Expression of Prostaglandin D₂ Synthase in Activated Eosinophils in Nasal Polyps

Sawako Hyo, MD; Ryo Kawata, MD; Keiichi Kadomyama, PhD; Naomi Eguchi, PhD; Takahiro Kubota, PhD; Hiroshi Takenaka, MD; Yoshihiro Urade, PhD

Objective: To clarify the relationship between prostaglandin D₂ production and eosinophil accumulation.

Design: Screening and diagnostic tests.

Subjects: Nineteen patients with chronic rhinosinusitis.

Interventions: Nasal polyps were obtained from 19 patients at endoscopic sinus surgery. Eosinophils in nasal polyps were counted after hematoxylin-eosin staining and immunostaining with antibodies against 2 eosinophil markers—major basic protein and EG2. Hematopoietic prostaglandin D₂ synthase (HPGDS) expression was examined by semi-quantitative Western blot analysis and by immunohistochemical staining with anti-HPGDS antibody.

Results: Nasal polyps were divided into 3 groups by the degree of eosinophilic infiltration. Western blot analysis revealed that HPGDS was more intensely and frequently expressed in the group with high infiltration than in the groups with low or medium infiltration. Hematopoietic prostaglandin D₂ synthase was immunohistochemically found in a subpopulation of EG2-positive eosinophils that had accumulated in the nasal polyps but not in the EG2-negative resting eosinophils. The ratio of HPGDS-positive eosinophils to EG2-positive eosinophils in the group with high eosinophil infiltration (mean ± SD, 64.8% ± 19.2%) was twice that in the group with low eosinophil infiltration (30.5% ± 13.8%).

Conclusion: Prostaglandin D₂ was actively produced by an EG2 and HPGDS double-positive subpopulation of activated eosinophils that had infiltrated into nasal polyps.

Arch Otolaryngol Head Neck Surg. 2007;133(7):693-700

NASAL POLYPOSIS IS A CONDITION INVOLVING CHRONIC AIRWAY INFLAMMATION OF THE PARANSAL SINUS MUCOSA, LEADING TO PROTRUSION OF BENIGN EDematous polyps from the meatus into the nasal cavities. Histologically, nasal polyps typically show the presence of a chronic inflammatory infiltrate with a large number of eosinophils. Patients with asthma, acute recurrent or chronic sinusitis, aspirin-induced asthma, or allergy require more polypectomies and more topical corticosteroid treatments than do patients without these diseases. Both secretion eosinophilia and tissue eosinophilia were found most often in patients with aspirin-induced asthma, and these patients had the most active nasal polyps, as judged by the degree of sinus involvement and number of reoperations and use of medication.

Prostaglandin D₂ (PGD₂) is the major prostanoid produced at sites of inflammation and infection and has an important role in the inflammatory response. Prostaglandin D₂ synthase (PGDS) contributes to the production of PGD₂ in antigen-presenting cells and mast cells in a variety of tissues and is involved in the activation and differentiation of mast cells as well as in chemotaxis or prolongation of cell survival of eosinophils. In an asthmatic model of knockout mice for D prostanoid 1, a receptor for PGD₂, the infiltration of eosinophils was substantially reduced. In addition, PGD₂ prolongs eosinophil survival by suppressing eosinophil apoptosis through the DP receptor. However, to our knowledge, no previous study has focused on the relationship between PGD₂ production and eosinophil accumulation in nasal polyps.

We investigated the expression and cellular localization of HPGDS in nasal polyps with high and low degrees of eosinophil infiltration and found that HPGDS was localized in a subpopulation of EG2-positive (activated) eosinophils in nasal polyps. Our findings indicate that PGD₂ is actively produced by EG2 and HPGDS double-positive activated eosinophils and suggest that the production of PGD₂ likely contributes to the recurrence of nasal polyps.
METHODS

ANTIBODIES

Polyclonal rabbit antibodies against human cyclooxygenase (COX)—1, HPGDS, and lipocalin-type PGD synthase and monoclonal mouse anti-human COX-2 antibody were obtained from a commercial supplier (Cayman Chemical Co, Ann Arbor, Michigan). Monoclonal mouse antibodies against human eosinophil major basic protein (MBP) (clone BMK13; Biosidex International, Saco, Maine), eosinophil cationic protein (clone EG2; Pharmacia, Uppsala, Sweden), mast cell tryptase (Chemicon International, Temecula, California), CD68 (DAKO Corp, Carpenteria, California), avidin-biotin-peroxidase complex kit, 3,3′,5,5′-tetramethylbenzidine peroxidase substrate kit, and normal rabbit and mouse immunoglobulins (all from Vector Laboratories Inc, Burlingame, California) were purchased from the manufacturers.

