragment was digested with EcoR1 and BamH1, followed by ligation into the EcoR1/BamH1-predigested vector plasmid pEGFP-N1 (Clontech, palo Atto, CA). This plasmid construct, the ribozyme was directly linked upstream of the EGFP gene to form a fusion transcript (Figure 1), so that the Fas-L<sub>ribozyme</sub> can be monitored indirectly by the expression of EGFP protein. Candidate clone was verified by DNA sequencing.

**DNA transfection**

Plasmid DNA was delivered into cells using the lipofection method with a ratio of 1 μg DNA/20 μl lipofectamine (Qiagen, Hilden, Germany). Plasmid pEGFP-N1 served as the non-ribozyme control. After DNA transfection, cells were grown in regular 20% FCS/DMEM for 48 h, and then selected with geneticin (G418, Sigma, St Louis, MO) at an effective concentration of 0.5 μg ml<sup>-1</sup> for at least 3 months before subjected to further study. Stable transfectants derived from U-118MG and U-118MG carrying either Fas-L<sup>-ribozyme</sup> or pEGFP-N1 plasmid were designated as Fas-L<sub>ribozyme</sub> (U-373MG), Fas-L<sub>ribozyme</sub> (U-118MG), EGFP(U-373MG) and EGFP(U-118MG), respectively.

**Semi-quantitative reversed transcription- polymerase chain reaction (RT-PCR)**

Total RNA was purified using the RNeasy Kit followed the manufacturer’s instruction (Qiagen) and converted to cDNA by StrataScript™-H-reverse transcriptase with oligo-dT primer in the presence of RNAsin (Stratagen, La Jolla, CA). RT-PCR for Fas-L, Fas, Bcl-2, FAP-1 and β-actin were performed as described previously (Cleary et al, 1986; Sato et al; 1995; Yang et al, 1998). The generated cDNA was subjected to 35-40 cycles of PCR amplification on a DNA thermal Cycler (Hybaid Omnigene, Middlesex, UK). The existence of ribozyme in stable cells was verified by Western blot

Cells were extracted with a buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF and 0.1 U ml<sup>-1</sup> leupeptin. Proteins were separated in SDS-polyacrylamide gel and electroblotted onto polyvinyl difluoride membrane (MSI, Westboro, MO). The proteins bounded on the membrane were probed with mouse antibody (Ab)-recognizing Fas-L (clone33; Transduction Laboratories, Lexington, KY) followed by a sheep anti-mouse IgG conjugated with horseradish peroxidase (Dako Corp, Carpinteria, CA). The Fas-L was made visible by fluorography with enhanced chemiluminescence detection kit (Amersham/Pharmacia Biotech, UK). Duplicate blot was probed with α-tubulin-specific Ab (clone DMIA; NeoMarker, Fremont, CA) and served as a protein-loading control.

**Detection of apoptotic cells**

Apoptotic cells in 3-day-old culture were stained with mercocyanine 540 (MC540, Sigma) according to the published procedures (Reid et al, 1996; Yang et al, 1999). Apoptotic cells became susceptible to MC540 binding due to alteration of surface membrane and showed FL2 values more than 230 detected by flow cytometry analysis. Apoptotic cells in tumour nodules or liver tissues were detected by TUNEL labelling detecting free 3′-OH groups in fragmented DNA in situ (Apop Tag-peroxidase in situ apoptosis detection kit, Oncor, MD). Paraffin-embedded, slide-mounted tissue sections were deparaffinized, and treated with proteinase K for 15 min followed by 3% H₂O₂ for 5 min. After nick end labelling with digoxigenin-deoxyuridine triphosphate by deoxynucleotidyl transferase, immunostaining was
performed using peroxidase-conjugated antidigoxigenin Ab for 30 min. Apoptotic cells were visualized with diaminobenzidine substrate and became a dark brown colour. Specimen was counterstained with 0.3% methyl green or haematoxylin/eosin.

