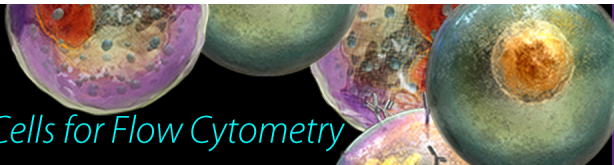


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# Fas Ligand and Fas Are Expressed Constitutively in Human Astrocytes and the Expression Increases with IL-1, IL-6, TNF- $\alpha$ , or IFN- $\gamma$

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Fas ligand (FasL) and Fas are mediators of apoptosis, which are implicated in the peripheral deletion of autoimmune cells, activation-induced T cell death, and cytotoxicity mediated by CD8<sup>+</sup> T cells. Fas is also believed to be involved in several central nervous system diseases, but until now, the effector cells expressing FasL in the brain have not been identified. We investigated the expression levels of Fas and FasL with the stimulation of cytokines and the possible effector cells targeting Fas-bearing cells. Our data demonstrated that: 1) FasL is expressed constitutively on astrocytes taken from a fetus or an adult and that its expression increases when these cells are treated with IL-1, IL-6, or TNF- $\alpha$  in which the pretreatment of IFN- $\gamma$  triggers astrocytes to express more FasL; 2) astrocytes induce apoptosis in MOLT-4 cells through FasL; 3) Fas is also expressed constitutively and is up-regulated by IL-1, IL-6, or TNF- $\alpha$  in which the pretreatment of IFN- $\gamma$  triggers astrocytes to express more Fas; 4) apoptosis occurs when fetal astrocytes are treated with agonistic anti-Fas IgM Ab after culture with IFN- $\gamma$  and TNF- $\alpha$ ; and 5) TNF-related apoptosis inducing ligand is up-regulated in fetal astrocytes with stimuli of IL-1 or TNF- $\alpha$ . These findings suggest a possible role of astrocytes in the induction of apoptosis in central nervous system diseases. *The Journal of Immunology*, 1999, 162: 1889–1895.

Interaction between Fas and Fas ligand (FasL)<sup>2</sup> is involved in cytotoxic effector mechanisms, clonal down-regulations of activated T cells and apoptosis of lymphocytes in immune privileged organs. Apoptosis is involved in several central nervous system (CNS) diseases. Confocal microscopic evaluation of fluorescein-labeled TUNEL-positive cells revealed the nuclei of glial cells with morphological characteristics of apoptosis in multiple sclerosis (MS; Ref. 1). Fas is believed to be involved in MS (2, 3), Alzheimer-type dementia (4, 5), parkinsonian disease (6), and postischemic situations (7). In *lpr* or *gld* mice the mutations of Fas or FasL dramatically ameliorated clinical signs of experimental allergic encephalitis, which indicates that functional Fas and FasL are important in the progression of clinical disease (8).

Fas can be induced by TNF- $\alpha$  and IFN- $\gamma$  in glial cells (9, 10), but Fas cannot be detected in uninjured brain cells (7, 10). In contrast to Fas, the expression of FasL is much more restricted and often requires cell activation (11). In the brain, FasL is expressed in oligodendroglial cells in multiple sclerotic plaque (2, 12) and in parenchymal microglia in mice with experimental allergic encephalitis (12, 13).

Astrocytes are the most prominent glial cells of CNS. Processes of astrocytes expand to the surface of CNS to constitute a superficial glial membrane and this may or may not encapsulate neurons. Hypertrophy of astrocytes has been reported in ischemic necrosis (14) and parkinsonian animals (15). In addition, apoptotic astrocytes and oligodendroglial cells have been found in MS (16).

To address the effector cells targeting Fas-bearing cells in the brain, the expression levels of Fas and FasL were investigated with the stimulation of inflammatory cytokines.

## Materials and Methods

### *Culture of astrocytes and induction of apoptosis*

Fetal astrocytes were isolated from three human fetuses (one was 20 wk old and two were 25 wk old) while adult astrocytes were isolated from a 30-year-old female and a 40-year-old male. The samples were obtained from therapeutic abortion or surgery for epilepsy, but they were from normal brain tissue. All the samples showed similar results and the representative results were shown as data. Astrocytes were cultured in 5% FCS-DMEM (Life Technologies, Grand Island, NY) containing 1% nonessential amino acids (Sigma, St. Louis, MO). Culture medium was changed every week. The cultures were maintained up to 1.5 mo. The indirect fluorescence staining for glial fibrillary acidic protein revealed that most of the cultured cells (>99%) were astrocytes. For induction of Fas or FasL, the astrocytes were cultured with 100 pg/ml of IL-1 (Genzyme, Cambridge, MA), 300 U/ml of IL-2 (Genzyme), 250 U/ml of IL-6 (Genzyme), and 750–1500 U/ml of TNF- $\alpha$  (Genzyme) for 8 h (for RNA preparation) or 24 h (for immunostaining). A total of 100 ng/ml of PMA (Sigma) and 500 ng/ml of ionomycin (Calbiochem, Cambridge, MA) were added together. Astrocytes were pretreated with IFN- $\gamma$  (Genzyme) at 100 U/ml for 18 h before adding cytokines. Agonistic anti-Fas IgM mAb CH-11 (Medical and Biological Laboratories, Watertown, MA) was added for 48 h at a concentration of 250 ng/ml to induce apoptosis. All the results showed similar patterns in fetal astrocytes or adult astrocytes, respectively, and the representative data were shown.