TISSUE HANDLING

Nasal polyps were obtained in 19 patients with chronic sinusitis undergoing endoscopic polypectomy. Informed consent was obtained from all patients. Patients were excluded from the study if they had taken systemic corticosteroid or nasal corticosteroid agents during the month before the study. Each nasal polyp was divided in half. One specimen was snap-frozen in liquid nitrogen and kept at −80°C and the other was fixed in a 10% neutral formaldehyde solution and embedded in paraffin. The paraffin-embedded blocks were then cut into 4-µm-thick consecutive sections.

IMMUNOHISTOCHEMICAL ANALYSIS

Paraffin sections were incubated for 1 hour with 10% goat serum to mask the nonspecific binding sites and then at 4°C overnight with antibodies against COX-1 (1:1000), lipocalin-type PGD synthase (1:5000), HPGDS (1:10000), COX-2 (1:1000), EG2 (1:1000), MBP (1:100), mast cell tryptase (1:1000), or CD68 (ready-to-use solution). The sections were then reacted with biotinylated secondary antibodies against rabbit and mouse IgG. Thereafter, they were incubated for 30 minutes with the avidin-biotin-peroxidase complex kit and the signal was visualized with diaminobenzidine tetrahydrochloride as a chromogen. Negative controls consisted of normal rabbit or mouse immunoglobulin or the antibody absorbed with an excess amount of the recombinant HPGDs.

In double staining, MBP or CD68 was immunostained in brown with diaminobenzidine tetrahydrochloride and EG2 or HPGDS, and in blue with 3,3′,5,5′-tetramethylbenzidine. For double immunofluorescence staining, the slides were incubated with rabbit antibody against HPGDS and mouse antibody against mast cell tryptase, MBP, EG2, or CD68 and then with Alexa Fluor 488–labeled anti-rabbit IgG and Alexa Fluor 546–labeled anti-mouse IgG antibodies (diluted 1:500; Molecular Probes, Invitrogen Corp, Carlsbad, California). Red and green fluorescences were observed with a confocal fluorescence microscope (Radiance 2000; Bio-Rad Laboratories Inc, Hercules, California).

WESTERN BLOT ANALYSIS

Nasal polyps were homogenized in phosphate-buffered saline solution (1 mL/100 mg wet weight of tissues). After centrifugation of the homogenates at 100 000 g for 1 hour, proteins in the resultant supernatant were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in a 10%:20% gradient gel, and microsomal proteins in a 4%:20% gel. Proteins were transferred onto polyvinyl difluoride membranes (Immobilon; Millipore Corp, Bedford, Massachusetts) electrothermally at 100 mA for 1 hour. After blockage of nonspecific binding sites for 1 hour at 25°C with phosphate-buffered saline solution containing 5% skim milk and 0.1% polysorbate 20 (Tween 20; Wako Junyaku, Osaka, Japan), the membranes were incubated at 4°C overnight with antibodies against human COX-1, HPGDS, lipocalin-type PGD synthase (1:5000), or COX-2 (1:1000) followed by horseradish peroxidase–coupled antibodies against mouse or rabbit IgG (1 mg/mL; Jackson ImmunoResearch Laboratories Inc, West Grove, Pennsylvania). The blot was then incubated with an electrochemiluminescence detection reagent (Amersham International PLC, Buckinghamshire, England) and subsequently exposed to an autoradiographic film (Kodak XOMAT AR film; Eastman Kodak Co, Rochester, New York).

