Expression of ICAD-L and ICAD-S in Human Brain Tumor and Its Cleavage upon Activation of Apoptosis by Anti-Fas Antibody

Jun Masuoka, Tetsuya Shiraishi,[1] Makoto Ichinose, Toshihiro Mineta and Kazuo Tabuchi

Department of Neurosurgery, Saga Medical School, 5-1-1 Nabeshima, Saga 849-8501

ICAD/DFF is a downstream molecule of caspases, participating in nuclear DNA fragmentation during apoptosis. ICAD/DFF binds CAD/DFF40 and inhibits its DNase activity. ICAD/DFF has two alternative isoforms, long isoform (ICAD-L/DFF45) and short isoform (ICAD-S/DFF35). We have studied the presence and functional status of ICAD/DFF in human glioma cell lines. All cell lines tested expressed both ICAD-L and ICAD-S. When the cultured glioma cells were exposed to anti-Fas antibody, these isoforms were degraded prior to the fragmentation of the nuclear DNA, indicating that the ICAD/DFF expressed in cultured glioma cells was potentially functional. In primary brain tumors and normal brain tissues, there was a difference in the expression level between ICAD-L and ICAD-S. In glioblastomas, ICAD-S was more abundant than ICAD-L. In contrast, ICAD-L was more abundant than ICAD-S in medulloblastomas. The present findings suggest that primary brain tumors and normal brain constitutively express ICAD/DFF, and that there is a difference between the expression levels of ICAD-L and ICAD-S.

Key words: ICAD/DFF — CAD/DFF40 — Brain tumor — Apoptosis

Apoptotic cell death plays an important role in physiologic and pathologic processes.[1, 2] Apoptosis can be induced by a variety of insults, such as death factors, chemotherapeutic drugs, ultraviolet or X-ray irradiation, and growth factor withdrawal.[3–6] The morphological changes of a cell during apoptosis include condensation of nuclei and cytoplasm, cytoplasmic membrane blebbing, and fragmentation of the cell into apoptotic bodies.[7, 8] Nuclear DNA fragmentation into nucleosomal levels is believed to be a biochemical hallmark of apoptosis.[9, 10] Recent studies have clarified that DNA fragmentation factor (DFF) is actually involved in nuclear DNA fragmentation during apoptosis. ICAD/DFF is a downstream molecule of caspases, participating in nuclear DNA fragmentation during apoptosis. ICAD/DFF binds CAD/DFF40 and inhibits its DNase activity. ICAD/DFF has two alternative isoforms, long isoform (ICAD-L/DFF45) and short isoform (ICAD-S/DFF35).[11–16] Caspases, which are activated during the apoptotic process, can cleave ICAD-L, and the CAD released from its inhibitor ICAD-L initiates the DNA fragmentation of a cell.[11, 12] Since blockades in the pathways responsible for apoptotic cell death can directly contribute to neoplastic transformation and resistance to chemotherapy,[17, 18] it is possible that defects in the apoptotic signaling pathway might exist in neoplastic cells.

The Fas-Fas ligand system (also known as Apo-1/Apo-1 ligand or CD95/CD95L) has been recognized as a major signaling pathway for the induction of apoptosis in lymphoid cells.[19, 20] Fas ligand, a member of the tumor necrosis factor superfamily, is expressed in activated T cells in addition to natural killer cells, and induces apoptosis in target cells by binding to its receptor, Fas.[3, 21] Although we have shown that anti-Fas antibody unequivocally induces apoptosis in the glioma cells expressing Fas,[22] it is unknown whether ICAD/DFF contributes to Fas-induced apoptosis in glioma cells. To address this question, we investigated the expression of ICAD/DFF in human glioma cell lines, surgical specimens of primary brain tumors and normal brain tissues, and examined whether ICAD/DFF functioned during apoptosis in glioma cells.