For the induction of apoptosis in MOLT-4 cells in response to astrocytes, MOLT-4 cells were cocultured for 24 h with fetal astrocytes that were not treated with cytokines. The ratio of E:T cells were 1:1, 2:1, or 5:1.

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<sup>2</sup> Abbreviations used in this paper: FasL, Fas ligand; CNS, central nervous system; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling; TRAIL, TNF-related apoptosis-inducing ligand; MS, multiple sclerosis.

Target cells were harvested carefully and the assay for DNA fragmentation was done. The staining for annexin V and propidium iodide was also performed. During the induction of apoptosis, cells were cultured in 5% FCS-DMEM (Life Technologies).

For the induction of apoptosis in fetal astrocytes or LN 215 cells in response to CH-11, target cells were cultured in both 750 U/ml of TNF- $\alpha$  and 100 U/ml of IFN- $\gamma$  for 18 h. Then target cells were cultured in serum-free DMEM containing 250 ng/ml of CH-11. After 6, 12, and 24 h, DNA was separated. LN 215 is an astrocytoma cell line and expresses Fas, which is confirmed by RT-PCR (data not shown). LN 215 cell were kindly provided by Dr. E. G. Van Meir (Department of Neurosurgery, Laboratory of Tumor Biology and Genetics, Lausanne, Switzerland). Jurkat cells were used as the positive control.

#### RNase protection assay, RT-PCR, and Southern blot analysis

Total RNA was isolated by a RNeasy kit (Qiagen, Santa Claris, CA). RNase protection assay for Fas and TNF-related apoptosis inducing ligand (TRAIL) was done using RiboQuant multiprobe RNase protection assay kit (PharMingen, San Diego, CA) with 10  $\mu$ g of total RNA.

Total RNA, 9  $\mu$ g, was used to synthesize cDNA with 0.1 OD of random hexamer (Pharmacia, Uppsala, Sweden) and 200 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies). RT-PCR was done using primers 5'-Cgg Agg ATT gCT CAA CAAC-3', 5'-TTg gTA TTC Tgg gTC Cg-3' for Fas; and 5'-ATg TTT CAg CTC TTC CAC CTA CAg AAg gA-3', 5'-CAG AgA gAg CTC AgA gAC gTT gAC-3' for FasL. RT-PCR controls included mutant forms of Fas DNA or  $\beta$ -actin. The conditions for PCR were as follows: denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min. PCR buffer conditions were 10 mM Tris-HCl (pH 10), 2.0 mM MgCl<sub>2</sub>, and 50 mM KCl with 1.25 U of *Taq* polymerase (Bioneer, Taejeon, Korea). After 20–21 cycles, an additional extension at 72°C for 10 min was conducted. For quantitative RT-PCR, the mutant forms of Fas and  $\beta$ -actin were constructed using a kit (Clontech, Palo Alto, CA). Southern blotting was done using an enhanced chemiluminescence kit (Amersham, Little Chalfont, U.K.) after the hybridization of FasL, Fas, and  $\beta$ -actin probes.

#### Viability assay

A total of 100  $\mu$ l of 0.3 mg/ml  $\beta$ -nicotinamide-adenine dinucleotide phosphate (NADH), and 25  $\mu$ l of 22.7 mM pyruvate was added into culture supernatant or remaining cells treated with Triton X-100. OD was read under A<sub>340</sub> immediately.

#### Fluorescence staining, flow cytometry analysis, and annexin V staining

For indirect fluorescence staining of anti-glial fibrillary acidic protein mAb (Sigma) and anti-FasL mAb, NOK-1 (PharMingen) were applied for 30

min at 4°C for 1  $\times$  10<sup>5</sup> astrocytes. Then anti-mouse rabbit serum, conjugated with FITC or phycoerythrin (Becton Dickinson, San Jose, CA), were treated. The cells were observed by the confocal microscope (Leica, Heidelberg, Germany) at  $\times$ 100 or  $\times$ 200 magnification. For flow cytometric analysis, astrocytes were detached by trypsinization and 1  $\times$  10<sup>5</sup> cells were stained with anti-Fas mAb, ZB4 (Becton Dickinson), and analyzed by FACStar (Becton Dickinson). Annexin V-FITC (Trevigen, Gaithersburg, MD) was used to stain apoptotic cells and propidium iodide (Sigma) was used to stain dead cells.