STATISTICAL ANALYSIS

Data comparison within different groups was performed with the Kruskal-Wallis test. The significance of differences between 2 groups was calculated using the Mann-Whitney test for unpaired data. P < .05 was considered statistically significant.

RESULTS

PATIENT CHARACTERISTICS

Clinical characteristics of the 19 patients are given in the Table. Patients were divided into 3 groups, as follows: low eosinophilic infiltration group, characterized by eosinophil infiltration representing less than 10% of the total inflammatory cell infiltrate in the polyp; medium eosinophilic infiltration group, in which eosinophils composed 10% to 20% of the total infiltrate; and high eosinophilic infiltration group, in which eosinophils constituted more than 20% of the total inflammatory cells. There were no significant differences between the 3 groups for age, sex, or prevalence of allergic rhinitis.

Although the percentage of peripheral blood eosinophils was statistically the same in low and high eosinophilic infiltration groups, the number of eosinophils in the polyps increased from (mean ± SD) 57.3 ± 48.3/mm² in the low eosinophilic infiltration group to 775 ± 393/mm² in the high eosinophilic infiltration group, indicating that eosinophilic accumulation is tissue-specific.

EXPRESSION OF HPGDS IN NASAL POLYPS WITH HIGH AND LOW EOSINOPHILIC INFILTRATION

Western blot analysis revealed that the HPGDS immunoreactive protein was expressed in all samples (n = 7 for each group) of nasal polyps (Figure 1). The intensity of the HPGDS–positive band was weak for 3 samples (L2, L3, and L4) and strong for 2 samples (L6 and L7) in the low eosinophilic infiltration group and for 6 samples (H2 to H7) in the high eosinophilic infiltration group, indicating that HPGDS was more frequently and intensely expressed in polyps with high infiltration compared with polyps with low infiltration. For optical density values...
obtained by densitometric analysis, the density in the high eosinophilic infiltration group was higher than in the low eosinophilic infiltration group; however, there was no statistical difference between the groups. COX-2 expression was high in 2 samples (L4 and H4) and was not detected in 4 samples (L6, H2, H3, and H5). COX-1 immunoreactivity was observed in all samples. There were no significant differences in expression of COX-1 and COX-2 between the 2 groups. Therefore, expression of HPGDS was characteristic of nasal polyps with high eosinophilic infiltration, yet not specific to them because HPGDS was often observed in polyps with low eosinophilic infiltration.

Table. Clinical Characteristics of Patients and the Ratio of Colabeled Eosinophils

<table>
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<tr>
<th>Sample</th>
<th>Sex/</th>
<th>Age, y</th>
<th>Peripheral Eosinophils, %</th>
<th>AR or Non-AR</th>
<th>Eosinophil Infiltration, Cells/mm²</th>
<th>EG2+/MBP+ Cells, %</th>
<th>HPGDS+/MBP+ Cells, %</th>
<th>HPGDS+/EG2+ Cells, %</th>
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<td>140</td>
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<td>0.4</td>
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<tr>
<td>L2</td>
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<td>1.6</td>
<td>Non-AR</td>
<td>50</td>
<td>13</td>
<td>1.4</td>
<td>11</td>
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<tr>
<td>L3</td>
<td>M/34</td>
<td>4.2</td>
<td>AR</td>
<td>95</td>
<td>23</td>
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<tr>
<td>L4</td>
<td>M/64</td>
<td>0.7</td>
<td>Non-AR</td>
<td>65</td>
<td>35</td>
<td>8.4</td>
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<td>L5</td>
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<th>Peripheral Eosinophils, %</th>
<th>AR or Non-AR</th>
<th>Eosinophil Infiltration, Cells/mm²</th>
<th>EG2+/MBP+ Cells, %</th>
<th>HPGDS+/MBP+ Cells, %</th>
<th>HPGDS+/EG2+ Cells, %</th>
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<td>56</td>
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<th>Sample</th>
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<th>Age, y</th>
<th>Peripheral Eosinophils, %</th>
<th>AR or Non-AR</th>
<th>Eosinophil Infiltration, Cells/mm²</th>
<th>EG2+/MBP+ Cells, %</th>
<th>HPGDS+/MBP+ Cells, %</th>
<th>HPGDS+/EG2+ Cells, %</th>
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<tr>
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<td>M/76</td>
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<td>Non-AR</td>
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<tr>
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<td>AR/AIA</td>
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<tr>
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<td>Non-AR/AIA</td>
<td>575</td>
<td>92</td>
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<td>H6</td>
<td>F/26</td>
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<td>AR/AIA</td>
<td>1150</td>
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<td>H7</td>
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<td>8</td>
<td>AR</td>
<td>570</td>
<td>99</td>
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Abbreviations: AIA, aspirin-induced asthma; AR, allergic rhinitis; HPGDS, hematopoietic prostaglandin D₂ synthase; MBP, major basic protein; +, positive.

