Synthetic double-stranded RNA enhances airway inflammation and remodelling in a rat model of asthma

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Summary

Respiratory viral infections are frequently associated with exacerbations of asthma. Double-stranded RNA (dsRNA) produced during viral infections may be one of the stimuli for exacerbation. We aimed to assess the potential effect of dsRNA on certain aspects of chronic asthma through the administration of polyinosine-polycytidylic acid (poly I:C), synthetic dsRNA, to a rat model of asthma. Brown Norway rats were sensitized to ovalbumin and challenged three times to evoke airway remodelling. The effect of poly I:C on the ovalbumin-induced airway inflammation and structural changes was assessed from bronchoalveolar lavage fluid and histological findings. The expression of cytokines and chemokines was evaluated by real-time quantitative reverse transcription PCR and ELISA. Ovalbumin-challenged animals showed an increased number of total cells and eosinophils in bronchoalveolar lavage fluid compared with PBS-challenged controls. Ovalbumin-challenged animals treated with poly I:C showed an increased number of total cells and neutrophils in bronchoalveolar lavage fluid compared with those without poly I:C treatment. Ovalbumin-challenged animals showed goblet cell hyperplasia, increased airway smooth muscle mass, and proliferation of both airway epithelial cells and airway smooth muscle cells. Treatment with poly I:C enhanced these structural changes. Among the cytokines and chemokines examined, the expression of interleukins 12 and 17 and of transforming growth factor-β1 in ovalbumin-challenged animals treated with poly I:C was significantly increased compared with those of the other groups. Double-stranded RNA enhanced airway inflammation and remodelling in a rat model of bronchial asthma. These observations suggest that viral infections may promote airway remodelling.

Keywords: airway remodelling; asthma; Brown Norway rats; double-stranded RNA; poly I:C

Introduction

Asthma is a chronic airway inflammatory disease associated with bronchial hyper-responsiveness and reversible airflow limitation. In addition to airway inflammation, structural changes, termed airway remodelling, are also evident within the Airways3 and are thought to result from chronic inflammation. Another feature of asthma is acute exacerbations. Respiratory viral infections are frequently associated with exacerbations of asthma,2–4 but the association between exacerbations of asthma and airway remodelling is poorly understood. The most frequently isolated pathogens during viral infection-induced exacerbations are RNA viruses including rhinovirus, parainfluenza virus and respiratory syncytial virus.2–4 RNA virus synthesizes double-stranded RNA (dsRNA) during viral replication and dsRNA activates the innate immune system through several receptors including Toll-like receptor 3 (TLR3), retinoic-acid inducible gene-1 (RIG-I), and melanoma differentiation-associated gene-5 (MDA-5).5,6 As a result dsRNA induces the secretion of inflammatory cytokines and chemokines and should be considered as one of the factors potentially responsible for virus-induced asthma exacerbations.
Many studies have shown an effect of dsRNA on the secretion of cytokines and chemokines. For example, intravenous injection of dsRNA induces the production of type I interferon (IFN), IFN-γ, and interleukin-12 (IL-12) in mice. Treatment with dsRNA induces the production of type I IFN by mast cells. Bronchial epithelial cells treated with dsRNA show increased secretion of several chemokines and mucous cell metaplasia. Furthermore, it has been demonstrated that dsRNA augments the differentiation of fibroblasts to myofibroblasts. In a rat model of asthma, dsRNA enhances airway inflammation and bronchial hyper-responsiveness. In a mouse model of asthma, dsRNA also induces airway inflammation with a low dose inducing T helper type 2 (Th2) cytokines and a high dose inducing Th1 cytokines. There are no reports concerning the effect of dsRNA on airway remodelling.

The Brown Norway rat develops airway remodelling consistent with asthma after multiple allergen challenges. In this model, repeated allergen exposure causes structural alterations including increased airway smooth muscle mass and goblet cell hyperplasia. Furthermore, repeated intratracheal administrations of dsRNA are feasible in this model. As a result, dsRNA can directly affect airway epithelium mimicking actual airway infections. The aim of this study was to examine the effect of innate immune responses on allergic airway remodelling using this experimental model of asthma and synthetic dsRNA, polyinosine-polycytidylic acid (poly I:C).

