The Exact Expression of Glial Fibrillary Acidic Protein (GFAP) in Trigeminal Ganglion and Dental Pulp

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Received September 28, 2001

Summary. The expression in various cell types of peripheral tissues of glial fibrillary acidic protein (GFAP), first discovered as an intermediate filament specific for astrocytes, remains controversial owing to numerous reports of a wide distribution for GFAP-immunoreactivity in various cells. The present study employed immunohistochemistry to investigate the precise expression of GFAP in the dental pulp and trigeminal ganglion of adult rats and wild-type mice as well as GFAP-knockout mice. The exhibition of GFAP-immunoreactivity in the trigeminal ganglion was further examined by a reverse transcription polymerase chain reaction (RT-PCR) technique, and in situ hybridization histochemistry using a specific cRNA probe prepared by us. The immunoreaction for GFAP was recognizable in the axons, Schwann cells, and the fibroblasts in the dental pulp of rats and wild-type littermate mice. However, mice with null mutations in the GFAP gene remained immunoreactive for GFAP in all these locations. Intense GFAP-immunoreactivity was found in a small number of satellite cells in the trigeminal ganglion in all animals examined in this study. RT-PCR analysis demonstrated bands for the GFAP gene corresponding to the length expected from the primer design in the samples of trigeminal ganglion and dental pulp. In situ hybridization histochemistry also showed intense signals for GFAP mRNA in some satellite cells of the trigeminal ganglion, but never in the neurons. These data suggest that the GFAP-immunoreactive molecules in the pulpal axons and fibroblasts react non-specifically with the polyclonal antibody and are probably a closely related type of intermediate filament.

Gliarial fibrillary acidic protein (GFAP) was first discovered as an astrocyte-specific intermediate filament (Eng et al., 1971; Eng, 1980). Since then, many reports have shown the expression of GFAP in Schwann cells such as enteric Schwann cells (Jessen and Mirsky, 1983; Fields and Yan, 1985), terminal Schwann cells at the neuromuscular junction following denervation (Georgiou et al., 1994), Schwann cells associated with the sciatic nerve (Yan and Fields, 1981; Eng, 1985; Kelly et al., 1992), and cultured Schwann cells (Bianchini et al., 1992). A wide distribution of GFAP also has been demonstrated in non-glial cells (Hatfield et al., 1984; Gard et al., 1985; Achttatter et al., 1986; Viale et al., 1991; Byers, 1995) by the use of immunostaining and immunoblotting analyses. In addition, the enteric nerves and the dental nerves innervating the dental pulp, periodontal ligament, and gingiva have been reported to show GFAP-immunoreactivity (Jessen and Mirsky, 1983; Fields and Yan, 1985; Jessen et al., 1990; Byers, 1995; Byers et al., 1995). These immunocytochemical data suggest diverse expression patterns for GFAP in the peripheral tissue. However, these GFAP-expression patterns appear to vary according to the kind of antibody used, species, and different functional and/or maturation stages of the cells (Fliegener and Liem, 1991; Xu et al., 1994), indicating that the precise distribution of GFAP in peripheral tissue remains undetermined.

The present study was thus undertaken to examine GFAP-immunoreactivity in the dental pulp of molars in normal rats and mice as well as GFAP-knockout

*This study was supported by Grants-in-Aid for Exploratory Research and Multi-disciplinary Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.
mice by immunocytochemistry. Further efforts were made to demonstrate the expression of the GFAP gene in the rat trigeminal ganglion and dental pulp by a reverse transcription polymerase chain reaction (RT-PCR) technique and in situ hybridization histochemistry using a specific cRNA probe that we prepared as dental nerves originate in the trigeminal ganglion (Arwill et al., 1973; Fink et al., 1975; Byers, 1984).

MATERIALS AND METHODS

All experiments were performed following the guidelines of the Niigata University Intramural Animal Use and Care Committee.

Immunohistochemistry for GFAP

Five male Wistar rats (about 80 g, 4 weeks old), three GFAP knockout, and two wild-type littermate mice (about 20 g) (Gomi et al., 1995) were used in this study. Under deep anesthesia with an intraperitoneal injection of chloral hydrate (400 mg/kg), the animals were transcardially perfused with physiological saline, followed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion fixation, the trigeminal ganglia, upper jaws, and cerebellum (used as control) were immersed in the same fixative for an additional 24 h. The upper jaws including the molars teeth were decalcified with 10% EDTA-2Na solution with slight agitation at 4°C for 3 weeks. Following immersion fixation and/or decalcification, all tissue blocks were soaked in 30% sucrose/phosphate buffered-saline (PBS) for cryoprotection. Serial sections were cut at a thickness of 20 μm in a cryostat (HM 500, Micron, Germany).