MATERIALS AND METHODS

Cell culture   Seven human glioma cell lines (T98G, U87, U118, U138, U251 A172 and U373) were used in this study. T98G, U87, U118, U138 and U373 were obtained from American Type Culture Collection (Rockville, MD). A172 was obtained from Health Science Research Resources Bank (Osaka). U251 was purchased from Riken Cell Bank (Tsukuba). All cell lines were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and 100 units/ml penicillin-streptomycin in a humidified incubator at 37°C and 5% CO_2.
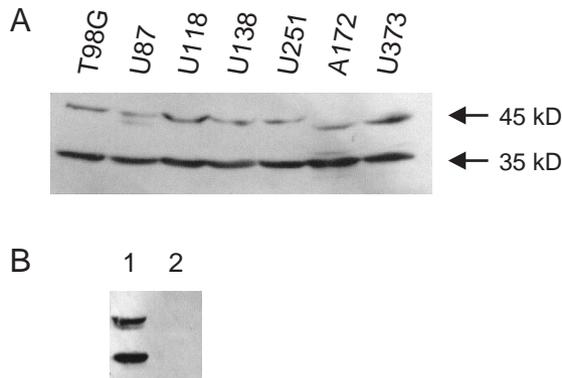
Tissue samples   Primary brain tumor specimens and adjacent unaffected brain tissues were obtained at the time of surgery and were snap-frozen in liquid nitrogen for protein extraction. In addition, portions of unaffected brain and tumor tissues were fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemical as well as histological examinations. Histological evaluation

was based on the WHO classification[23] and included 8 glioblastomas, 7 medulloblastomas and 8 meningiomas.

Reagents Murine monoclonal anti-human ICAD antibody (clone 6B8), anti-human Fas antibody (clone CH-11) and recombinant human caspase-3 were obtained from Medical & Biological Laboratories Co. (Nagoya).

Western blot analysis Protein was extracted from either cultured cells or tissue samples with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl, 1% NP40, 20 mg/ml aprotinin, 20 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation for 10 min at 14 000 rpm at 4°C, the lysate supernatant was assayed for protein concentration using a BCA protein assay kit (Pierce, Rockford, IL). Samples were electrophoresed on 10% or 15% SDS-polyacrylamide gels and subsequently transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) by electroblotting. After the electroblotting, non-specific binding was blocked by incubation with 5% non-fat dry milk in TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.05% Tween 20] overnight at 4°C. After washing in TBST, the membranes were probed with anti-ICAD antibody diluted 1:1000 in TBST for 1 h at room temperature. The membranes were then washed with TBST and incubated for 1 h at room temperature with anti-mouse immunoglobulin peroxidase-conjugated antibody. After extensive washing, protein bands were visualized using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) and quantified using NIH Image 1.61 software. Values are shown as the mean±SE.

Immunofluorescent staining T98G cells were fixed in 4% paraformaldehyde for 10 min, washed with phosphate-buffered saline (PBS) three times, and permeabilized with 0.1% Triton X-100 in PBS for 20 min. Following incubation with PBS containing 10% normal goat serum for 20 min, cells were further incubated with anti-ICAD antibody diluted 1:40 in PBS supplemented with 1% bovine serum albumin for 30 min. After the cells were washed three times with PBS, the antibody was labeled with anti-mouse IgG-fluorescein (Boehringer Mannheim Biochemica, Mannheim, Germany) diluted 1:40 in PBS for 30 min. A confocal laser scanning microscope (Fluoview IX70; Olympus, Tokyo) was used for fluorescence microscopy. All procedures were carried out at room temperature.