Materials and methods

Animals

All animals used in this study were 8- to 12-week-old male Brown Norway rats purchased from SankyoLabo Corp. (Tokyo, Japan). Experimental procedures were approved by the institutional animal care committee.

Sensitization, challenges and treatments

Ovalbumin (OVA) sensitization was performed by concurrent subcutaneous injection of 1 mg OVA (grade V; Sigma-Aldrich, St Louis, MO) adsorbed to 4.28 mg of aluminium hydroxide, and intraperitoneal injection of 6 × 10^6 heat-killed Bordetella pertussis bacilli as adjuvant on day 1. On days 8, 15, 20 and 25, rats were sedated with xylazine and anaesthetized with pentobarbital sodium intraperitoneally. Orotracheal intubation was then performed using a 6-cm length of polyethylene tubing. Rats were divided into four groups. Two groups of rats received 400 μg synthetic dsRNA, poly I:C (Sigma-Aldrich) dissolved in 200 μl PBS and delivered through the endotracheal tube and the other two groups received 200 μl PBS in the same way. On days 15, 20 and 25, the rats were challenged by aerosol with 5% OVA solution [poly I:C-treated/OVA-challenged group (poly I:C/OVA, n = 7) and PBS-treated/OVA-challenged group (PBS/OVA, n = 8)] or with PBS [poly I:C-treated/PBS-challenged group (poly I:C/PBS, n = 6) and PBS-treated/PBS-challenged group (PBS/PBS, n = 6)].

Bronchoalveolar lavage fluid and cellular analysis

Bronchoalveolar lavage fluid (BALF) was collected by instilling and immediately withdrawing five aliquots of 5 ml PBS. Total cell count in BALF was determined with a haemocytometer. Cytospin slides of BALF were stained with Diff-Quik (International Reagents Corp., Kobe, Japan) and differential cell counts were determined by counting 300 cells. Absolute numbers of each cell type were calculated.

Assessment of airway inflammation

After bronchoalveolar lavage, left lungs were used for preparation of the histological sections. Paraffin-embedded 5-μm lung sections were stained with haematoxylin & eosin to assess airway inflammation.

Goblet cell hyperplasia

Goblet cell hyperplasia was assessed by histological analysis of lung sections stained with periodic acid–Schiff (PAS). The PAS staining was performed with a PAS staining kit (Merck, Darmstadt, Germany). Quantification of goblet cell hyperplasia was accomplished by counting the number of PAS-positive cells, and standardizing for airway size by dividing the count by the perimeter of the basement membrane (PBM). PBM was measured with the image analysis software WinROOF (Mitani Corporation, Fukui, Japan).

Measurement of airway smooth muscle mass

Airway smooth muscle (ASM) was detected by α-smooth muscle actin (α-SMA) immunohistochemistry. Paraffin sections were deparaffinized with xylene and rehydrated. Antigen retrieval was performed by heating in antigen retrieval solution (H3300; Vector Laboratories, Burlingame, CA). Non-specific binding was blocked with 5% normal horse serum (S-2000; Vector Laboratories) and 1% BSA in PBS. Cross-reaction with endogenous biotin and avidin was blocked with an Avidin/Biotin blocking kit (SP-2100; Vector Laboratories). Sections were then incubated with primary antibody, monoclonal anti-α-SMA antibody (Clone 1A4; Sigma-Aldrich) diluted to 1 : 2000, overnight at 4°C. After washing, sections were incubated with the secondary antibody, which was biotinylated horse anti-mouse IgG (BA-2000; Vector Laboratories) diluted to
1:500 for 60 min at room temperature. The signal was detected with Vectastain ABC-AP kit (AK-5000; Vector Laboratories) and Vector Red (SK-5100; Vector Laboratories).

The α-SMA-positive area was measured with image analysis software WinROOF. Quantification of the amount of ASM was accomplished by summing α-SMA-positive areas in the wall of each airway and standardizing for airway size by dividing the total area by the square of the perimeter of the basement membrane ($P_{BM}^2$).

**Analysis of the proliferation of airway epithelial and smooth muscle cells**

Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was performed to detect cell proliferation. Antigen retrieval and blocking of non-specific binding were performed as described above. Sections were incubated with a monoclonal primary anti-PCNA antibody (NA03; Calbiochem, Darmstadt, Germany) at a dilution of 1:200. After washing, sections were incubated with the secondary antibody, which was biotinylated horse anti-mouse IgG at a dilution of 1:500. The signal was detected with a Vectastain ABC-AP kit and BCIP/NBT chromogen substrate (SK-5400; Vector Laboratories). Sections were then rinsed in PBS and subsequently α-SMA immunohistochemistry was performed as described above.