#### DNA fragmentation

Cells were treated by lysis buffer (200 mM HEPES (pH 7.5), 2% Triton X-100, 400 mM NaCl, 20 mM EDTA) and were digested with RNase A for 45 min. Samples were extracted with phenolchloroform and precipitated by ethanol. DNA electrophoresis was performed using 1.8% agarose gels.

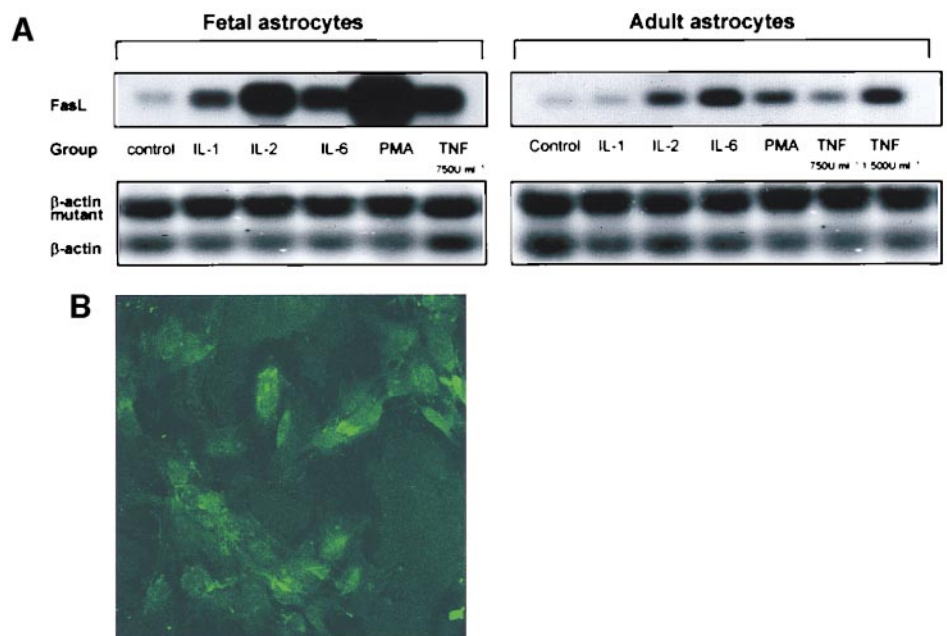
## Results

### FasL is expressed constitutively on astrocytes and is up-regulated with IL-1, IL-6, and TNF- $\alpha$

To determine the expression of FasL, quantitative RT-PCR and the subsequent Southern blotting of PCR products were performed using primary cultures of astrocytes from fetal and adult brains. As a result, FasL was expressed on fetal and adult astrocytes (Fig. 1A). The immunofluorescence staining for fetal astrocytes revealed the constitutive expression of FasL (Fig. 1B). To address the effect of cytokines, astrocytes were treated with IL-1, IL-6, or TNF- $\alpha$ , respectively, for 24 h. We observed that IL-1, IL-2, IL-6, TNF- $\alpha$ , and PMA up-regulated the expression of FasL (Fig. 1A).

### FasL of astrocytes induces apoptosis in MOLT-4 cells

To address the induction of apoptosis by FasL in astrocytes, MOLT-4 cells were reacted with fetal astrocytes and were analyzed by flow cytometry. Annexin V staining showed apoptotic cells at the proportion of 24.7%, 30.5% and 37.8% in MOLT-4 cells at the E:T ratio of 1:1, 2:1, and 5:1, respectively (Fig. 2, A and B). Propidium iodide staining showed 6.1%, 7.2%, and 8.6% at the same E:T ratio (Fig. 2A). Negative control showed 16.4% of annexin V positive cells. Positive control showed 39.7% of annexin V positive cells and 12.3% of propidium iodide positive cells.



**FIGURE 1.** Expression of FasL in astrocytes. *A*, Southern blot analysis of quantitative RT-PCR products was performed in fetal astrocytes and adult astrocytes. Hybridization of the same blot with quantitative RT-PCR products of  $\beta$ -actin is shown at the bottom to demonstrate the starting RNA in each lane. *B*, Indirect immunostaining of FasL in fetal astrocytes. Fetal astrocytes were pretreated with IFN- $\gamma$  (100 U/ml) for 18 h and were stimulated with TNF- $\alpha$  (750 U/ml) for 24 h.

*Fas* is expressed constitutively on astrocytes and is up-regulated with *IL-1*, *IL-6*, and *TNF- $\alpha$*

To determine the expression of Fas, RNase protection assay was performed in fetal astrocytes and adult astrocytes. Quantitative RT-PCR and a subsequent Southern blotting of PCR products was also performed. Fas was also constitutively expressed on both fetal astrocytes and adult astrocytes (Fig. 3, A–C).

