Hematopoietic Prostaglandin D Synthase and DP\textsubscript{1} Receptor Are Selectively Upregulated in Microglia and Astrocytes Within Senile Plaques From Human Patients and in a Mouse Model of Alzheimer Disease

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Abstract
Prostaglandin (PG) D\textsubscript{2} is produced in activated microglia by the action of hematopoietic PGD synthase (HPGDS) and plays important roles in neuroinflammation. Because of the fact that neuro-inflammation accelerates progression of Alzheimer disease (AD) has been documented, we investigated whether PGD\textsubscript{2} is also important roles in neuroinflammation. Because the fact that neuro-inflammation accelerates progression of Alzheimer disease (AD) has been documented, we investigated whether PGD\textsubscript{2} is also involved in the pathology of AD. Here, we report that the level of the mRNA of the receptor for PGD\textsubscript{2} (DP\textsubscript{1}) was increased in AD brains compared with the level in non-AD brains. Immunocytochemical analysis showed HPGDS expression to be localized in the microglia surrounding senile plaques. In situ hybridization studies revealed that DP\textsubscript{1} mRNA was specifically localized in microglia and reactive astrocytes within senile plaques of AD brains. In the brain of Tg2576 mice, a model of AD, HPGDS and DP\textsubscript{1} proteins were mainly localized immunocytochemically in microglia and astrocytes in the plaques, and the levels of their mRNAs increased in parallel with amyloid \(\beta\) deposition. These results indicate that PGD\textsubscript{2} may act as a mediator of plaque-associated inflammation in AD brain and may explain the pharmacologic mechanisms underlying the favorable response of patients with AD to nonsteroidal anti-inflammatory drugs.

Key Words: Alzheimer disease, Amyloid \(\beta\), Gliosis, Neuroinflammation, Nonsteroidal anti-inflammatory drugs (NSAIDs), Prostanoid, Tg2576 mouse.

INTRODUCTION
Alzheimer disease (AD) is the most common form of dementia. The cognitive decline in patients with AD is associated with neuronal degeneration, the appearance of neurofibrillary tangles, and the formation of senile plaques (1). It has been reported that inflammatory responses, including the production of proinflammatory cytokines and prostaglandins by activated microglia and astroglia, play important roles in the pathology of AD (2–5). Senile plaques arise from the excessive accumulation, aggregation, and deposition of amyloid \(\beta\) (A\(\beta\)) peptide, which triggers the activation of microglia and astrocytes around the senile plaques. Tg2576 mice overexpressing human APP695 with the “Swedish” mutation (a murine model of AD) develop memory deficits and senile plaques as they age (6). These animals show a rapid increase in A\(\beta\) deposition starting around 6 months and amyloid plaques beginning at 9 to 12 months (7).

We recently reported that activated microglia express hematopoietic prostaglandin (PG) D synthase (HPGDS) and that PGD\textsubscript{2} produced by HPGDS promotes neuroinflammation in a mouse model of Krabbe disease (8). These discoveries...
prompted us to clarify whether PGD\(_2\) is also involved in the plaque-associated inflammation in the AD brain. A determination of the mRNA contents of all known brain prostanoid synthases and receptors showed that HPGDS and DP1 mRNAs were selectively expressed to a higher level in AD brains compared with their levels in age-matched control patients. This overexpression was localized to reactive astrocytes and microglia closely associated with senile plaques, suggesting that HPGDS/PGD\(_2\)/DP\(_1\) signaling may accelerate chronic local inflammation around plaques in AD. We also found that HPGDS and DP\(_1\) mRNAs were selectively expressed in reactive astrocytes and microglia closely associated with senile plaques in the AD brains and brains of Tg2576 mouse. Our findings suggest that HPGDS/PGD\(_2\)/DP\(_1\) signaling may accelerate chronic local inflammation around senile plaques in the AD brain.

**MATERIALS AND METHODS**

**Human Tissue Source**

Human brain tissues were obtained from Tokyo Metropolitan Brain Bank for Aging Research according to the neuropathologic protocols described elsewhere (9). We obtained tissue fresh at autopsy and stored it at \(-80^\circ\)C, and, to avoid RNA degradation, we selected samples with as short a postmortem interval before autopsy as possible. The storage period was 2 to 32 months and 2 to 31 months for AD and control brains, respectively, and the storage period was not significantly different between the 2 groups.