The sections were processed for the avidin-biotin-complex method (Issú et al., 1981). After the inhibition of endogenous peroxidase activity with absolute methanol containing 0.3% H2O2 for 30 min, they were incubated with a polyclonal antibody to GFAP (Dako Japan, Japan) overnight at room temperature. The sections were then reacted with two consecutive incubations with biotinylated goat anti-rabbit IgG (1:100; Vector, CA), followed with the ABC complex (ABC kits; Vector, CA) at room temperature for 90 min each. The antigen-antibody reaction sites were made visible by incubation with a mixture of 3,3'-diaminobenzidine (0.04%) and H2O2 (0.003%) in 0.05 M Tris-HCl buffer (pH 7.6). The immunostained sections were counter-stained with 0.03% methylene blue, dehydrated through an ascending series of ethanol, and cover-slipped with Permount (Fisher Scientific, NJ).

Immunospecificity control

Immunohistochemical controls for GFAP were performed by: 1) an absorption test; 2) replacing the primary antibody with non-immune serum or PBS; or 3) omitting the anti-rabbit IgG or the ABC complex. In an adsorption test, we used GFAP-antibody incubated overnight at 4°C with the antigen (10 μg/ml diluted antibody).

The immunocounter experiments did not show any specific immunoreaction in the rats or wild-type mice.

RT-PCR technique

Under deep anesthesia in the same manner, an additional five rats were decapitated. The upper molars were extracted, and then the dental pulp was extirpated from the pulp cavity after being cut into halves with a chisel and mallet. The trigeminal ganglia, dental pulp and cerebellum (used as control) were removed, immediately frozen in liquid nitrogen, and stored in a deep freezer at −80°C prior to use.

Total RNAs were isolated by a modified acid-guanidinium thiocyanate-phenol-chloroform method using TRIzol (Gibco-BRL, MD) reagents according to the manufacturer’s instructions. They were digested with RNase-free DNase I (Boehringer-Mannheim Biochemica, Germany). DNase I-treated total RNA (2.5 μg) was reverse transcribed using 200 units of Superscript II reverse transcriptase and 250 ng of random hexamer primers (Takara, Kusatsu, Japan) in a 25 μl reaction at 42°C for 60 min. cDNA (5 μl) was subjected to PCR in a 50 μl reaction containing 10× PCR buffer (5 μl), 4 μl dNTP, 100 μM of the primer set (1 μl), and 1 unit Taq DNA polymerase (0.25 μl). Synthetic oligonucleotides were purchased from Nihon Gene Research Laboratories (Sendai, Japan) corresponding to bases 13-32 (primer 1, 5'-GAAGCAGGCAAGATGAGGAC-3') and complementary to bases 276-296 (primer 2, 5'-GCTGTCCAGGAAGCGGCAAT-3') of the rat GFAP mRNA sequence (Feinstein et al., 1992). PCR thermal cycling was carried out in a PCR thermocycler (GeneAmp PCR system 9700, Applied Biosystem, Foster City, CA). Amplified products were separated by electrophoresis in 1.5% agarose gels, with DNA molecular marker 5 (Nihon Gene) used to provide size standards. The gels were stained with ethidium bromide and examined by ultraviolet transillumination (Funkoshi, Tokyo, Japan).

cRNA probe preparation of rat GFAP

Rat GFAP primers used for RT-PCR were also utilized to prepare a GFAP cRNA probe for in situ hybridization. Briefly, 5 μg purified rat GFAP cDNA
Fig. 1. Photomicrographs showing GFAP-immunoreactivity in the rat molar. The ABC method using a polyclonal antibody to GFAP. Counter-stained with methylene blue. a. The dental pulp of the normal rat molar contains many GFAP-immunoreactive structures. Arrows indicate the subodontoblastic nerve plexus of Raschkow. b. Higher magnification of pulp horn (asterisk) shown in a. GFAP-immunoreactive nerve fibers are seen to consist of thick and thin fibers. Frequently, Schwann cells (arrow) show GFAP-immunoreactivity in the dental pulp of the normal rat molar. D dentin. a: ×70, b: ×320