Tumour formation in nude mice
Male BALB/c-nu mice were purchased from the National Laboratory Animal Breeding and Research Center, Taiwan, ROC and maintained under specific pathogen-free condition. Mice at 8 weeks old were randomly divided into groups and 3–5 mice in a group. Approximate 10⁶ stable cells in 0.3 ml PBS were injected subcutaneously into the upper flanks of each mouse. Tumour formation was examined every other day. Injected cells were considered as tumorigenic if tumour nodule with a size more than 3 mm developed at the injection site. Mice were sacrificed one week after tumour nodules were formed. Tumour nodules and liver tissue were surgically obtained, fixed in neutral-buffered formalin, pH 7.2, and then subjected to analyses on tumour-infiltrating cells and apoptotic cells. Infiltrating macrophages and granulocytes were morphologically identified based on the ratio of nuclear to cytoplasm and the presence or absence of granules. EGFP-expressing cells in those tissues were immunostained using rabbit anti-EGFP Ab (Clontech) followed by peroxidase-conjugated goat anti-rabbit IgG Ab (Calbiochem, La Jolla, CA). Sections were counterstained and mounted with glycerol gelatin before examination.

Statistical analysis
Results were analysed by Student’s t-test. Differences with P < 0.05 were judged significant.

RESULTS
Fas-L ribozyme plasmid reduced the expression of Fas-L in glioma cells
After growing in geneticin-containing medium for 3 months, more than 90% of stable transfectants carrying either Fas-L ribozyme or pEGFP-N1 plasmid emitted green light under fluorescent microscopy and were morphologically similar to the parental cells. Bulk culture of DNA-transfected cells was used in this study to avoid any bias deduced from cell lines having unwanted gene backgrounds generated during selection. The chimeric transcripts containing Fas-L-specific ribozyme and EGFP sequence were detected in Fas-L ribozyme-carrying cells by RT-PCR as a 411 bp DNA fragment (Figure 2A). A 400 bp PCR fragment was amplified from the cDNA of vector controls, which represents the authentic transcript of the EGFP gene. Transfection of Fas-L ribozyme did not affect the amount of Fas, Bcl-2 and FAP-1 transcripts in both U-118MG and U-373MG cells (Figure 2B). A decrease in the expression of Fas-L was observed in Fas-L ribozyme-carrying cells as compared to those in vector controls at both mRNA (Figure 2B) and protein (Figure 2C) levels.

Fas-L ribozyme enhanced slightly the growth rate but reduced spontaneous apoptosis of glioma cells in vitro
Growth rate of stable transfectants was determined at 24, 48 and 72 h after subculture in fresh medium (Figure 3). Fas-L ribozyme(U-373MG) showed enhanced growth rate in vitro than EGFP(U-373MG). In general, Fas-L ribozyme-carrying cells grew slightly faster than vector controls, although elevation in growth rate in Fas-L ribozyme(U-118MG) was not statistically significant. In addition, less floated cells and apoptotic bodies appeared in 72-h culture of Fas-L ribozyme, glioma cells than that of vector controls. The viability of the transfectants in 72-h culture, in which cells grew to confluence, was estimated by MTT assay (Figure 4). Fas-L ribozyme(U-373MG) showed significantly better viability than EGFP(U-373MG). In consistent with the growth rate, cellular viability of Fas-L ribozyme(U-118MG) was not significant different from that of EGFP(U-118MG). To further quantify the apoptosis, cells were stained with (MC540) (Figure 5). The proportion of MC540-positive cells were 8.7% in culture of Fas-L ribozyme(U-373MG) as compared to 38.5% in culture of EGFP(U-373MG). Similarly, MC540-positive cells in Fas-L ribozyme(U-118MG) were about 3-fold less than...
EGFP(U-118MG) (8.7% versus 22.2%). These results indicate that as a result of Fas-L down-regulation, the Fas-L ribozyme transfec-
tants were more resistant to spontaneous cell death under con-
fluent growth condition.

Tumour formation of EGFP/Fas-L ribozyme stable cells

U-118MG produced tumours by 2–3 weeks after subcutaneous
inoculation into nude mice. Parental U-373MG cells were less
tumorigenic and produced tumours in mice around 8–9 weeks
after cell injection. To determine whether endogenous tumour Fas-
L involves in tumorigenesis, 10^6 cells, with or without Fas-L ribozyme,
were injected subcutaneously into the dorsal flanks of nude mice.
Results are summarized in Table 1. Fas-L ribozyme(U-118MG) and
EGFP(U-118MG) produced tumours in all animals by 2 weeks
post-injection with diameters of 3–5 mm and 2–4 mm, respec-
tively. EGFP(U-373MG), as expected, produced tumours of small
size (1.5–2 mm) and only 2 out of 3 mice developed tumours first
at 7.5 and 10 weeks post-injection. Interestingly, Fas-L ribozyme(U-
373MG) produced more tumours in all injected mice with diame-
ters of 3–4 mm about 3–5 weeks earlier.