Immunohistochemical staining After deparaffinization and rehydration in graded alcohols, the sections were
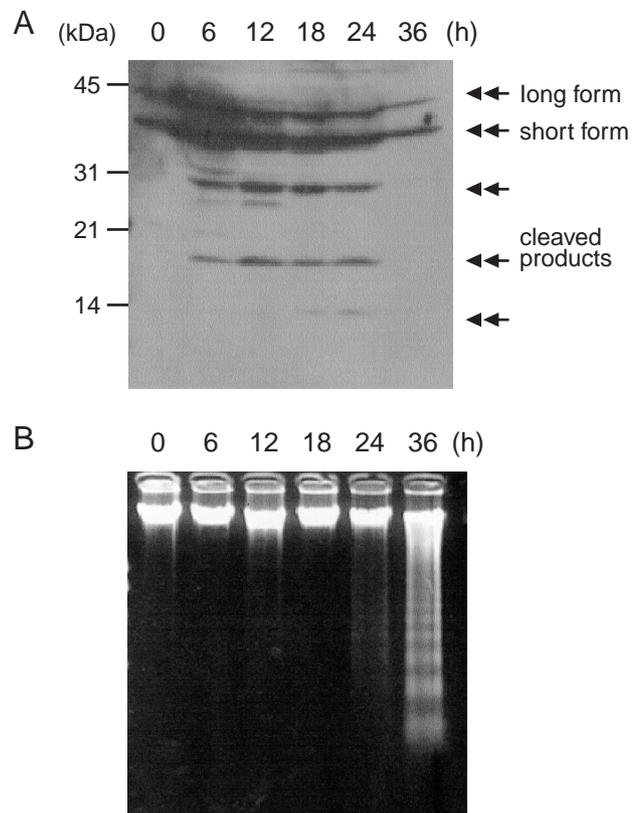
Fig. 2. DFF45/ICAD cleavage in T98G cells undergoing apoptosis. A: Western blot analysis of T98G cells treated with anti-human Fas antibody. Equal amounts of total protein (80 μg/lane) electrophoresed on 15% SDS-PAGE. B: DNA fragmentation assay. Ten micrograms of DNA purified from each sample loaded onto 2% agarose gel. The two bands of ICAD/DFF were cleaved into fragments of 30 and 17 kDa after 6 h treatment with anti-Fas antibody.

Fig. 1. Western blot analysis of DFF45/ICAD in human glioma cell lines. A: Equal amounts of total protein (50 μg/lane) were electrophoresed on 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide-gel electrophoresis). B: Total cell lysates from T98G cells incubated in the absence (lane 1) or presence (lane 2) of active recombinant caspase-3 at 37°C for 2 h. All glioma cell lines examined revealed two distinct bands, 35 and 45 kDa in size. When the cell lysate from T98G was incubated with active recombinant caspase-3, both bands disappeared.

heated and boiled for 10 min by microwaving in 10 m*M* citrate buffer, pH 6.0. To diminish endogenous peroxidase activity, each section was treated with 3% hydrogen peroxide for 15 min. After rinsing in PBS, the sections were blocked with 10% normal rabbit serum for 15 min. Anti-ICAD antibody diluted 1:200 in TBST was added to the sections and incubated overnight at 4°C. Non-immune mouse IgG was used as the negative control under equivalent conditions in place of the primary antibody. Immuno-histochemistry was performed using the streptavidin-biotin method according to the manufacturer's instructions [HISTOFINE SAB-PO(M) kit, Nichirei Corp., Tokyo]. Biotinylated rabbit anti-mouse antibody was used as the secondary antibody and diaminobenzidine was used as the chromogen. Sections were counterstained with hema-toxylin.

DNA fragmentation assay DNA fragmentation was ana-lyzed by a previously reported method.[22] In brief, T98G cells were continuously exposed to anti-Fas antibody (200 ng/ml) for various time periods. All cells (both attached and detached cells) were collected and then incubated at 37°C overnight in 500 μl of lysis buffer containing 100 m*M* Tris (pH 8.5), 5 m*M* EDTA, 200 m*M* NaCl, 0.2% w/v SDS, and 0.2 mg/ml proteinase K. DNA was precipitated with ethanol, treated with 200 μg/ml RNase A and the concentration of DNA was determined in terms of the UV absorbance at 260 nm. DNA (10 μg/lane) was loaded onto a 2% agarose gel, and electrophoresis was conducted at 50 V for 2 h. The gel was stained with 2 mg/ml ethidium bromide for 15 min, destained with distilled water for 1 h, and visualized under UV light.