Fifteen brains each from AD patients and control patients without clinicopathologic evidence of AD were the basis of the present work. The diagnosis of AD was based on the following criteria: 1) clinical dementia rating of \(\geq 1\) (10); 2) the topographical distribution of senile plaques matching Braak stage C; and 3) of neurofibrillary tangles equal to or above stage IV, as reported elsewhere (9). The selection criterion for control patients was a clinical dementia rating score of 0, senile plaque stage 0 or A, and a neurofibrillary tangle stage lower than stage II. The profiles of AD and control patients are listed in Table 1. Subjects with AD were 70 to 93 years old (82.6 years on average), and the postmortem delay ranged from 1.5 to 17 hours (average of 8.5 hours). The age of non-AD control patients was 71 to 91 years (79.5 on average), and the postmortem delay was 2.3 to 14.8 hours (average of 12.1 hours).

The tissues were either snap-frozen in liquid nitrogen orpowdered dry ice or immediately fixed in 4% paraformaldehyde (Sigma, St. Louis, MO) for 2 days and processed into paraffin blocks. Paraffin or frozen sections of 6-μm thickness from the frontal cortex and the hippocampus were cut with a cryostat (CM1850; Leica Microsystems, Wetzlar, Germany) and microtome (RM 2035; Leica), and used for immunohistochemical studies. Snap-frozen tissues, that were en face to the paraffin sections, were also used for quantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis of mRNA expression. The frontal cortex was selected for this purpose, because the difference in the number of senile plaques was most prominent there between AD and control groups. This study was approved by the institutional review boards of Osaka University Graduate School of Medicine, Tokyo Metropolitan Geriatric Hospital, Tokyo Metropolitan Institute of Gerontology, and Osaka Bioscience Institute.

**Mice**

All animal experiments were performed in accordance with Japanese law for the protection of experimental animals and conformed to the regulations issued by the National Institutes of Health and the Society for Neuroscience. Homozygous Tg2576 mice were purchased from Taconic (Hudson, NY) and maintained by interbreeding.

**Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis of mRNA**

By quantitative RT-PCR analysis, we determined the mRNA contents of enzymes involved in prostanoid synthesis, including cyclooxygenase (COX)-1, COX-2, HPGDS, lipocalin-type PGD synthase (L-PGDS), microsomal PGE synthases (mPGES-1 and mPGES-2), cytosolic PGE synthase (cPGES), PGF synthase, prostacyclin synthase, thromboxane (TX) A synthase, and those of prostanoid receptors for PGD\(_2\) (DP\(_1\) and DP\(_2\)), PGE\(_2\) (EP\(_1\), EP\(_2\), EP\(_3\), and EP\(_4\)), PGF\(_{2\alpha}\) (FP), PGI\(_2\) (IP), and TXA\(_2\) (TP), as well as the mRNA content of cytosolic phospholipase A\(_2\) (cPLA\(_2\)) and peroxisome proliferator-activated receptor (PPAR)-γ. After removal of the pia-arachnoid membrane, total RNA was prepared from the frontal cortex of 17 AD patients and 12 control patients by using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. In the mouse model, 3 each of Tg2576 and age-matched wild-type mice at ages 6 months, 1 year, and 2 years were subjected to quantitative RT-PCR for determination of COX-1, COX-2, HPGDS, DP\(_1\), and DP\(_2\) mRNA levels. All primers were synthesized by Sigma-Aldrich Japan (Tokyo, Japan). First-strand cDNAs were synthesized from 1 μg of total RNA by using avian myeloblastosis virus reverse transcriptase (Takara, Kyoto, Japan) and oligo dt-adaptor primer (Takara) at 50°C for 40 minutes after denaturation at 72°C for 3 minutes. The cDNA was amplified by use of a real-time PCR LightCycler system (Roche Diagnostics, Indianapolis, IN), a LightCycler-FastStart DNA Master SYBR Green I (Roche Diagnostics), and gene-specific primers under the following conditions: an initial denaturation at 94°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 15 seconds, annealing for 5 seconds, and extension at 72°C for 10 seconds. The oligonucleotide primers and annealing temperatures used are shown in Table 2. All oligonucleotide primers were synthesized by Sigma Genosys Japan (Tokyo, Japan). PCR products were evaluated by melting-curve analysis following the manufacturer’s instructions, checked after agarose gel electrophoresis, and sequenced. All values were corrected with reference to the value for glyceraldehyde-3-phosphate dehydrogenase (G3PDH), used as an internal standard.