Histopathology

Fas-L ribozyme altered the architecture of tumours. Tumour nodules of
Fas-L ribozyme-carrying cells showed loose margin and invasive char-
acteris (Figure 6). Immune cells showing morphology of mononuclear
cells/granulocytes accumulated in and around the tumour nodules
(Figure 7). There were less infiltrating cells in tumours produced by Fas-L ribozyme
 carrying cells than those of vector controls (Table 2). Notably, the infiltrating cells tended to accumulate near the margin
of tumour nodules produced by vector controls (Figure 7B).
Apoptotic cells showing fragmented nuclei and stained positive by
TUNEL staining (brown-coloured cells as shown in the represen-
tative pictures for U-373MG; Figure 8) were numerous in tumours
of EGFP(U-118MG) and EGFP(U-373MG). Apoptosis was signifi-
cantly reduced in tumour nodules of all Fas-L ribozyme-carrying cells as
compared to vector controls.

Liver damage in tumour-bearing mice

Multifocal, diffuse, necrotizing areas were frequently observed in
liver of mice bearing vector controls (Figure 9A, C), but less
in liver of mice bearing Fas-L ribozyme-carrying cells (Figure 9B).
Apoptotic cells showing morphological features of hepatocytes
were demonstrable in the liver lesion (Figure 9D). Take the advan-
tage that our established tumour cell lines express EGFP, the pres-
ence of tumour cells in situ was confirmed by histological

| Table 1 | Summary of the effects of Fas-L ribozyme on tumour formation |
|---------|------------------|
|         | U-373MG | U-118MG |
| Tumour size (mm^3) | Control | Fas-L ribozyme | Control | Fas-L ribozyme |
| 1.5–2 | 3–4 | 2–4 | 3–5 |
| Tumour number (total in 3 mice) | 2 | 7 | 5 | 5 |
| Tumour formation (weeks) | 7.5–10 | 4.5–5 | 2 | 2 |
| Infiltrating cells | +++ | + | +++ | + |
| Apoptotic cells | +++ | ++ | +++ | + |
| Liver damage | ++ | + | +++ | + |

Note: +++: severe; ++: moderate; +: mild; –/: few; –: not found.
immunostaining on EGFP protein (Figure 9E, F). Although all EGFP-positive glioma cells, both Fas-\textsuperscript{L ribozyme}-carrying cells and vector controls, did not form gross detectable tumour nodules in the liver by subcutaneous injection, a few EGFP-positive tumour cells were found among the liver lesions and were morphologically different from those in subcutaneous tumour nodules (comparing Figure 9E).

**DISCUSSION**

By using a hammerhead ribozyme we have successfully down-regulated the expression of Fas-L in human glioma cells at both mRNA and protein levels. Apoptosis in tumour cells was significantly reduced by Fas-L\textsuperscript{L ribozyme}. In nude mice, Fas-L\textsuperscript{L ribozyme}-carrying cells formed bigger tumours with significantly fewer infiltrating cells in the tumour areas than vector controls.

Fas/Fas-L signalling has been demonstrated to elicit apoptosis in glioma cells in certain culture conditions (Weller et al, 1994; Shinoura et al, 1998; Yang et al, 1999). Although cell cycle progression in glioma cells could be promoted by activating Fas antibody (CH-11) probably through the MEK-ERK pathway, however, apoptosis occurred at the same time and the net viable cell count was not increased (Shinohara et al, 2000). In addition, transfer of Fas-L gene into Renca cells, a Fas signal-sensitive renal cell carcinoma caused a suicidal destruction of tumour cells by apoptosis (Arai et al, 1997). In consistence with those findings, glioma cells carrying Fas-L\textsuperscript{L ribozyme} had reduced apoptosis both in vitro and in vivo. Since Fas-L\textsuperscript{L ribozyme} did not alter the expression of other death-related genes including Fas, Bcl-2, and FAP-1, suppression of Fas-L gene by Fas-L\textsuperscript{L ribozyme} resulting in less Fas/Fas-L ligation should account for the reduced apoptosis in