RESULTS

Total cell lysates from the glioma cell lines were ana-lyzed for the presence of ICAD/DFF by western blotting with anti-human ICAD monoclonal antibody. As shown in Fig. 1A, all glioma cell lines examined revealed the two distinct bands, 35 and 45 kDa in size. The expression level of the 35 kDa band (ICAD-S) was significantly higher than that of 45 kDa band (ICAD-L). The ratio of ICAD-L to ICAD-S was 0.35±0.09 (mean±SE). To confirm that these two bands were ICAD/DFF, we tested the cleavage of these bands in the absence or presence of active recom-binant caspase-3, because ICAD/DFF is a substrate for caspase-3.[15, 16] When the cell lysate from T98G cells was incubated with the active recombinant caspase-3, both bands disappeared (Fig. 1B), suggesting that both repre-sented ICAD/DFF.

To confirm that the ICAD/DFF in glioma functions appropriately with apoptosis-stimulating agents, we treated T98G cells with anti-human Fas antibody (CH-11). In addition, we compared the time course of DNA degrada-tion with that of ICAD/DFF cleavage. T98G cells were incubated with 200 ng/ml of anti-Fas antibody and cell lysates were prepared at 6, 12, 18, 24 or 36 h following the antibody treatment. As shown in Fig. 2A, after a 6 h treatment with anti-Fas antibody, the two bands of ICAD/
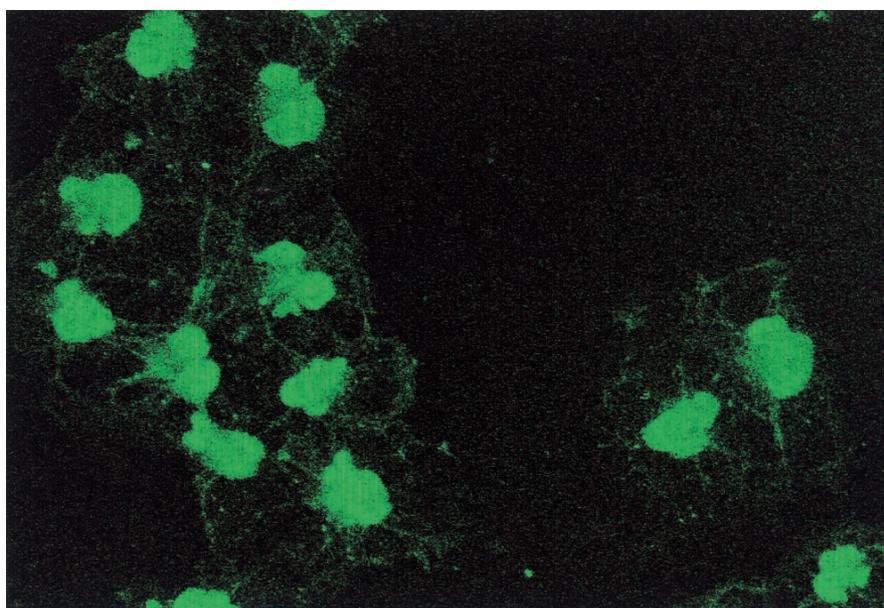

Fig. 3. Immunofluorescence staining of DFF45/ICAD in T98G cells. Immunoreactivity with the anti-DFF45/ICAD antibody is detected mainly in the nuclei and weakly in the cytoplasm. Original magnification, ×400.

DFF in T98G cells were cleaved into fragments of 30 and 17 kDa, respectively. At later time points, an 11 kDa fragment was observed. DNA fragmentation was demonstrated after 24 h of incubation with the anti-Fas antibody and the extent of fragmentation was most notable after 36 h of incubation (Fig. 2B).

The localization of ICAD/DFF in T98G cells was investigated using a confocal laser scanning microscope. Immunoreactivity with the anti-ICAD monoclonal antibody was detected mainly in the nuclei and weakly in the cytoplasm (Fig. 3).