**Immunocytochemistry**

Rabbit polyclonal antibodies against human HPGDS, L-PGDS, and mPGES-1 (11), and rabbit polyclonal
(0.1 μg/mL) and rat monoclonal (0.2 μg/mL) anti-mouse HPGDS antibodies (12) were raised and purified in Osaka Bioscience Institute. The specificity of each antiserum (1:1000 dilution) was confirmed by the disappearance of immunoreactivity after incubation of the antiserum with an excess amount of the corresponding purified recombinant immunogen. The other primary antibodies used in this study were as follows: anti-cow glial fibrillary acidic protein (GFAP) (1:5000 dilution; DakoCytomation, Glostrup, Denmark) for astrocytes; anti-human CD68 antibody (1:100 dilution; DakoCytomation) and anti-Iba1 (a generous gift of Dr. Shinichi Kousaka, National Center of Neurology and Psychiatry, Japan) for microglia/macrophages; and anti-human Aβ (11-28) antibody (1:100 dilution; IBL, Gunma, Japan) for detecting amyloid plaques.

Tg2576 and control mice were used for immunocytochemical analysis. Under deep ether anesthesia, the mice were perfused via the heart with physiologic saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) for 10 minutes. The brains were removed and postfixed in the same fixative overnight. Coronal slices were taken and routinely embedded in paraffin. Additionally, 2 each of Tg2576 and control mice were perfused with physiologic saline only and processed for the preparation of fresh-frozen sections. Both paraffin and frozen sections (5-μm thickness) were mounted on 3-aminopropyltriethoxysilane-coated slides. Deparaffinized sections were preincubated with 0.3% H2O2 (Wako, Osaka, Japan) in methanol followed by PBS containing 0.2% Triton X-100 (Nakarai Tesque, Kyoto, Japan). After pretreatment with 0.1% trypsin (Sigma) at 37°C for 15 minutes, they were sequentially incubated with primary antibody, biotinylated secondary antibody (2 μg/mL; Vector Laboratories, Burlingame, CA), and avidin-biotin-complex (ABC) by using an ABC elite system (Vector Laboratories) according to the manufacturer’s protocol.

For double immunostaining, deparaffinized sections were incubated at 4°C overnight with either anti-GFAP or anti-CD68 antibody together with rabbit anti-human HPGDS.
The sections were then reacted with alkaline phosphatase-conjugated anti-mouse IgG antibody (200 μg/mL; Aurora, Cambridge, UK) and biotin-conjugated anti-rabbit IgG antibody (Vector Laboratories), followed by ABC. The reaction products of horseradish peroxidase and alkaline phosphatase activities were visualized with diaminobenzidine (Dotite, Kumamoto, Japan) and naphthol AS-BI phosphate (Sigma) coupled to hexazotized new fuchsin (Merck, Darmstadt, Germany), respectively, as substrates.

All sections were analyzed, and images were obtained with an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP50 digital camera. The digital images were adjusted to an appropriate figure size required but were otherwise not processed.