Table 2  Tumour-infiltrating cells

|                  | U-118MG | U-373MG |
|------------------|---------|---------|
|                  | Ribozyme | Vector  | Ribozyme | Vector  |
| 1/T ratio\textsuperscript{a} | 0.085 ± 0.059 | 0.162 ± 0.061\textsuperscript{*} | 0.041 ± 0.023 | 0.140 ± 0.058\textsuperscript{*} |
| G/T ratio        | 0.008 ± 0.004 | 0.053 ± 0.017\textsuperscript{*} | 0.002 ± 0.004 | 0.053 ± 0.036\textsuperscript{*} |
| M/T ratio        | 0.037 ± 0.012 | 0.091 ± 0.049\textsuperscript{*} | 0.037 ± 0.021 | 0.082 ± 0.033\textsuperscript{*} |

\textsuperscript{a}I/T: infiltrating cells/total cells; G/T: granulocytes/total cells; M/T: macrophages/total cells. Each value represents mean ± SD obtained from 3 mice of all groups except U-373MG/vector, the latter were obtained from 2 tumour-bearing mice. \textsuperscript{*}Significant difference between ribozyme-carrying glioma cells and control groups (P < 0.05).

**Figure 6**  Tumour formation of U-118MG- and U-373MG-derived cells in nude mice. Approximate 10\textsuperscript{6} stable cells, EGFP(A:U-118MG, C:U-373MG) or Fas-L\textsuperscript{L ribozyme}(B:U-118MG, D:U-373MG), were injected subcutaneously into dorsal flanks of nude mice. Tumour tissues were formalin-fixed, paraffin-embedded, and stained with haematoxylin/eosin.
those Fas-L ribozyme-carrying glioma cells. Down-regulation of Fas-L by Fas-L ribozyme caused an increase in growth rate, improved cell viability and speeded up tumour formation indicating that the endogenous tumour Fas-L might not be growth promoting for glioma cells in all. Recently, we had observed that delivering Fas-L ribozyme into murine melanoma or Ras-activated NIH3T3 malignant cell lines also effectively reduced apoptosis in those cells (our unpublished data). It seems that the suicidal effect triggered by Fas-L occur widely in many malignant cells.

The proinflammatory effect of Fas-L has been recognized for days and is attributed partly to local recruitment and activation of neutrophils (Seino et al, 1997; Chen et al, 1998). Apoptotic body by itself is a potent chemotactic agent for immune cells (Horino et al, 1998). Besides, when phagocytes engulf apoptotic body, they can effectively initiate T-cell immunity (Chattergoon et al, 2000). Therefore, the Fas-L ribozyme-associated suppression in tumour-infiltrating cells in nude mice observed in our system could be due to reduced amount of apoptotic body generated by Fas-L ribozyme- carrying cells. Alternatively, Fas/Fas-L engagement between tumour and immune cells in subcutaneous environments will trigger survival favouring signal for cells constituting innate immunity as those cases reported for T cells or tumours (Alderson et al, 1993, Owen-Shaub et al, 1994). This possibility waits to be proven by additional study. It is noteworthy that U-373MG, U-118MG and their derivatives tested did not form large tumours in nude mice. It substantiates the effectiveness of the remaining innate immunity in nude mice for tumour control and partly explains the negative correlation between cell infiltration and tumour size in this study. Taken together, Fas-L ribozyme could modify the tumorigenesis of human glioma cells in nude mice by several ways. First, Fas-L ribozyme accelerated tumour cell growth by improving cell viability. Second, Fas-L ribozyme reduced the innate immune reaction against tumour.

The appearance of glioma cells in the liver of tumour-bearing mice was an unexpected finding. Even though tumour nodules were not detected grossly in the liver, EGFP-expressing tumour cells were detectable in this tissue. The glioma cells in liver lesion were with atypical shape, mixed with apoptotic hepatocytes and numerous inflammatory cells indicating rigorous local immune responses. Growth of Fas-L+ tumour cells in syngeneic murine host has been shown to induce toxicity in Fas+ organs probably through the production of soluble Fas-L (Zeytum et al, 2000). Hepatocytes are known to express Fas and are very sensitive to Fas-L-mediated cytotoxicity (Ogasawara et al, 1993; Rensing-Ehl et al, 1995). The expression of Fas-L in human colon cancer has been correlated with the hepatic metastasis and the apoptosis of hepatocytes in tumour lesion (Shiraki et al, 1997). In our study model, Fas-L ribozyme reduced the severity of liver damage in tumour-bearing mice. Therefore, the killing of hepatocytes in glioma-bearing mice was obviously mediated by a contact with
either metastatic tumour cells or the infiltrating immune cells. Overall, this work using a loss-of-function model appears to complement previous Fas-L overexpression study and implies a possible immune-stimulating function of Fas-L.

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