Quantification of PCNA-positive epithelial cells was accomplished by counting the number of PCNA-positive cells in the epithelium, standardizing for airway size by dividing the count by $P_{BM}$. The PCNA-positive ASM cells were also counted and standardized for airway size by dividing the count by $P_{BM}^2$.

**Assessment of cytokine and chemokine expression in the lung**

Real-time quantitative reverse transcription- (RT-) PCR was performed to assess mRNA expression of cytokines and chemokines in lung tissues. Total RNA was extracted from right lung tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA) and purified using an RNeasy mini kit (Qiagen). Complementary DNA was synthesized using Superscript III first-strand synthesis supermix (Invitrogen) and the PCR was performed with the Miniopticon real-time detection system (Bio-Rad, Hercules, CA) using IQ SYBR Green Supermix (Bio-Rad). The sequences and annealing temperatures of rat primers are shown in Table 1.17–24 The size of each PCR product was confirmed by agarose gel electrophoresis. The PCR products were extracted and purified with QIAquick Gel Extraction kit (Qiagen, Tokyo, Japan). Standard was made of extracted material by step dilution and used to construct efficiency curves for each gene. The relative amount of mRNA was calculated by $C_t$ value and PCR amplification efficiency and was normalized by the housekeeping gene, cyclophilin A. Results were calculated as fold induction over the PBS/PBS group.

**Table 1.** PCR primer sequences, annealing temperatures, and the expected sizes of PCR products

| Gene (references) | Forward primer | Reverse primer | Annealing temperature (°C) | Product size (bp) |
|-------------------|----------------|----------------|-----------------------------|------------------|
| Cyclophilin A (17) | 5'-ATGGTCACCCCCACCGTG-3' | 5'-CGTGTGAAGTCACCACCT-3' | 57 | 206 |
| IL-12p35 (18) | 5'-TGATGATGACCCTGTGCCTT-3' | 5'-GCATGGACGAGATACAGAC-3' | 69 | 250 |
| IFN-γ (19) | 5'-ATGATGTCACCCACCGTG-3' | 5'-GAGTTCAATCAACGCTTTGG-3' | 65 | 405 |
| IL-4 (19) | 5'-TGATGATGACCCTGTGCCTT-3' | 5'-CTTCTCAATGTTGATGACGT-3' | 70 | 378 |
| IL-13 (20) | 5'-ATGCGACTTGGGTGACTG-3' | 5'-GGCCATTCAATAATGCTTGG-3' | 57 | 277 |
| IL-17 (19) | 5'-ATGCGACTTGGGTGACTG-3' | 5'-TTAGGACGGATGAGCGAACAATAGA-3' | 67 | 453 |
| RANTES (21) | 5'-CCATATGCTCGAGGACACCA-3' | 5'-CCCCATCTCCCTTCTGTTG-3' | 69 | 168 |
| Eotaxin (22) | 5'-AACCTCACTCATCTACAGT-3' | 5'-TTCTGTCATGAGCTTTTCTG-3' | 62 | 202 |
| CINC-1 (23) | 5'-ACCACACAGGACATGCATG-3' | 5'-ACTGTCGCTTGGGCTTCCAC-3' | 67 | 112 |
| TGF-β1 (24) | 5'-CGAGGATGCCTTGAGGACACACATGC-3' | 5'-CTGCTCCTTGGGCTTGGGACACAC-3' | 57 | 405 |

IL-12, interleukin-12; IFN-γ, interferon-γ; RANTES, regulated on activation normal T cell expressed and secreted; CINC-1, cytokine-induced neutrophil chemoattractant 1; TGF-β1, transforming growth factor-β1.
Interleukin-17 and transforming growth factor-β1 (TGF-β1) levels in BALF supernatant were measured by ELISA (rat IL-17 ELISA kit, eBioscience, San Diego, CA; mouse/rat TGF-β1 ELISA kit, R&D systems, Minneapolis, MN).