In tissue samples, western blot analysis showed that ICAD/DFF was expressed in all primary brain tumors and normal brain tissues examined (Fig. 4A). However, the expression levels of ICAD-L and ICAD-S showed a significant variation according to the histological type of the tumors. In glioblastomas, the expression level of ICAD-S was higher than that of ICAD-L (Fig. 4B). In contrast, ICAD-L was more abundant than ICAD-S in medulloblastomas (Fig. 4C). The ratios of ICAD-L to ICAD-S (ICAD-L/S ratio) in glioblastomas and medulloblastomas were 0.57±0.16 and 2.26±0.35, respectively. In normal brain tissues (Fig. 4A) and meningiomas (Fig. 4D), these two bands were observed at similar levels (ICAD-L/S ratio: 0.97±0.08 and 1.41±0.09, respectively). To examine the *in situ* localization of ICAD/DFF, immunohistochemistry was carried out in primary brain tumors and normal brain tissues. Most of the tumor cells showed positive staining for ICAD/DFF (Fig. 5, C–E), whereas no staining was observed when non-immune mouse IgG replaced the anti-ICAD antibody in the staining protocol (Fig. 5F). Positive immunostaining was mainly observed in the nuclei, consistent with the finding of cultured cells obtained by immunofluorescence microscopy. In normal brain tissues, intense positive staining was observed in glial cells (Fig. 5A), and only weak or little staining in neurons (Fig. 5B). No difference in immunostaining intensity was found between normal glial and neoplastic cells.

DISCUSSION

Two basic apoptotic signaling pathways have been reported previously. The first involves the ligation of death receptors, resulting in the recruitment of adaptor proteins and caspase-8 as well as caspase-10, which then act to cleave and activate downstream caspases. In the second, cellular stress triggers mitochondrial release of cytochrome *c*, followed by activation of caspase-9. Both pathways converge on downstream effectors such as caspase-3, which leads to activation of DNA fragmentation factor.[24, 25]

In the present study, we demonstrated the expression and functional status of the ICAD/DFF in glioma cells. ICAD/DFF was expressed uniformly in all human glioma cell lines studied. Western blot analysis of glioma cell

lines demonstrated two distinct polypeptides, 35 and 45 kDa in size. Mouse ICAD exists in two isoforms, ICAD-L as the long isoform and ICAD-S as the short isoform, which are generated by alternative splicing.[12] These two forms possess two putative cleavage sites for caspase-3,[12] and the release of the C-terminal 11 kDa fragment is necessary to induce DNA fragmentation.[26] In the present study, ICAD/DFF in T98G cells was rapidly processed prior to the appearance of DNA fragmentation and finally cleaved to a small fragment of 11 kDa, which indicated
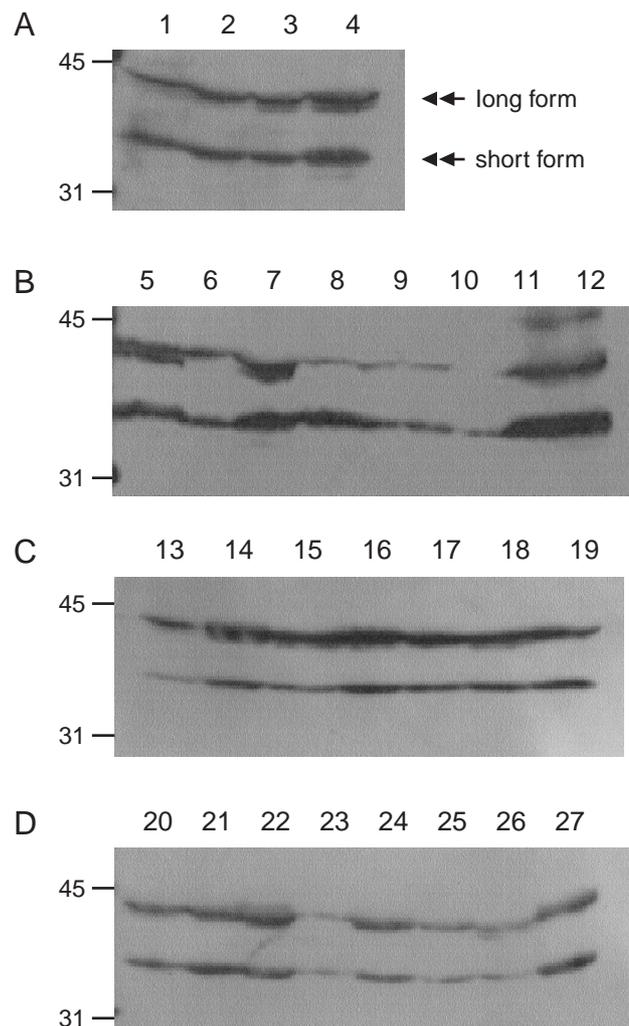