TNF- $\alpha$  induced Fas transcription clearly in fetal astrocytes and to a lesser degree in adult astrocytes in a dose-dependent manner (from 750–1500 U/ml) (Fig. 3, B and C). Pretreatment of IFN- $\gamma$  also up-regulated the expression of Fas in fetal astrocytes (Fig. 3B) and in adult astrocytes (Fig. 3C). The expression of Fas by cultured astrocytes was identified by indirect immunostaining (Fig. 4A). Flow cytometric analysis revealed most cells express Fas very weakly, but the median value shifted to the right in comparison with a negative control (Fig. 4B). And the expression of Fas was enhanced with the treatment of cytokines, especially with TNF- $\alpha$  (Fig. 4C).

*Membrane deterioration, but not DNA fragmentation, occurs with stimulation of IFN- $\gamma$ , TNF- $\alpha$ , and CH-11*

To address the function of Fas on astrocytes, a viability assay was done by measuring lactic dehydrogenase in culture supernatant and compared with that from viable cells. In the control group, cell death did not occur (Fig. 5A). Annexin V staining showed few apoptotic cells with the treatment of agonistic Ab, CH-11, by flow cytometric analysis (Fig. 5B, left panel). However, cell death and membrane deterioration occurred in fetal astrocytes when they were treated with TNF- $\alpha$ +IFN- $\gamma$ /CH-11. Immunofluorescence analysis of annexin V staining showed dying astrocytes (Fig. 5B,

right panel). Cell membranes of large dying reactive astrocytes were stained positive for annexin V (Fig. 5C). But DNA fragmentation was not detected at 6, 12, and 24 h (Fig. 5D) in fetal astrocytes even after treatment with CH-11. An astrocytoma cell line, LN 215, and Jurkat cells showed the DNA laddering.

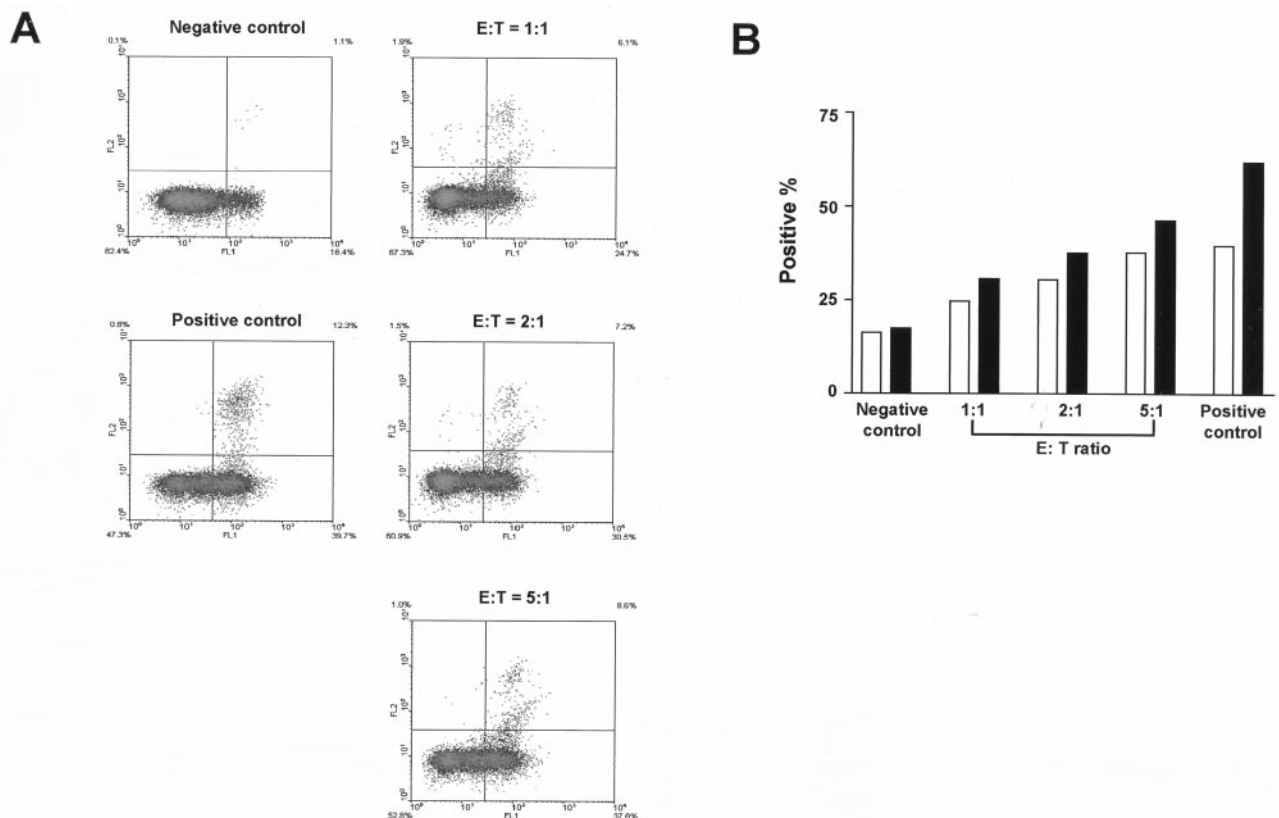
*TRAIL is up-regulated in the presence of IL-1 or TNF- $\alpha$*

TRAIL is expressed both in fetal astrocytes and adult astrocytes. The expression of TRAIL could be detected by RNase protection assay (Fig. 6). The up-regulation of TRAIL in fetal astrocytes was shown only in IL-1 or TNF- $\alpha$ -treated groups. In adult astrocytes, the up-regulation of TRAIL was not found.