Mean age, 51.7 years; age range, 23 to 76 years.

Figure 1. Western blot analysis of hematopoietic prostaglandin D₂ synthase (HPGDS), and cyclooxygenase 2 (COX-2) and COX-1 in polyps with low (L1-7) and high (H1-7) eosinophilic infiltration. The applied total protein was 5 µg in each lane. M1-5 indicates nasal polyps with medium eosinophilic infiltration; and P, positive control.

**IMMUNOHISTOCHEMICAL LOCALIZATION OF HPGDS IN INFLAMMATORY CELLS OF NASAL POLYS**

All samples were examined by immunohistochemical staining with anti-HPGDS antibody and antibodies against several marker proteins for eosinophils (MBP and EG2), monocytes (CD68), and mast cells (tryptase). **Figure 2** shows typical results of hematoxylin-eosin staining and immunoperoxidase staining for MBP, EG2, CD68, and tryptase, and HPGDS in nasal polyps in the high and low eosinophilic infiltration groups. Results of confocal double-immunofluorescence staining of polyps with low
Figure 2. Staining with hematoxylin-eosin (HE) and immunoperoxidase for hematopoietic prostaglandin D₂ synthase (HPGDS), CD68, mast cell tryptase, major basic protein (MBP), and EG2 in nasal polyps from sample L1 with low eosinophilic infiltration (A, C, E, G, and I) and sample H3 with high eosinophilic infiltration (B, D, F, H, and J). Results are shown for HE staining (A and B), double immunostaining for MBP (brown) and EG2 (blue; C and D), immunostaining for mast cell tryptase (E and F), double immunostaining for CD68 (brown) and HPGDS (blue; G and H), and immunostaining with anti-HPGDS antibody (brown) and nuclear staining (blue; I and J) (original magnification for A-J, ×200). Insets in the upper right-hand corners of A through D and J and below I show high-magnification (original magnification ×400) views of typical positive cells. Insets in the lower left-hand corners of I and J show the results of staining with antibody preabsorbed with purified HPGDS. No positive staining was obtained with nonimmune IgG (data not shown). Arrows indicate mast cells; double arrows, monocytes; black arrowheads in I and J, eosinophils; white arrowheads in C and D, MBP-positive eosinophils; black arrowheads in B, MBP and EG2 double-positive eosinophils; white arrowheads in G and H, CD68-positive cells; black arrowheads in G and H, CD68 and HPGDS double-positive cells; black arrowheads in I and J, eosinophils; and bars, 10 µm.
and high eosinophilic infiltration with anti-CD68 or anti-mast cell tryptase antibody and anti-HPGDS antibody are shown in Figure 3, and with anti-MBP or EG2 antibody and anti-HPGDS antibody in Figure 4.

Hematoxylin-eosin staining revealed that monocytes, plasma cells, lymphocytes, and a few eosinophils had infiltrated into polyps with low eosinophilic infiltration (Figure 2A). In contrast, eosinophils were the dominant cells in the infiltrate in polyps with high eosinophilic infiltration (Figure 2B). We used 2 recognized eosinophilic markers, MBP and EG2, to differentiate resting from activated eosinophils. Anti-MBP antibody recognizes both resting and activated eosinophils, whereas EG2 antibody reacts selectively with activated eosinophils. In polyps with low eosinophilic infiltration (eg, L1), eosinophils stained positive with the anti-MBP antibody (brown in Figure 2C) but scarcely with the EG2 antibody (blue in Figure 2C), indicating that most of the eosinophils that had infiltrated into the polyp had not been activated. Approximately 60% of the MBP-immunoreactive eosinophils in polyps with high eosinophilic infiltration (eg, H3) stained positive with the EG2 antibody (Figure 2D), indicating that most of them were active.