Fig. 4.   Western blot analysis of DFF45/ICAD in surgical specimens. Equal amounts of total protein (50 μg/ml) electrophoresed on 10% SDS-PAGE. A, normal brain tissues (lanes 1–4); B, glioblastomas (lanes 5–12); C, medulloblastomas (lanes 13–19); D, meningiomas (lanes 20–27). ICAD/DFF was expressed in all primary brain tumors. The expression levels of ICAD-L and ICAD-S varied according to the histological type of the tumors.
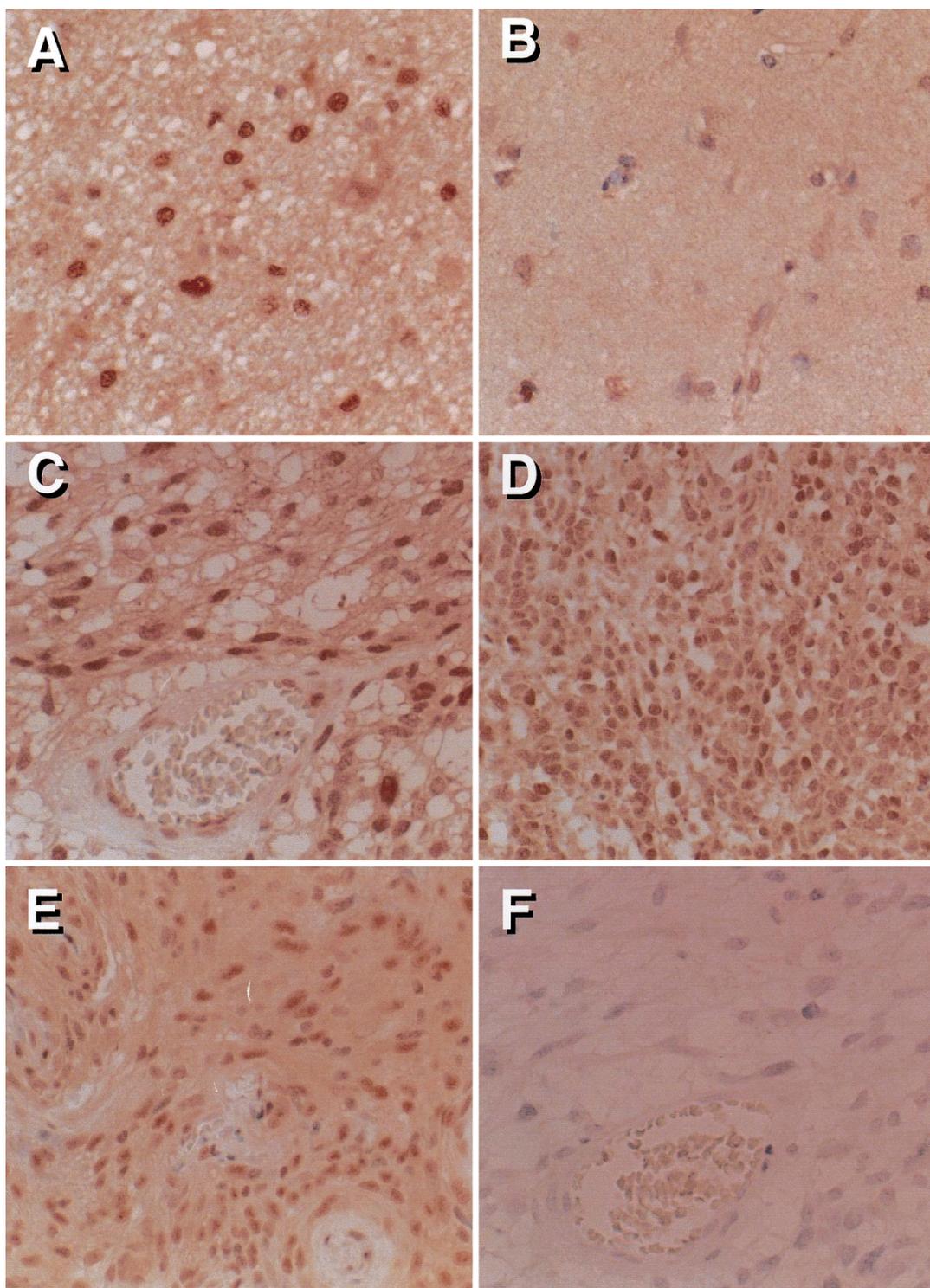
Fig. 5. Immunohistochemical analysis of DFF45/ICAD in normal brain and primary brain tumors. Nuclei are counterstained with hematoxylin. A, normal white matter; B, normal gray matter; C, glioblastoma; D, medulloblastoma; E, meningioma; F, control staining with non-immune mouse IgG. Original magnification, ×400. Positive immunostaining was observed mainly in the nuclei.