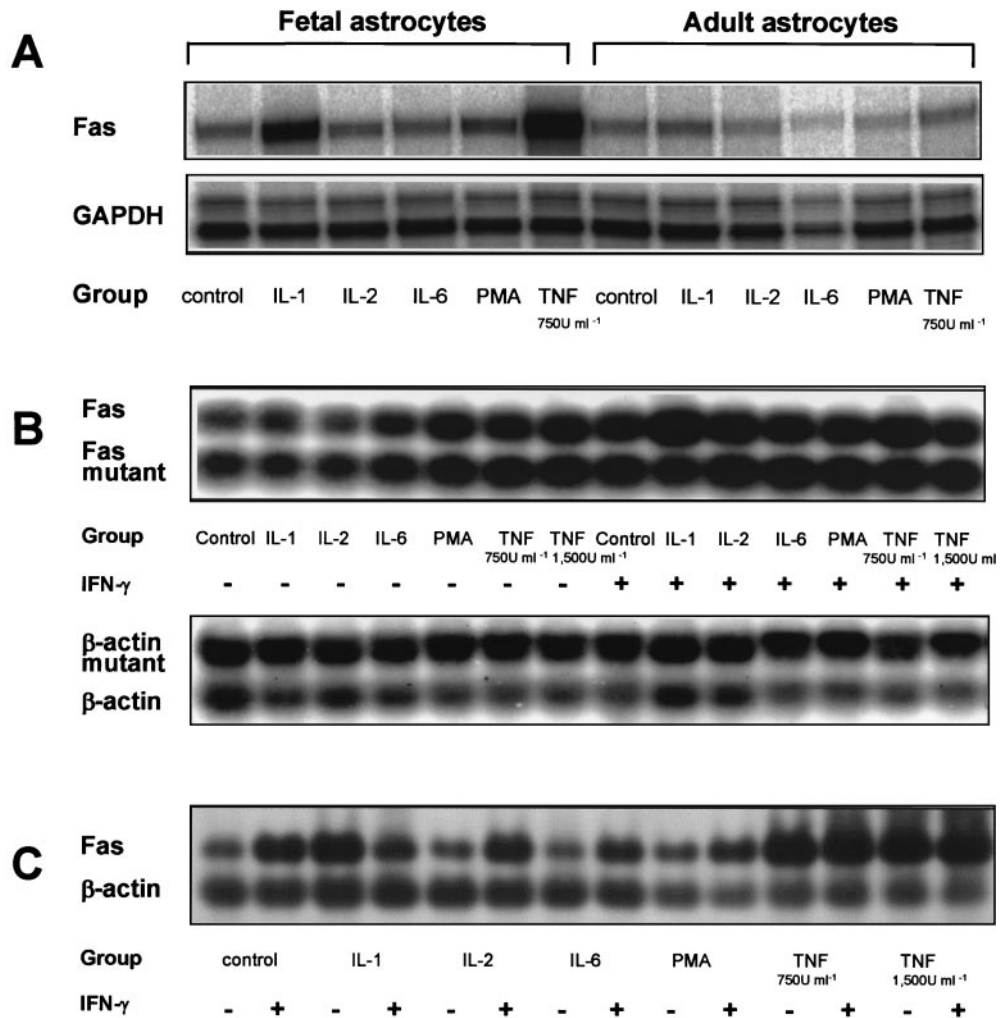
## Discussion

We have demonstrated that cultured fetal astrocytes and adult astrocytes constitutively express FasL and Fas. The presence of FasL on the surface of astrocytes suggests that the ligand may play a role as an effector molecule or that perhaps soluble FasL may be released from astrocytes to mediate pathological or physiological functions in CNS. It has been reported that glial cells in the MS brain and in brain tumors express FasL (15–17), which suggests that the interaction of Fas on target cells may contribute to the control of immune defense in the brain.