As can be seen in the Table, the percentage of activated eosinophils in the polyps, expressed by the ratio of EG2-positive cells to MBP-positive cells, varied from 0.7% to 90% in polyps with low eosinophilic infiltration and from 14% to 99% in polyps with high eosinophilic infiltration. The mean (±SD) ratio was not statistically different between the 2 groups (46.8%±29.2% and 65.8%±34.7%, respectively). There were no significant differences in the number of cells positive for mast cell tryptase or CD68 between the 2 groups (Figure 2E-H).

In polyps with low eosinophilic infiltration (Figure 2I), HPGDS immunoreactivity was found in monocytes, mast cells, and eosinophils, in equal proportions. The HPGDS-positive monocytes were colabeled with anti-CD68 antibody (Figure 3A), the HPGDS-positive mast cells with antitryptase antibody (Figure 3B), and HPGDS-positive eosinophils with anti-MBP antibody (Figure 4A). The HPGDS immunoreactivity was not detected in a subpopulation of MBP-positive or EG2-positive eosinophils (Figure 4A and B). In the high eosinophilic infiltration group (Figure 2J), HPGDS immunoreactivity was found in a large number of eosinophils, most of which were colabeled with anti-MBP antibody (Figure 4C) or EG2 antibody (Figure 4D). The EG2-negative eosinophils were rarely labeled with anti-HPGDS antibody in either group, indicating that HPGDS expression was a marker of activated eosinophils.
Figure 4. Confocal double immunofluorescence staining for hematopoietic prostaglandin D₂ synthase (HPGDS) and major basic protein (MBP) or EG2 in nasal polyps. A nasal polyp from 1 patient from each of the low (sample L2; A and B) and high (sample H3; C and D) infiltration groups was stained with anti-HPGDS antibody (green) and antibody against MBP (red; A and C) or EG2 (red; B and D). The merged images in A through D show colocalization (yellow) of HPGDS and MBP (solid arrowheads in A and C) or EG2 (D). Open arrowheads show the HPGDS-negative and MBP- or EG2-positive cells. Bars represent 10 µm.
HPGDS EXPRESSION IN A SUBPOPULATION OF EG2-POSITIVE ACTIVATED EOSINOPHILS IN NASAL POLYPS

The Table also gives the percentages of eosinophils simultaneously labeled with antibodies against either of the 2 eosinophilic markers MBP and EG2, and the anti-HPGDS antibody in all samples. The ratio of HPGDS and MBP double-positive cells to MBP-positive eosinophils was relatively low in the group with low eosinophilic infiltration (12.1%±13.3%) but was 3.5-fold higher in the group with high eosinophilic infiltration (43.8%±26.0%). The ratio of HPGDS-positive cells to EG2-positive eosinophils in the group with high infiltration (64.8%±19.2%) was twice that in the group with low infiltration (30.5%±13.8%). In both cases, the differences in these ratios between the 2 groups of patients was statistically significant (P<.002).

Our results show that HPGDS was not expressed in resting eosinophils (MBP-positive and EG2-negative cells) in the peripheral blood (data not shown) or in polyps. The HPGDS was expressed specifically in about 30% and 60% of the EG2-positive (activated) eosinophils in the groups with low and high eosinophilic infiltration, respectively. The percentage of EG2 and HPGDS double-positive cells in eosinophils was relatively higher in the group with high vs low eosinophilic infiltration; the high eosinophilic infiltration group had more active nasal polyposis. Together these results suggest that HPGDS was expressed in more activated eosinophils than EG2-positive eosinophils in nasal polyps and contributes to tissue-specific eosinophilia and the recurrence of nasal polyps.