Statistical analysis

Comparisons among groups were analysed using the Kruskal–Wallis test. When appropriate, a non-parametric post-hoc multiple comparisons test – the Steel–Dwass test – was used to evaluate differences between the groups. A P-value < 0.05 was considered to be significant.

Results

Treatment with poly I:C increased total cells and neutrophils in BALF

To evaluate the effects of dsRNA on a rat model of asthma, animals were sensitized and challenged with OVA, as shown in Fig. 1(a). We divided rats into four groups to compare OVA-challenged poly I:C-treated rats with the other control groups (Fig. 1b).

There was an increase in total cells of BALF in the PBS/OVA group when compared with two groups that were challenged with PBS (Fig. 2a). Intratracheal treatment with poly I:C significantly enhanced this increase. Although both neutrophils and macrophages in BALF increased in the PBS/OVA group, eosinophils accounted for the majority of the increase in cells (Fig. 2b). The numbers of neutrophils, eosinophils, lymphocytes and macrophages of BALF in the poly I:C/OVA group significantly increased when compared with the PBS/PBS group (Fig. 2c–e). The increase in BALF neutrophils was particularly noteworthy in the poly I:C/OVA group compared with the other three groups.

Haematoxylin & eosin staining was performed for histological analysis of the lungs. Remarkable differences were not observed in the histology between the PBS/PBS group and the poly I:C/PBS group (Fig. 3a,b). As we expected, the PBS/OVA group showed peribronchial inflammation (Fig. 3c) and this was even more marked in the poly I:C/OVA group (Fig. 3d).

Goblet cell number is enhanced by poly I:C treatment

One of the key epithelial changes observed in asthmatic lungs is goblet cell metaplasia. We performed PAS staining of lung sections to assess the effect of dsRNA on airway remodelling. Only relatively few and isolated PAS-positive cells were observed in the airway epithelium of the PBS/PBS group (Fig. 4a). Numbers of PAS-positive cells in the airway epithelium of the poly I:C/PBS group were slightly increased (Fig. 4b) and as previously reported, the PBS/OVA group showed an obvious increase in airway PAS-positive cells (Fig. 4c). This increase was even more apparent in the poly I:C/OVA group (Fig. 4d).

The increase in goblet cells was quantified and confirmed the observations made by visual inspection of the slides. The poly I:C/OVA group showed a significant increase in PAS-positive airway epithelial cell numbers compared with the other three groups (Fig. 4e).

Treatment with poly I:C enhanced the increase in ASM mass after repeated allergen challenges

A second alteration of airway structure characteristic of the chronic remodelling change in asthma is an increase in ASM mass. ASM was identified as ASM-specific α-actin-positive cells. The lungs of the poly I:C/PBS group were not visually distinguishable from those of the PBS/PBS group (Fig. 5a,b). Sensitization and repeated challenges with OVA led to more ASM-specific α-actin staining in lung sections compared with the two PBS-challenged control groups (Fig. 5c). In addition, the lung sections of poly I:C/OVA-treated animals showed prominent increases in ASM mass (Fig. 5d). The ASM mass of the poly I:C/OVA group significantly increased compared with that of the other three groups and showed a 1.8-fold increase over that of the PBS/OVA groups (Fig. 5e).
Treatment with poly I:C enhanced the proliferation of airway epithelial cells and ASM cells in response to repeated allergen challenges

One of the mechanisms believed to contribute to an increase in goblet cell numbers and in ASM mass is cell proliferation.\(^\text{25,26}\) We therefore evaluated the expression of PCNA in epithelial cells and in ASM cells. The airways of the PBS-challenged groups showed relatively few PCNA-positive epithelial cells and ASM cells (Figs 6a,b and 7a,b). Challenge with OVA led to an increase in the number of both PCNA-positive airway epithelial cells and ASM cells (Figs 6c and 7c). Furthermore, the poly I:C/OVA group showed a prominent increase in number of both PCNA-positive epithelial cells and ASM cells (Figs 6d and 7d). Quantification of PCNA-positive epithelial cells and ASM cells showed that repeated allergen challenges led to a significant increase in the number of PCNA-positive epithelial cells and ASM cells and these increases were significantly enhanced by poly I:C treatment (Figs 6e and 7e).