### TABLE 2. Primer Sequences and Annealing Temperatures Used for Quantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Annealing Temperature (°C)</th>
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<tbody>
<tr>
<td>Human</td>
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</tr>
<tr>
<td>HPGDS</td>
<td>Forward</td>
<td>5'-GAAATAGAACAAGCTGACTGGC-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGCCCAAATCTGTTTTTGG-3'</td>
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<tr>
<td>L-PGDS</td>
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<td>5'-CAGGAAAAACAGTGTGAGACC-3'</td>
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<td></td>
<td>Reverse</td>
<td>5'-AGAGGGTGGCGATGCGGAAG-3'</td>
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<tr>
<td>mPGES-1</td>
<td>Forward</td>
<td>5'-CTGCTGTTCAACTAGATGTA-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACACACCGTGGGCTTGG-3'</td>
</tr>
<tr>
<td>mPGES-2</td>
<td>Forward</td>
<td>5'-TACAGTACAAAGCTGTTG-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGCCAAATCTGTGTTTTTGG-3'</td>
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<tr>
<td>cPGES</td>
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<td></td>
<td>Reverse</td>
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<td>PGFS</td>
<td>Forward</td>
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<tr>
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<td>Reverse</td>
<td>5'-AATCTGACCTTGTCATCTTCCC-3'</td>
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<td>DP1</td>
<td>Forward</td>
<td>5'-CCCTCTGAAGAAGCAAGAC-3'</td>
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<td>Reverse</td>
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<td>DP2</td>
<td>Forward</td>
<td>5'-CTCTCCATCTTCTTCTCAACA-3'</td>
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<td>Reverse</td>
<td>5'-TTCAGGAGGACAGACATTTGTA-3'</td>
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<td>EP1</td>
<td>Forward</td>
<td>5'-AACCTGAGCTGCGGCGGCGGGA-3'</td>
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<td>Reverse</td>
<td>5'-AGAAAGCATGCAAGCCAGCGAAGA-3'</td>
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<td>5'-AAACAGCACACACCAGATTAC-3'</td>
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<td>G3PDH</td>
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<td>5'-TGAACGCGGAGCTCAGTG-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCCACCACCCCTTGGCTGTA-3'</td>
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<table>
<thead>
<tr>
<th>Mouse</th>
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<tbody>
<tr>
<td>COX-1</td>
<td>Forward</td>
<td>5'-AGTTAGATCTGGAAGCAGAT-3'</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-CATAGTCCACAGCTGAGATG-3'</td>
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<tr>
<td>COX-2</td>
<td>Forward</td>
<td>5'-AAGTGCATCGTCTTCTCAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CCAGGCAGGACAGTATTTGAGA-3'</td>
</tr>
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<td>HPGDS</td>
<td>Forward</td>
<td>5'-GAATAGAACAAGCTGACTGGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGCCAAATCTGTTTTTGG-3'</td>
</tr>
<tr>
<td>DP1</td>
<td>Forward</td>
<td>5'-TTGGGAGTGTGCAAGTACT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GCCATGGAGGCGAGTATA-3'</td>
</tr>
<tr>
<td>DP2</td>
<td>Forward</td>
<td>5'-TGCCCGTCTTCCACAGAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACGCAGTTGCGGAAATTCG-3'</td>
</tr>
<tr>
<td>G3PDH</td>
<td>Forward</td>
<td>5'-TGAACGCGGAGCTCAGTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCCACCACCCCTTGGCTGTA-3'</td>
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COX, cyclooxygenase; cPGES, cytosolic PGES; DP1 and DP2, prostanoid receptors for PGD2; EP1, EP2, EP3, and EP4, prostanoid receptors for PGE2; FP, prostanoid receptor for PGF2α; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HPGDS, hematopoietic prostaglandin (PG) D synthase; L-PGDS, lipocalin-type PGD synthase; mPGES, microsomal PGE synthase; PGFS, PGF synthase.
In Situ Hybridization

In situ hybridization was performed by using a digoxigenin (DIG)-anti-DIG technique. For preparation of the human DP1 riboprobe, a 484-base pair fragment was amplified by use of FastStart Taq DNA polymerase (Roche Diagnostics) with human DP1-specific primers (forward, 5' GGGGTACTCTGTGCTCTACTCCAG-3' and reverse, 5' ACTGGATTCCATGTTAGTGGAATTG-3'), subcloned into pGEM-T Easy vector (Promega, Madison, WI), and converted into the corresponding RNA. Five-micrometer-thick cryosections were fixed in 10% formalin (Wako) for 1 hour and hybridized at 58°C for 16 hours with the DIG-labeled riboprobe for DP1 in 50% formamide (Wako), 5 × saline sodium citrate, 5 × Denhardt’s solution, 0.25 mg/mL yeast tRNA, and 0.5 mg/mL herring sperm DNA. After the sections had been washed with PBS, the DIG-labeled RNA was detected by using a Genius DNA labeling and detection kit (Roche Diagnostics) according to the manufacturer’s protocol.

For identification of DP1-expressing cells, the sections were reacted with the DP1-specific riboprobe and then immunostained with either anti-human GFAP antibody, anti-human CD68 antibody, or anti-human Aβ (11-28) antibody detected by using either immunofluorescence or immunoperoxidase. For fluorescence labeling, a biotinylated riboprobe was used in place of the DIG-labeled riboprobe. For double immunofluorescence, we confirmed the colocalization by omitting 1 of the first antibodies during the process.