Fig. 2. GFAP-immunoreaction in the dental pulp of a mouse with a null mutation of GFAP gene. a. The dental pulp of a GFAP knockout mouse retains GFAP-immunoreactivity in the dental pulp. b. Higher magnification of the area in a shown by an asterisk. Many pulpal nerves show GFAP-immunoreactivity. c. Highly magnified view of a Schwann cell (arrow) immunopositive for GFAP in the dental pulp of a knockout mouse molar. D dentin. a: ×120, b: ×600, c: ×700
was amplified by Taq DNA polymerase using the PCR reaction described above. 284 bp fragments were purified from 3.0% TAE agarose gel with the Quantum Prep Gel Extraction Kit (BIO-RAD Lab., Hercules, CA) and subcloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA). Single-strand RNA probes labeled with digoxigenin-11-UTP were prepared from linearized templates which were transcribed from T7 or Sp6 promoters of the vector to generate sense and antisense probes using a DIG RNA Labeling kit (Boeringer-Mannheim Biochemica). The antisense probe did not have any significant homology with other nucleotide sequences, to the extent that we surveyed the databases (GenBank/EMBL).

In situ hybridization histochemistry
For in situ hybridization histochemistry, three additional rats were fixed in the same way as mentioned above. The upper molars were decalcified with 10% RNAase-free EDTA-2Na solution in the same way as above. The trigeminal ganglia and upper molars were embedded in paraffin by conventional methods. In situ hybridization histochemistry was performed according to Nomura et al. (1988) with slight modifications: deparaffinized sections were incubated with 2 μg/ml of proteinase K for 30 min at 37°C. They were then fixed with 4% paraformaldehyde in 0.1 M PB for 10 min at room temperature. Following acetylation, the sections were dehydrated by passage through PB and 70, 80, 90, and 100% ethanol for 15 sec each, and air-dried. The hybridization solution contained 50% deionized formamide, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1×Denhardt's solution, 600 mM NaCl, 10% dextran sulfate and 0.25% SDS. The digoxigenin-labeled antisense and sense probes were diluted with the hybridization solution to a final concentration of 0.1 μg/ml and then placed onto the sections. Incubation with hybridization probes was processed at 50°C for 16 h in a moisture chamber saturated with 50% formamide.

The signals for GFAP mRNA were detected by immunocytochemical technique. The hybridized sections were primarily reacted with a polyclonal antibody to digoxigenin (Boeringer-Mannheim Biochemica) diluted 1:250 overnight, and then consecutively incubated with biotinylated anti-rabbit IgG and HRP-conjugated streptavidin (Vector). An enzyme reaction was developed with 3,3'diaminobenzidine (0.04%) and H2O2 (0.003%) in 0.05 M Tris-HCl buffer (pH 7.6). The immunoreacted sections were counterstained with 0.03% methylene blue.

RESULTS
GFAP-immunoreactivity in dental pulp
No difference in immunostaining pattern was demonstrated with a polyclonal antibody to GFAP between normal rats and wild-type littermate mice.

Immunohistochemistry for GFAP was able to demonstrate many GFAP-immunopositive structures in the rat and mouse dental pulp (Fig. 1a).

The GFAP-positive nerve bundles ran along the blood vessels in the root pulp, but did not show extensive ramifications. As soon as GFAP-positive nerve bundles reached the coronal pulp, they began to arborize extensively to extend their branches throughout it. The ramified GFAP-immunoreactive nerve fibers gathered beneath the odontoblast cell layer to form the subodontoblastic nerve plexus of RASCHKOW (1835) (Fig. 1a). Some GFAP-immunopositive nerve fibers terminated near the odontoblast cell layer, but the majority climbed up between the odontoblasts towards the pulp-dentinal border (Fig. 1b).

However, these GFAP-nerve fibers did not penetrate into the predentin and dentin, which have been reported to receive a dense sensory innervation (Gunji, 1982; Maeda et al., 1987). In addition to pulpal nerve fibers, a small population of Schwann cells associated with the pulpal nerves also showed GFAP-immunoreactivity (Fig. 1b).

In contrast, the GFAP-immunoreactions in dental pulp of GFAP knockout mice did not differ from those in rats and wild type mice; the dental pulp of GFAP knockout mice retained GFAP-immunoreaction in the pulpal nerves (Fig. 2a, b), and some Schwann cells (Fig. 2c) were positive for GFAP-immunoreaction. However, their distributions and immunostaining patterns were identical to those in the rat and wild-type mouse.