Figure 2. Analyses of total cells and differential cell counts in bronchoalveolar lavage fluid. Total cells (a). Differential cell counts of eosinophils (b), neutrophils (c), macrophages (d) and lymphocytes (e). Data are presented as means (SEM). * \(P < 0.05\).

Figure 3. Representative haematoxylin & eosin-stained lung sections from PBS-treated PBS-challenged animals (a), polyinosine-polycytidylic acid (poly I:C) -treated PBS-challenged animals (b), PBS-treated ovalbumin (OVA) -challenged animals (c), and poly I:C-treated OVA-challenged animals (d).
Cytokine and chemokine expression analysis in the lung

We assessed the profile of cytokines and chemokines using RT-PCR and ELISA. The IL-12p35 and IFN-γ were investigated as representative Th1 cytokines using RT-PCR. Expression of IL-12p35 in the lung homogenates of the poly I:C/OVA group was significantly higher than in the other three groups (Fig. 8a). Expression of IFN-γ in the poly I:C/OVA group was significantly higher than that in the PBS/PBS group (Fig. 8b). Interleukin-4 and IL-13 were investigated as representative Th2 cytokines. Expression of IL-4 was comparable in the lungs of the four groups (Fig. 8c). However, IL-13 expression of both the PBS/OVA and the poly I:C/OVA groups was significantly higher than that of both the PBS/PBS and the poly I:C/PBS groups (Fig. 8d). Interleukin-17 expression of the PBS/OVA group increased compared with that of the PBS/PBS group (Fig. 8e). Treatment of animals with poly I:C before OVA challenges significantly augmented the expression of IL-17 compared with the three other groups.

Previous studies have shown that treatment with dsRNA increased the chemokine regulated on activation normal T cell expressed and secreted (RANTES) but not eotaxin in human primary epithelial cells and human airway epithelial cell lines.9,10 Our results on the expression of chemokines showed similar trends. Expression of RANTES in both groups treated with poly I:C tended to be higher than that in both groups treated with PBS, but a significant difference was observed only between the PBS/OVA group and the poly I:C/OVA group (Fig. 8f). There were no significant differences among the four groups in eotaxin expression and cytokine-induced neutrophil chemoattractant 1 (CINC-1) expression (Fig. 8g,h). We investigated also TGF-β1 expression. As expected, TGF-β1

Figure 4. Representative periodic acid-Schiff (PAS) staining of lung sections from PBS-treated PBS-challenged animals (a), polyinosine-polycytidylic acid (poly I:C)-treated PBS-challenged animals (b), PBS-treated ovalbumin (OVA)-challenged animals (c), and poly I:C-treated OVA-challenged animals (d). Goblet cell hyperplasia is determined by counting the number of PAS-positive cells per airway and is normalized for airway size by dividing by the perimeter of the basement membrane (PBM) (e). Data are presented as means (SEM). *P < 0.05.
expression of the PBS/OVA group was increased compared with that of the PBS/PBS group and was further augmented by treatment of animals with poly I:C before OVA challenges (Fig. 8i).

Levels of IL-17 and TGF-β1 proteins in BALF were assessed by ELISA. Consistent with mRNA expression, IL-17 and TGF-β1 were significantly increased in the poly I:C/OVA group compared with those of the other three groups (Fig. 8j,k).

Discussion

The present study demonstrated that repeated intratracheal administrations of dsRNA enhanced the effects of allergen challenge on airway remodelling in a rat model of asthma. Our results imply the potential effect of dsRNA on certain aspects of airway structural changes in asthma. Previous reports have shown that dsRNA enhances airway inflammation and airway hyper-responsiveness.13,14 But there are no reports showing the effect of dsRNA on airway remodelling in vivo. The present study indicates that dsRNA enhances not only airway inflammation but also airway remodelling induced by allergen challenge. These results suggest an explanation for respiratory virus-induced worsening of asthma and further suggest a possible association between acute exacerbations and airway remodelling.

Recently, the relationship between innate immunity and asthma has been intensively investigated. The TLR4 ligand lipopolysaccharide has been shown to exacerbate allergic airway inflammation in mice.27,28 In contrast, the TLR2 agonist Pam3CSK4 decreases airway hyper-responsiveness in mice and the TLR7/8 ligand S28463 prevents airway inflammation and remodelling in rats.26,29
association between infection and airway remodelling has also been investigated. Kuo et al. have demonstrated that rhinovirus infection increases extracellular matrix protein in human bronchial epithelial cells, fibroblasts and airway smooth muscle cells. Consistent with their results, which imply that viruses may contribute to airway remodelling, we have shown that polyI:C enhanced airway structural changes in asthma using an in vivo model.