Primary Cultures of Mouse Microglia and Astrocytes

We prepared primary cultures of microglia from wild-type mouse brains at postnatal day 1. Cerebral cortices were dissected, and the leptomeninges were completely removed.
The tissues were minced, suspended in PBS (GIBCO, Grand Island, NY) containing 0.05% trypsin (GIBCO) and 0.01% DNase I (Sigma), and then incubated for 10 minutes at 37°C. After incubation and centrifugation, the cell pellets were washed 3 times with PBS. The cells were then filtered through a 75-μm nylon mesh, centrifuged, suspended in Dulbecco’s modified Eagle’s medium (Nakalai Tesque) containing 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), 100 IU/mL penicillin (GIBCO), and 100 μg/mL streptomycin (GIBCO), and transferred to culture dishes. For microglial cultures, the medium was changed to Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum after a 24-hour culture period. This medium was exchanged for fresh medium twice weekly thereafter. The supernatants including microglial cells were collected and subcultured at 1 x 10^5 cells/well (6-well plate). After incubation in Dulbecco’s modified Eagle’s medium without fetal bovine serum for 6 hours, the microglia were stimulated by the addition of Aβ (1-40) (Peptide Institute, Osaka, Japan) for 24 hours. After the cells had been washed with PBS, RNA was extracted from them and subjected to quantitative RT-PCR.

Data Analysis

The difference of means among groups of data was analyzed by analysis of variance. Statistical significance was established at the level of p < 0.05 or 0.01.

RESULTS

Expression of Prostanoid-Producing Enzymes in Alzheimer Disease Brains

Using the frontal cortex obtained from AD and control patients, we examined the mRNA expression level of all enzymes involved in prostanoid synthesis. Quantitative

FIGURE 2. (A-G) mRNA expression of prostanoid receptors in the frontal cortex of Alzheimer disease (AD) (n = 17) and control brains (n = 12), as determined by quantitative RT-PCR analysis. The numbers of copies relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) are shown. The mean values and SE are indicated by the crossbars. *, p < 0.05.
RT-PCR analysis revealed the mRNA levels of HPGDS (Fig. 1A) and L-PGDS (Fig. 1B) to be elevated in AD brains compared with those of the control brains. However, the mRNA levels of other prostanoid-producing enzymes including COX-1 and -2 (Fig. 1C, D), PGESs including mPGES-1, mPGES-2, and cPGES (Fig. 1E–G), PGF synthase (Fig. 1H), and TXA synthase (data not shown), as well as the mRNA level of cPLA₂ (data not shown), were similar between control and AD brains. Prostacyclin synthase mRNA was not detected in either AD or control samples (data not shown).

**Increased DP₁ Expression in Alzheimer Disease Brains**

Next, we examined the mRNA expression level of all prostanoid receptors thus far identified (Fig. 2) and found that the expression of DP₁ was significantly upregulated in AD brains as compared with that in control brains (Fig. 2A). In 6 of the 17 AD patients, the mRNA level was higher than 2 SD from the mean of control brains, and these values were 2.5- to 16-fold greater than this mean. The mRNA level of DP₂, which is another PGD₂ receptor, was also increased, but not significantly, in AD brains (Fig. 2B). In contrast, the mRNA levels of other prostanoid receptors were almost the same between the 2 groups (Fig. 2C–G) except for the approximately 2-fold increases in EP₂ (Fig. 2D) and FP (Fig. 2G) in AD brains. In addition, the mRNA level of PPARγ, which is a nuclear receptor for PGD₂ metabolites (13), was not significantly different between AD and control brains (data not shown).

**Expression of Hematopoietic Prostaglandin D Synthase and DP₁ Is Associated with Senile Plaques in Alzheimer Disease Brains**

Because the mRNA levels of HPGDS and DP₁ mRNA were increased in AD brains, as judged from the results of RT-PCR analysis, we investigated the cellular localization of these messages.