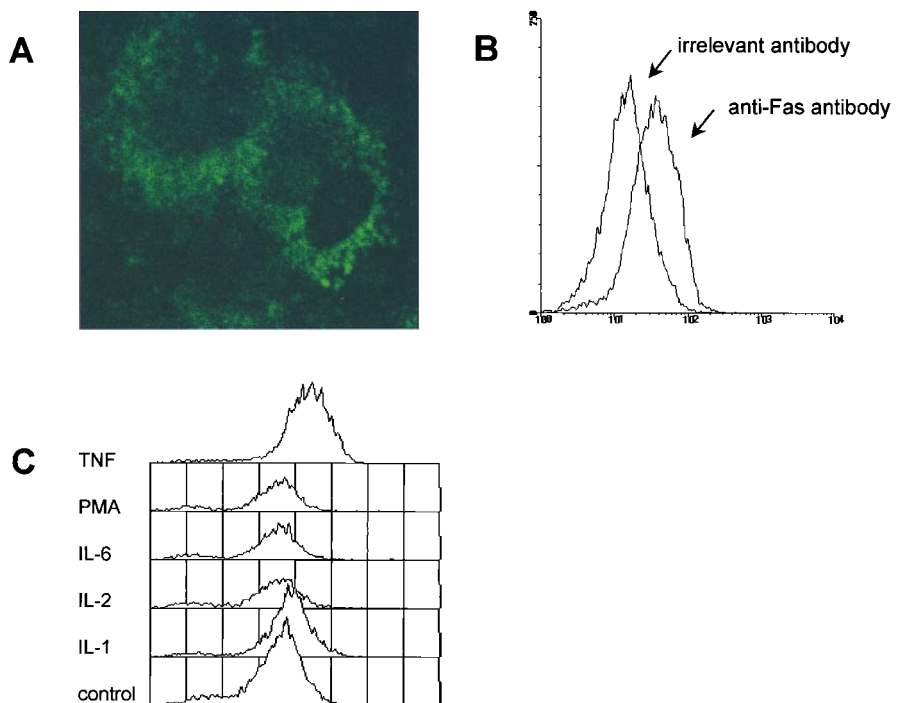
The expression of Fas or FasL may be the result of a unique phenomenon during fetal development because normal adult astrocytes were reported not to express either Fas (16) or FasL (10, 17). Only astrocytoma cell lines (18–20) and human astrocytoma in vivo (19–22) have been known to express FasL, which delivers a death signal to Fas-bearing cells including infiltrating leukocytes and astrocytoma cells themselves. However, our study clearly



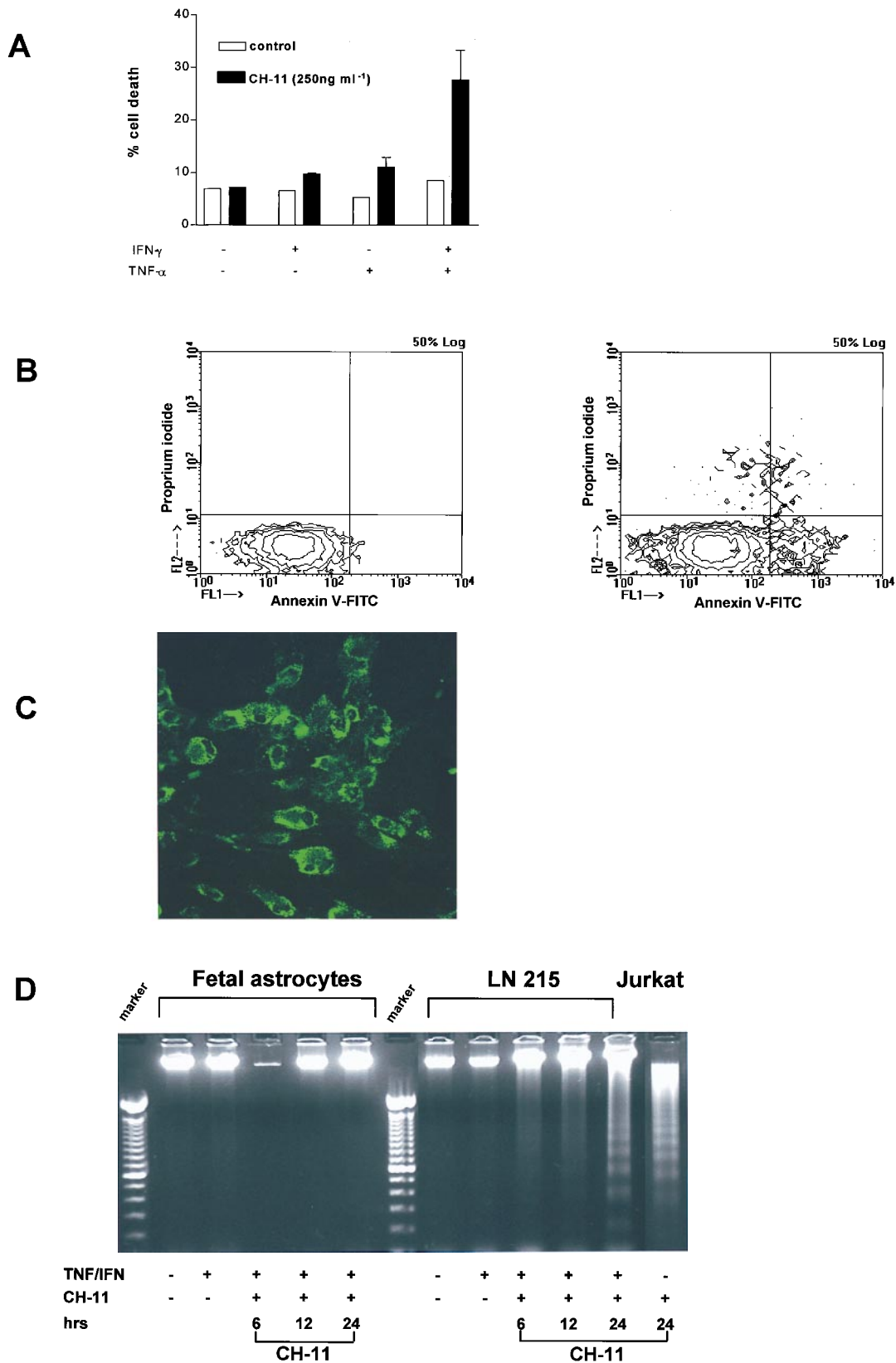
**FIGURE 2.** Induction of apoptosis in MOLT-4 cells. A, Target cells were cocultured with astrocytes at the E:T ratio of 1:1, 2:1, or 5:1 in 5% FCS-DMEM. A, After 24 h, MOLT-4 cells were stained with annexin V-FITC (FL1) and propidium iodide (FL2). B, Open bars represent the percentage of cells stained by annexin V. Filled bars represent the percentage of cells stained by both annexin V and propidium iodide.