Among the receptors for dsRNA, TLR3 and MDA-5 can recognize long dsRNA whereas RIG-I can detect short dsRNA. Hence the biological consequences of exposure to dsRNA may vary as a function of the length of dsRNA. Most of the RNA viruses that cause respiratory infection, including rhinovirus, coronavirus, parainfluenza virus and respiratory syncytial virus, have long single-stranded RNA. These RNA viruses produce long dsRNA during their replication. Therefore, TLR3 and MDA-5 are considered as the main receptors of dsRNA during respiratory viral infections. Because it has been demonstrated that a synthetic dsRNA poly I:C consists of long-length dsRNA and serves as a ligand for TLR3 and MDA-5, we chose poly I:C to simulate infection with these long-RNA respiratory viruses.

We have demonstrated that dsRNA enhances airway remodelling including goblet cell hyperplasia, an increase in ASM mass, and the proliferation of airway epithelial cells and smooth muscle cells. We have also shown that treatment with dsRNA enhances the expression of TGF-β1, which plays an important role in promoting the structural changes of tissue remodelling. These data suggest that virus-induced exacerbations of asthma may result in airway remodelling through activation of dsRNA receptors and TGF-β1. Consistent with this idea, it has been reported that activation of TLR3 by dsRNA induces production of α-SMA and extracellular matrix protein including fibronectin and collagen through increase of TGF-β1 in fibroblasts.

![Figure 6. Representative airways stained by both proliferating cell nuclear antigen (PCNA, purple) and airway smooth muscle-specific α-actin (red) from PBS-treated PBS-challenged animals (a), polyinosine-polycytidylic acid (poly I:C)-treated PBS-challenged animals (b), PBS-treated ovalbumin (OVA)-challenged animals (c), and poly I:C-treated OVA-challenged animals (d). Quantification of PCNA-positive epithelial cells was performed by counting the number of PCNA-positive epithelial cells and normalizing for airway size was performed by dividing by the perimeter of the basement membrane (PBM) (e). Data are presented as means (SEM). *P < 0.05.](image)
Another important characteristic of the animals treated with dsRNA is an increased expression of IL-12 and IL-17. The Th2 hypothesis of asthma pathogenesis proposes that asthma is caused by an increase in Th2 response in combination with a decrease in Th1 response. However, some studies have indicated that Th1 plays a role in severe asthma. Other studies have shown that neutrophilic inflammation in severe asthma is related to Th1 cytokine expression. Recently the role of IL-17 has been investigated in relation to asthma, in particular in neutrophilic asthma. Our data suggest that dsRNA produced during viral infections enhances the expression of Th1 and Th17 cytokines in the airways and may therefore lead to promoting a severe phenotype of asthma. Consistent with our results, previous studies have demonstrated that dsRNA induces neutrophilic airway inflammation and can cause Th1, Th2, or Th17 responses depending on the dose and timing of administration.

In conclusion, a rat asthma model treated with dsRNA demonstrated enhanced recruitment of neutrophils in the airways, both Th1 and Th17 cytokine expression, and strong airway remodelling. Our results provide a potential explanation for virus-induced asthma worsening and suggest an association between these exacerbations and augmentation of airway remodelling. The dsRNA and its receptors may be worthwhile therapeutic targets in patients with virus-induced asthma exacerbations and airway remodelling.

**Author’s contributions**

Satoshi Takayama conceived the study, participated in its design and all analysis, and drafted the manuscript. Meiyo...
Tamaoka conceived the study and participated in its design and animal treatment. Koji Takayama and Kimi-take Tsuchiya participated in the animal treatment and histological analysis. Kaori Okayasu participated in the assessment of cytokine and chemokine mRNA expression. Yasunari Miyazaki participated in the coordination of the study. James G. Martin participated in the assessment of cytokine and chemokine mRNA expression and the review of the paper. Yuki Sumi and Naohiko Inase participated in the coordination of the study and the review of the paper. All authors have read and approved the final manuscript.

Disclosures
The authors have no financial conflicts of interests.
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