Immunocytochemical analysis revealed that anti-HPGDS-immunoreactive cells were greater in number in the AD brain than in the control brain (Fig. 3A, B). Immunostaining for HPGDS (C) and amyloid β (Aβ) (D) associated with the senile plaques in an AD brain. The same cells in the serial sections are indicated by arrows. Scale bar = 20 μm. (E, F) Double immunostaining for HPGDS (purple) and glial fibrillary acidic protein (GFAP) (pink, E) or CD68 (pink, F) in an AD brain. HPGDS-positive/GFAP- (E) or CD68-positive (F) and HPGDS-negative/GFAP- (E) or CD68-positive (F) cells are indicated by arrows and arrowheads, respectively. Scale bar = 20 μm.
in reactive astrocytes and microglia within the senile plaques. Immunocytochemical analysis revealed that L-PGDS-expressing cells having the same morphologic characteristics as oligodendrocytes, as reported previously (14), were increased in number and were more intensely stained in AD than in control brains; however, the distribution of L-PGDS-positive oligodendrocytes was almost ubiquitous and seemingly unrelated to that of senile plaques (data not shown). mPGES was localized in some neurons of both AD and control brains, but there was no significant difference in intensity of staining or in the number of immunoreactive neurons between the 2 (data not shown).

Upregulation of DP1 mRNA in AD brains was confirmed by in situ hybridization with a DP1-specific antisense riboprobe, because antibody specific for human DP1 is still unavailable. DP1 mRNA was hardly detected in the control brain (Fig. 4A); however, high signal density was detected in AD brains (Fig. 4A). When we hybridized the sections with the DIG-labeled antisense riboprobe in the presence of a 500-fold excess of nonlabeled antisense riboprobe or with sense riboprobe, no signal was observed in either control or AD brain sections (Fig. 4A, sense). Double labeling for Aβ and DP1 hybridization revealed that the distribution of the DP1 signal was closely associated with Aβ immunoreactivity (Fig. 4B). However, the intensity of the DP1 signal differed among the senile plaques; and the signal was less preferentially observed in large-sized senile plaques (Fig. 4C). Furthermore, double staining with antibodies against glial markers revealed that the DP1 signal was localized in GFAP-positive reactive astrocytes (Fig. 4D, F) and CD68-positive microglia (Fig. 4E, G) within senile plaques, similar to the distribution of HPGDS shown in Figure 3E and F. These results indicate that expression of HPGDS and DP1 was increased in reactive astrocytes and microglia within the senile plaques in AD brain and suggest that PGD2 produced by HPGDS exerts its function through binding to DP1 in a paracrine or autocrine manner. The preferential expression of these PGD2-related molecules in the small-sized senile plaques suggests the contribution of PGD2 to neuroinflammation in the early phase of plaque evolution.

Upregulation of Hematopoietic Prostaglandin D Synthase and DP1 Expression in Tg2576 Mouse Brain

Next we examined the Tg2576 mouse brain to clarify the relevancy of our findings on human AD brains. In 2-year-old mice, there were numerous amyloid plaques in the cortex of Tg2576 brains (Fig. 5A) but not in that of the wild-type mouse brain (Fig. 5B). The results of the combination of Congo red staining and immunocytochemistry for Iba1, a marker for activated microglia, are shown in Figure 5C and D. Surrounding the amyloid plaques were many Iba1-positive activated microglia (Fig. 5D, arrowheads), which were not seen in the wild-type control. Double labeling with Congo red and anti-GFAP showed that fibers from activated astrocytes surrounded and enclosed amyloid plaques (Fig. 5E, F). These findings indicate that inflammatory responses such as microglial activation and astrogliosis were remarkable around early amyloid plaques in the Tg2576 mouse brain.

Next we investigated the localization of HPGDS and DP1 in the Tg2576 mouse brain. As in the case of the human

![FIGURE 4. (A) In situ hybridization for DP1 in control and Alzheimer disease (AD) brains. Control (left) and AD brain (middle) sections were incubated with antisense riboprobe. Another AD brain section was incubated with sense riboprobe (right). Scale bar = 100 μm. (B, C) In situ hybridization for DP1 (purple) and immunocytochemistry for amyloid β (Aβ) (pink) in an AD brain. The DP1-positive cells are uniformly intermingled with Aβ immunoreactivity in the senile plaque (B), and a large-sized DP1-negative senile plaque is shown (C). Scale bar = 20 μm. (D, E) Double staining by in situ hybridization for DP1 (purple) and immunocytochemistry for glial fibrillary acidic protein (GFAP) (brown, D) or CD68 (brown, E). Scale bar = 50 μm. (F, G) Double fluorescence labeling for DP1 (green) and GFAP (red, F) or CD68 (red, G). Double-positive cells are indicated by arrows. Scale bar = 30 μm.](http://jnen.oxfordjournals.org/)

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AD brains, HPGDS-positive microglia were common in relatively small plaques (Fig. 5G) but not in the large burned out class of plaques (Fig. 5H). HPGDS and Iba1 double immunofluorescence revealed that HPGDS was expressed in a certain number of Iba1-positive activated microglia (Fig. 5I). DP1 and GFAP double immunofluorescence revealed that DP1 was expressed on the astrocytic processes close to the amyloid plaques (Fig. 5J).