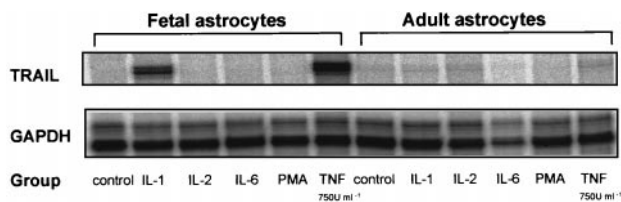
**FIGURE 3.** Expression of Fas in astrocytes. *A*, RNase protection assay using 10 μg total RNA was performed in fetal astrocytes and adult astrocytes with stimulation of IL-1, IL-6, and TNF-α. *B*, Quantitative RT-PCR using Fas mutant and β-actin mutant was done in fetal astrocytes with stimulation of IL-1, IL-6, and TNF-α. IFN-γ was pretreated in some groups. Southern blotting of PCR products was done. β-actin was used as an internal control. Hybridization of the same blot with β-actin is shown at the bottom to demonstrate the starting RNA in each lane. *C*, Semiquantitative RT-PCR using Fas mutant and β-actin mutant was done in adult astrocytes with stimulation of IL-1, IL-6, and TNF-α.



**FIGURE 4.** Flow cytometric analysis and immunostaining for Fas. *A*, Fetal astrocytes from the primary culture were stained by anti-Fas Ab without stimuli. *B*, Fetal astrocytes with stimulations of IL-1, IL-6, or TNF-α were stained by anti-Fas Ab. The control shows the cells stained with anti-Fas Ab in which Fas was expressed constitutively. *C*, Indirect immunostaining was done using anti-Fas mAb. Fetal astrocytes were pretreated with IFN-γ (100 U/ml) for 18 h and were stimulated with TNF-α (750 U/ml) for 24 h.



**FIGURE 5.** Viability assay, annexin V staining, immunostaining, and DNA laddering. *A*, For viability assay, lactic dehydrogenase activity in culture supernatant was measured and compared with those from viable cells treated with Triton X-100. *B*, Annexin V-FITC staining for astrocytes was done without stimulation (*left panel*) and with stimulation of TNF- $\alpha$  (750 U/ml) for 24 h after the pretreatment of TNF- $\alpha$  (750 U/ml) for 18 h (*right panel*). *C*, Direct immunostaining of astrocytes with annexin V was observed by confocal microscopy. Astrocytes were cultured with stimulation of TNF- $\alpha$  (750 U/ml) for 24 h after the pretreatment of IFN- $\gamma$  (100 U/ml) for 18 h, then agonistic mAb, CH-11, was added and the culture was maintained for 8 h. Large dying reactive astrocytes containing numerous internalized nuclei and their cytoplasm stained diffusely positive for annexin V. *D*, Fetal astrocytes or LN 215 cells were cultured in both 750 U/ml of TNF- $\alpha$  and 100 U/ml of IFN- $\gamma$  for 18 h. Then 250 ng/ml of CH-11 was added. After 6, 12, and 24 h, DNA laddering was observed. Jurkat cells were used as the positive control.