GFAP-immunoreactivity in trigeminal ganglion and cerebellum
The neurons and trigeminal axons in the rat and wild-type mouse trigeminal ganglion did not show any GFAP-immunoreaction (Fig. 3a). However, some flat satellite cells surrounding the trigeminal neurons were positive for GFAP-immunoreaction, appearing as satellite rings (Fig. 3a). The Schwann cells located both in the trigeminal ganglion and the peripheral root lacked any immunoreaction for GFAP.

Immunostaining with a polyclonal antibody to GFAP could demonstrate numerous astrocytes in the cerebellum of normal rats and wild-type mice. These
Fig. 3. GFAP-immunoreactivity in the trigeminal ganglion of a rat (a) and knockout mouse (b). ABC method counter-stained with methylene blue. a. In the rat trigeminal ganglion, some satellite cells (arrows) surrounding the immunonegative neurons show GFAP-immunoreactivity. They are flat in shape, appearing as satellite rings. b. No specific immunoreaction for GFAP is seen in the neurons as well as in the satellite cells in the trigeminal ganglion of the GFAP-knockout mouse. a: ×120, b: ×210

Fig. 4. GFAP-immunoreactivity in the cerebellum of a wild type (a) and knockout mouse (b). Immunostaining with a polyclonal antibody to GFAP, counter-stained with methylene blue. a. The cerebellum of the wild type mouse contains numerous GFAP-immunoreactive astrocytes, which possess elaborate and slender cytoplasmic process. Their dendritic cell processes reach the endothelial walls to form a blood-brain barrier (arrows). b. A depletion of the GFAP gene diminishes all immunoreaction from the cerebellum of a GFAP-knockout mouse. Arrows indicate immunonegative Purkinje cells. a: ×160, b: ×120
positive astrocytes displayed dendritic profiles with slender cytoplasmic processes (Fig. 4a), most of which reached the GFAP-immunonegative endothelial cells in the cerebellum to form the blood-brain barrier.

The mice with a null mutation of the GFAP did not show any GFAP-immunoreaction in the trigeminal ganglion (Fig. 3b) or the cerebellum (Fig. 4b), compared with positive reactions in the wild-type controls (Figs. 3a, 4a). In all other respects these tissues had the same structure in the knockout and wild-type mice.

The GFAP-immunostaining pattern between knockout mice and wild-type littermates/rats is summarized in Table 1.

<table>
<thead>
<tr>
<th>Tissue or cell type</th>
<th>Normal rat and wild-type mouse</th>
<th>GFAP-KO mouse</th>
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<tbody>
<tr>
<td>Astrocyte</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Trigeminal neuron</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Trigeminal satellite cell</td>
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<tr>
<td>Pulpal axon</td>
<td>+</td>
<td>+</td>
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<td>Pulpal Schwann cell</td>
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Expression of GFAP gene in rat trigeminal ganglion, dental pulp and cerebellum by RT-PCR

Primers 1 and 2 were used to amplify the corresponding region of clone rGFA15, a 5.7-kb rat cDNA clone that contains the entire rat GFAP sequence.

Figure 5 shows the ethidium bromide-staining pattern of amplified RT-PCR products, demonstrating clear bands for GFAP genes corresponding to the length expected from the primer design in each lane of samples from the trigeminal ganglion, dental pulp, and cerebellum. Furthermore, no product was yielded from samples of the cerebellum designed as negative controls of RT-PCR without reverse transcription. These results confirmed that our RT-PCR yielded specific products solely derived from GFAP mRNA.

**In situ** hybridization histochemistry in rat trigeminal ganglion

**In situ** hybridization histochemistry using a specifically prepared cRNA probe showed signals for GFAP mRNA in the satellite cells appearing as a satellite ring surrounding the neurons, but without any neuronal signals in the rat trigeminal ganglion (Fig. 6a). On the other hand, the negative control experiment using a labeled sense probe for GFAP mRNA did not show any specific signals in the trigeminal ganglion (Fig. 6b), verifying the specificity of the hybridization in the sections.