Quantitative RT-PCR was performed to evaluate the level of PGD2-related molecules in Tg2576 mouse brains and age-matched controls at the age of 6 months, 1 year, and 2 years. The expression of COX-1 mRNA was not different between Tg2576 and control mice (Fig. 6A), but that for COX-2 was higher in the Tg2578 mouse starting from the age of 6 months (Fig. 6B). The level of HPGDS mRNA was the same between Tg2576 and wild-type controls until they reached 2 years of age, at which time its level was 1.5 times higher in the Tg2576 mice than in the controls (Fig. 6C). The level of DP1 mRNA progressively increased in the Tg2576 mouse brain and reached a level 5-times higher than that in the control mice at the age of 2 years (Fig. 6D). In the case of DP2, its mRNA level progressively increased starting before the age of 1 year compared with that of the age-matched controls (Fig. 6E). These lines of evidence show that HPGDS and DP1 expression was associated with the small senile plaques and that the increase in DP2 receptors was more marked than that of HPGDS in the mouse model as in human AD.

To examine whether Aβ could directly stimulate the production of HPGDS in microglia, we performed an in vitro study using primary cultures of microglia obtained from normal neonatal mouse brains. We measured the level of HPGDS mRNA in these cultures before and after stimulating the cells with Aβ(1-42) at concentrations from 0 to 1000 nM. As shown in Figure 6F, the level of HPGDS mRNA did not increase when a 1 nM concentration of Aβ(1-42) was tested but did significantly increase when a 10 nM concentration or higher was used. However, no

**FIGURE 5.** (A, B) Amyloid β (Aβ) immunostaining of primary motor cortex of Tg2576 (A) and wild-type (B) brains from 2-year-old mice. Scale bar = 200 μm. (C, D) Combination of Congo red staining (pink) and Iba1 immunocytochemistry (brown) in a Tg2576 mouse brain. (D) The arrowheads and asterisk indicate Iba1-positive activated microglia and an amyloid plaque, respectively. Scale bars = (C) 200 μm; (D) 20 μm. (E, F) Combination of Congo red staining (pink) and glial fibrillary acidic protein (GFAP) immunocytochemistry (brown) in a Tg2576 mouse brain. (F) Arrowheads and asterisk indicate processes of GFAP-positive astrocytes and an amyloid plaque, respectively. Scale bars = (E) 100 μm; (F) 20 μm. (G, H) Hematopoietic prostaglandin D synthase (HPGDS) immunocytochemistry on a Tg2576 mouse brain. HPGDS-positive cells surround a small reactive amyloid plaque (G) but not the large burned out class of plaques (H). Asterisks indicate amyloid plaques. Scale bar = 20 μm. (I) Double immunofluorescence for HPGDS (pink) and Iba1 (green). Some of the Iba1-positive microglia express HPGDS. Scale bar = 50 μm. Inset shows a high magnification view of double-positive cells showing morphologic characteristics of ameboid microglia. (J) Double immunofluorescence for DP1 (red) and GFAP (green). DP1 is expressed on the processes of reactive astrocytes that have extended toward the center of this amyloid plaque. Scale bar = 10 μm.
further increase was observed at concentrations higher than 10 nM (Fig. 6F).

DISCUSSION

HPGDS/PGD$_2$/DP$_1$ Signaling Pathway Plays Important Roles in Inflammatory Reactions Within the Senile Plaques

We demonstrated that expression of HPGDS and DP$_1$ was increased in reactive astrocytes and microglia encircling senile plaques in the AD brain. In contrast, the mRNA levels of all other prostanoid synthases and receptors were almost the same between AD and control groups. A previous study by Iwamoto et al (15) demonstrated that the amounts of PGD$_2$ and TXB$_2$ were significantly increased in the brains of Alzheimer-type dementia patients, although other prostaglandin metabolites in their AD group showed no significant changes from normal patients. In this study, we used a more specific and sensitive criterion for AD (9) and determined the levels of prostaglandin synthases and receptors instead of direct prostaglandin determination, because the latter is subject to a large postmortem artifact (16). Ages and the postmortem interval of the patients at autopsy were not significantly different between AD and control groups.