the release of the C-terminal 11 kDa fragment of ICAD-L. Therefore, it is strongly suggested that ICAD-L in glioma cells is functional.

A recent study reported functional differences between the long and short isoforms of ICAD/DFF.[27] ICAD-L inhibits the DNase activity of CAD/DFF40 by binding to it, and works as a chaperone for CAD/DFF40, facilitating its correct folding during synthesis. However, ICAD-S is thought to be a suppressor of ICAD-L function by competitive binding to CAD/DFF40, decreasing the ability to work as a chaperone for CAD/DFF40. The present findings demonstrated that glioma cell lines and tissue samples expressed both the long and short isoforms of ICAD/DFF, and interestingly, there was a difference between the expression levels of ICAD-L and ICAD-S. In the glioma cell lines and glioblastoma tissues, ICAD-S was expressed more abundantly than ICAD-L. In contrast, ICAD-L was more abundant than ICAD-S in medulloblastoma tissues.

No direct evidence is available that shows whether the ratio of these two distinct variants of ICAD/DFF could have an impact upon the cell death signal. Future studies should be planned to investigate the molecular mechanism of functional correlation between ICAD-L and ICAD-S.

The present study revealed that ICAD/DFF was mainly localized in the cell nuclei by both immunofluorescence (*in vitro*) and immunohistochemical (*in situ*) analyses. It was previously proposed that ICAD/DFF is a cytoplasmic factor and that a major function of ICAD/DFF is to restrain CAD/DFF40 in the cytoplasm in living cells.[11, 13]

The present observation of the nuclear localization of ICAD/DFF is consistent with the findings of an immunofluorescence study by Liu *et al.*[14] and those obtained using GFP-ICAD fusion protein by Samejima and Earnshaw,[28] but contrasts with the cytosolic retention model of Enari *et al.*[11] With regard to the discrepancy in the localization of ICAD/DFF, Liu *et al.* concluded that ICAD/DFF was isolated from cytosol because the protein leaked from nuclei during the extract preparation, and that ICAD/DFF was cleaved as a result of the nuclear translocation of active caspase-3. ICAD/DFF does not have a predicted nuclear localization signal,[12] suggesting that the nuclear ICAD/DFF may be transported into the nucleus in a complex with CAD/DFF40, which possesses a functional nuclear localization signal.[15]

In conclusion, we have demonstrated that primary brain tumors and normal brain constitutively express ICAD/DFF, and that there is a difference between the expression levels of ICAD-L and ICAD-S. Future studies should elucidate the clinical significance of differential expression of ICAD-L/ICAD-S.

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