**Fig. 5.** RT-PCR analysis of the rat trigeminal ganglion (lane 1), dental pulp (lane 2), negative control (lane 3) and cerebellum (lane 4) of rats. Amplified products for rat GFAP mRNA are seen at about 290 bp (arrow) in the samples except for the negative control. The negative control using the sample without reverse transcription shows no specific products. M marker DNA.
DISCUSSION

The GFAP-expression in peripheral tissue has been controversial due to differences in the kinds of antibodies, species differences, and to the different functional/maturational stages of the cells (cf. Fliegener and Liem, 1991; Xu et al., 1994). In trigeminal ganglion, it has been thought that GFAP-immunoreactivity in the satellite cells is absent or very weak under normal conditions. On the other hand, nerve injury has been reported to evoke the up-regulation of GFAP in astrocytes (Morrison et al., 1985; Canady et al., 1990, 1994a,b; Steward et al., 1991), in satellite cells of the trigeminal (Stephenson and Byers, 1995; Chueller et al., 1997) and dorsal root ganglion (Woodham et al., 1989), and in Schwann cells at the motor end plate (Georgiou et al., 1994). The up-regulation of GFAP after nerve injury shows two phase patterns: rapid (4–6 h) and delayed (3 days or more) time courses, this response being triggered by the altered ionic environment (Canady et al., 1990, 1994a,b; Steward et al., 1991), or by hormones and growth factors (Morrison et al., 1985). In this study, we succeeded in demonstrating a constant expression of GFAP mRNA by the RT-PCR technique as well as signals for GFAP mRNA by in situ hybridization histochemistry in the trigeminal satellite cells. Furthermore, mice with null mutation in the GFAP gene did not show any specific immunoreaction in the satellite cells of the trigeminal ganglion. Taken together, these findings lead us to conclude that the trigeminal satellite cells possess GFAP even under normal conditions, although it was first discovered as an astrocyte-specific intermediate filament (Eng et al., 1971; Eng, 1980).

The pulpal nerve fibers have been reported to show GFAP-immunoreactivity (Byers, 1995; Byers et al., 1995), as shown in this study. Previous experimental studies seeking to resect the inferior alveolar nerve showed the nerves in the dental pulp to be sensory in nature except for the vasomotor nerves, originating from the trigeminal ganglion (Arwill et al., 1973; Fink et al., 1975; Byers, 1984). However, this immunohistochemical and in situ hybridization histochemistry failed to demonstrate any immunoreaction for GFAP or signal for GFAP mRNA in the rat trigeminal ganglion neurons. This finding strongly indicates that the GFAP in the pulpal axons is a non-specific immunoreaction. Although immunohistochemistry for intermediate filaments has been utilized for the identification of cell type and origin, some immunohistochemical reports are available regarding me cross-reactivity between GFAP and other intermediate filaments (Achtstatter et al., 1986; Kelly et al., 1992). For instance, Hansen et al. (1989), using immunohistochemistry with an antibody preabsorbed with pure neurofilaments, found the absence of GFAP-immunoreactivity in non-glial elements but their persistence in astrocytes, leading to the possibility that the GFAP-antibody cross-reacts with other intermediate filaments under certain conditions. Indeed, the pulpal nerve fibers have intense neurofilament-immunoreactivity (Maeda et al., 1987). It is reasonable to consider that GFAP-immunoreaction in the pulpal axons at least under normal conditions is caused by the specificity of the GFAP-antibody.

The present RT-PCR analysis demonstrated a band for amplified products of the GFAP gene, though the GFAP-immunoreaction in the dental pulp can be regarded as false positive, as suggested by the
findings on GFAP-knockout mice. This immunohistochemical observation showed a small population of GFAP-immunoreactive Schwann cells which were also found in the knockout mice. Although we failed to demonstrate signals for GFAP gene in dental pulp by in situ hybridization histochemistry in our preliminary study probably due to technical problems, some of the pulpal Schwann cells seemed to have GFAP, consistent with electron microscopic immunocytochemistry showing some pulpal Schwann cells with GFAP-immunoreaction (Byers et al., 1995). However, the functional significance of GFAP remains unclear in the pulpal Schwann cells.

In conclusion, the expression of GFAP-immunoreaction in the peripheral tissues except for a part of trigeminal satellite cells and pulpal Schwann cells — was non-specific at least under normal conditions, probably due the specificity of the antibody to GFAP, despite its wide distribution that has been reported to date.

Acknowledgements. The authors thank Messrs Kiichi Takeuchi and Masaaki Hoshino for their histological assistance.

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