Senile plaques have different characteristics depending on their stages (17); for example, there are primitive, neuritic (classic), and burned out class of plaques in various stage of human diseases. α-Macroglobulin and anti-chymotrypsin are expressed in the neuritic-type plaques but not in the burned out ones (18, 19). Although plaques homologous with neuritic plaques were not recognized in Tg2576 mouse brains, we observed that HPGDS expression was limited mainly to the relatively small plaques with surrounding activated microglia and was diminished in the burned out class of plaques in both human and mouse AD brains. These observations may explain why some patients did not show HPGDS mRNA upregulation and may suggest that HPGDS is responsible for early inflammation caused by Aβ accumulation in the evolution of amyloid plaques.

The question as to which is first, plaques or inflammation, still remains to be clarified. We previously demonstrated that secondary demyelination was suppressed by inhibition of PGD$_2$ production (8). We consider, therefore, that once the plaque is formed, microglia and astrocytes are activated and produce cytokines, which cause further neuronal injury and plaque formation. To clarify this, additional investigation is needed.

New Potential Therapies for Alzheimer Disease Based on Inhibition of the Hematopoietic Prostaglandin D Synthase/Prostaglandin D$_2$/DP$_1$ Signaling Pathway

Many epidemiologic and animal studies have revealed that nonsteroidal anti-inflammatory drugs (NSAIDs) are
beneficial for AD patients (20–24). For example, a large, prospective and population-based cohort study confirmed that the relative risk of developing AD was significantly reduced in long-term users of NSAIDs compared with nonusers (25). Furthermore, orally administered ibuprofen produced significant diminution of the ultimate number of amyloid deposits as well as significantly reduced gliosis in Tg2576 mice (24).

PGD₂ is formed from arachidonic acid, which is released from the lipid portion of membranes by cPLA₂, by successive enzyme reactions mediated by COX and PGD synthases. COXs, expression of which is upregulated in the AD brain (26, 27), are the major targets of NSAIDs. NSAIDs may reduce the plaque pathologic by either their anti-inflammatory actions or suppression of γ-secretase activity (28, 29) or both. The latter reduces the level of the 42-amino acid form of Aβ protein (Aβ42), the most toxic form of the protein. NSAIDs inhibit the constitutively expressed COX-1, as well as the inducible COX-2, both of which produce PGE₂, a common precursor of PGD₃, PGE₂, PGF₂α, prostaoyclin (PGI₂), and TXA₂ (30). PGD₂ is well known as an inflammatory mediator: it augments vascular permeability (31), regulates chemotaxis (32) and antigen presentation (33), and inhibits platelet aggregation (34). The biologic actions of PGD₂ are elicited through binding to its receptors, DP₁ (35) or DP₂ (32). Regarding the anti-inflammatory property of NSAIDs, 3 molecular targets of these drugs have been identified so far in AD: COX-1, COX-2, and PPARγ. NSAIDs not only inhibit the activity of COXs but also activate the nuclear PPARγ (36), which inhibits the production of pro-inflammatory cytokines. All 3 of these NSAID targets seem to be expressed by activated microglia (5). In this study, the level of PPARγ was not changed in AD brains, although this finding does not exclude the possibility of PPARγ-mediated neuroprotection by NSAIDs.

COX-2 has been the major target of anti-inflammatory therapy and a previous study showed an approximately 25% increase in the COX-2 level in the frontal cortex of AD brains (n = 17) compared with that in control brains (n = 12), as determined by densitometric analysis of Northern blots (37). We detected upregulation of COX-2 mRNA in Tg2576 brains (n = 17) compared with that in control brains (n = 12), increase in the COX-2 level in the frontal cortex of AD-mediated neuroprotection by NSAIDs. Although this finding does not exclude the possibility of PPARγ-mediated neuroprotection by NSAIDs.

In conclusion, we propose that PGD₂ is a novel inflammatory mediator in the AD brain and that the efficacy of HPGDS inhibitors and DP₁ antagonists should be investigated for halting the devastating course of AD.

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