**FIGURE 6.** Expression of TRAIL in astrocytes. Astrocytes were cultured with stimulation of IL-1, IL-2, IL-6, or TNF- $\alpha$  for 8 h. Ten micrograms of total RNA was used for RNase protection assay.

showed that Fas and FasL are expressed in both fetal astrocytes and adult astrocytes during primary culture. The inflammatory cytokines, IL-1 and TNF- $\alpha$ , up-regulate the transcription of *fas*. The regulatory mechanism may occur by activating the transcription of NF- $\kappa$ B (23). Pretreatment of IFN- $\gamma$  also up-regulated the expression of Fas in fetal astrocytes and in adult astrocytes. In Hashimoto's thyroiditis, Fas is induced by IL-1 and this induction results in the loss of thyroid hormone-producing cells because FasL is expressed constitutively in thyroid tissue (24). IL-1 is also known to activate transcription of NF- $\kappa$ B (24). The expression of Fas by mesangial and tubular cells is increased with IFN- $\gamma$ , IL-1, and TNF- $\alpha$  (25). During pathologic conditions such as acute inflammation (ischemia and traumatic injury) or during chronic neurodegeneration (amyotrophic lateral sclerosis and scrapie), IL-1, IFN- $\gamma$ , and TNF- $\alpha$  are produced by invading lymphocytes, macrophages, or microglia (26). This may induce the expression of Fas by astrocytes. Whether NF- $\kappa$ B is directly involved in the transcriptional regulation of Fas or FasL needs to be clarified because the NF- $\kappa$ B binding site was recognized upstream of the FasL chromosomal gene (27). Flow cytometric analysis also revealed that most cells express Fas very weakly, but the expression of Fas was enhanced with the treatment of cytokines, especially with TNF- $\alpha$ .

Our data showed that cell death did not occur in the control group. Annexin V staining showed few apoptotic cells with the treatment of agonistic Ab, CH-11, by flow cytometric analysis. However, cell death and apoptosis occurred in fetal astrocytes when they were treated with TNF- $\alpha$ +IFN- $\gamma$ /CH-11, which was detected by immunofluorescence analysis of annexin V. These findings suggested that astrocytes are resistant to spontaneous apoptosis despite detectable levels of Fas and FasL expression. However, cell death and apoptosis occurred in astrocytes when they were treated with cytokines, especially TNF- $\alpha$  and IFN- $\gamma$ . So the Fas molecules on astrocytes may be functional, although the stimulation of cytokines is needed. Whether the same phenomenon occurs in vivo is not known at present. In our study, the membrane deterioration of astrocytes was confirmed by annexin V staining, but the DNA fragmentation was not detected. One other recent study showed that the evidence of Fas-engaged apoptosis in fetal astrocytes was not found even though Fas was expressed constitutively (28). In that study, only the TUNEL assay was done to detect apoptosis so the early event-like membrane change could not be ruled out. In astrocytes, cell death without DNA fragmentation may occur. One other report showed that Fas and FasL gene expression were detectable in myelin basic protein-reactive T cells and in glial cells and that when T cells interacted with glial cells, death could be induced in both cells (29). Cell lysis by Fas engagement without DNA fragmentation was reported on adult human oligodendroglia (12). Such a phenomenon may also occur in cultured human astrocytes and the discrepancy of membrane deterioration and DNA fragmentation in cell death of astrocytes remains to be clarified. The role of cytokines is not identified in the

induction of apoptosis but other mechanisms such as *c-myc*-related apoptosis (30) should also be considered.

FasL expressed in astrocytes are functional in our study. Fetal astrocytes induced apoptosis in MOLT-4 cells in the E:T ratio-dependent manner. MOLT-4 cells express Fas but not death receptor 4 (DR4), the TRAIL receptor 1, which was confirmed by RT-PCR (data not shown).

The expression of TRAIL, which shares the highest degree of homology among the TNF family (31), was up-regulated by IL-1 and TNF- $\alpha$ . This finding suggests that another proximal signaling pathway may exist in addition to the Fas signal to transmit the death signal in the brain. This phenomenon occurred only in fetal astrocytes, not in adult astrocytes. Immune cells including lymphocytes are known to express TRAIL-R3, the decoy receptor, and it has been suggested they may be resistant to TRAIL-induced apoptosis (32, 33). However, the decoy activity of TRAIL-R3 appears to be transient (35). TRAIL-R4, which shows widespread tissue distribution including thymocytes and splenocytes, also binds TRAIL and inhibits TRAIL-induced apoptosis. Still, the phosphatidylinositol 1 (PS1) cell line expressing TRAIL-R4 is fully susceptible to TRAIL-induced apoptosis (35). It may be possible to deduce that TRAIL has a role in shielding CNS from the immune effector system during fetal development.

Finally our findings indicate that the Fas-FasL interaction of astrocytes may have an important role in the state of immune privilege or in the pathogenesis of various CNS diseases when IL-1, IL-6, IFN- $\gamma$ , or TNF- $\alpha$  are involved.

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