COMMENT

To our knowledge, this is the first study to find that HPGDS was expressed in a subpopulation of EG2-positive (activated) eosinophils that had infiltrated into human nasal polyps. We found no previous report of the localization of HPGDS in eosinophils, although HPGDS was previously found in antigen-presenting cells, mast cells, and megakaryocytes in the peripheral tissue and in helper T cells in the peripheral blood. In 2004 reported that HPGDS was not detected in eosinophils in human nasal polyps. We also found that HPGDS was not expressed in MBP-positive but EG2-negative eosinophils, which were dominant in most of the samples with low eosinophilic infiltration. For example, in sample L1 with low infiltration of a polyp, many eosinophils accumulated in the polyp (140 cells/mm²) but the percentage of HPGDS-positive eosinophils was low (0.4%); thus, HPGDS-negative eosinophils were dominant in this case. It would seem that Nantel and colleagues examined the low infiltration polyps rich in preactivated EG2-negative eosinophils. However, even in the low infiltration group, samples L6 and L7 with slight and mild eosinophilic infiltration (5 and 40 cells/mm², respectively) showed strong expression of HPGDS-immunoreactive protein, as determined at Western blot analysis, and a high percentage of HPGDS-positive activated eosinophils (20% and 38%, respectively) that had infiltrated into the polyp. Commonly, 8% to 70% of MBP-positive eosinophils were positive for HPGDS. The percentage of HPGDS-positive cells in EG2-positive (activated) eosinophils was higher, being twice that in polyps with high vs low infiltration. These results suggest that HPGDS is not only a marker of eosinophilic inflammation but also a sign of activation of infiltrating eosinophils.

Prostaglandin D₂ exerts its actions by binding to 2 types of receptors: DP (DP1) and CRTH2 (chemotaxant receptor–homologous molecule expressed on Th2 [DP2]). Both of these receptors are expressed on eosinophils. We also detected DP messenger RNA (mRNA) in the nasal polyps, the level of which was slightly higher than that of CRTH2 mRNA (data not shown). The level of HPGDS mRNA was significantly correlated with that of DP mRNA but not with that of CRTH2 mRNA (data not shown). Prostaglandin D₂ induces chemotaxis of eosinophils, basophils, and Th2 cells that is CRTH2-mediated. Stimulation of DP delays the onset of apoptosis of cultured eosinophils. Our findings, together with previous observations, suggest that the local production of PGD₂ by HPGDS-positive activated eosinophils is likely of great importance for further infiltration of eosinophils and more prolonged survival of tissue eosinophils in nasal polyps. The results of this study indicate that HPGDS is expressed in a subpopulation of activated eosinophils that accumulate in nasal polyps. Furthermore, our data suggest that PGD₂ produced by HPGDS-positive activated eosinophils may be an important prognostic factor for the clinical course of nasal polyposis.

Submitted for Publication: March 30, 2006; final revision received September 9, 2006; accepted September 28, 2006.

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Author Contributions: Dr Hyo had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Hyo, Kawata, Kadoyama, Eguchi, Kubota, Takenaka, and Urade. Acquisition of data: Hyo, Kawata, Kadoyama, and Urade. Analysis and interpretation of data: Hyo, Eguchi, Kubota, Takenaka, and Urade. Drafting of the manuscript: Hyo and Eguchi. Critical revision of the manuscript for important intellectual content: Hyo, Kawata, Kadoyama, Kubota, Takenaka, and Urade. Statistical analysis: Hyo, Kawata, and Eguchi. Obtained funding: Hyo, Kawata, Kadoyama, and Urade. Administrative, technical, and material support: Kawata, Kadoyama, Kubota, and Urade. Study supervision: Kubota, Takenaka, and Urade.

Financial Disclosure: None reported.

Funding/Support: This study was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, by a research grant from the Japan Foundation for Applied Enzymology, and by TAIHO Pharmaceutical Co Ltd.

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Role of the Sponsor: TAIHO Pharmaceutical Co Ltd had no role in the design and conduct of the study; in the collection, analysis, and interpretation of the data; or in the preparation, review, or approval of the manuscript.

Additional Contributions: Ms Shigeko Matsumoto and Nanae Nagata provided expert technical support, and Michitoshi Araki, MD, and Yasuhiro Hattori, MD, provided advice on the clinical data.

REFERENCES