**α1-Acid Glycoprotein Attenuates Adriamycin-Induced Nephropathy via CD163 Expressing Macrophage Induction**

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**Abstract**

**Background** Recent clinical studies have shown that proteinuria is a critical factor in the progression of CKD and onset of cardiovascular disease. Inflammation and infiltration of macrophages into renal tissue are implicated as causes of proteinuria. α1-Acid glycoprotein (AGP), an acute-phase plasma protein, is leaked into the urine in patients with proteinuria. However, the relationship between urinary leakage of AGP, renal inflammation, and proteinuria remains unclear.

**Methods** Human AGP (hAGP) was exogenously administrated for 5 consecutive days to adriamycin-induced nephropathy model mice.

**Results** Adriamycin treatment increased urinary AGP, accompanied by decreased plasma AGP in mice. Exogenous hAGP administration to adriamycin-treated mice suppressed proteinuria, renal histologic injury, and inflammation. hAGP administration increased renal CD163 expression, a marker of anti-inflammatory macrophages. Similar changes were observed in PMA-differentiated THP-1 cells treated with hAGP. Even in the presence of LPS, hAGP treatment increased CD163/IL-10 expression in differentiated THP-1 cells.

**Conclusions** AGP alleviates proteinuria and renal injury in mice with proteinuric kidney disease via induction of CD163-expressing macrophages with anti-inflammatory function. The results demonstrate that endogenous AGP could work to protect against glomerular disease. Thus, AGP supplementation could be a possible new therapeutic intervention for patients with glomerular disease.

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**Introduction**

Approximately 13% of the worldwide population suffer from CKD, and the number of patients is increasing year by year (1). CKD not only leads to ESKD, but is also associated with a high risk of death from cardiovascular disease (CVD) (2). In previous clinical trials, proteinuria was reported as a critical factor involved in the progression of CKD and onset of CVD (2–4). In fact, nephrotic patients with massive proteinuria have a 5.5-fold higher risk of CVD than matched controls (5). It has been reported that inflammation and infiltration of immune cells into renal tissue are a cause of proteinuria. In addition, the onset of proteinuria further induces inflammation in tubular cells (6,7). Therefore, it is important to stop the vicious cycle induced by persistent proteinuria and inflammation, for the prevention of CKD progression and onset of CVD.

Recently, the involvement of macrophages in inflammation in renal pathologies has been reported (8,9). Macrophages have phenotypes that are classify them as M1 macrophages (classically activated macrophages) or M2 macrophages (alternatively activated macrophages). In general, M1 macrophages promote inflammation and play a central role in host defense during infection. In contrast, M2 macrophages are involved in anti-inflammation and tissue remodeling (10). These phenotypes can be changed in response to a disease state. M1 macrophages predominate in the early injury phase, whereas M2 macrophages predominate in the recovery and repair phase (11). Indeed,
previous reports have demonstrated that administration of M2 macrophages induced by IL-4 or IL-4/IL-13 is effective against renal injury resulting from ischemia reperfusion or adriamycin (ADR)-induced nephropathy in mice (11,12). These studies suggest that regulation of macrophage phenotypes in the kidney could be important in reducing renal inflammation and proteinuria.

\( \alpha_1 \)-Acid glycoprotein (AGP), also known as orosomucoid, is a heavily glycosylated serum protein with five branched \( N \)-glycans containing negatively charged sialic acids (13,14). Therefore, AGP has the lowest pI value of all plasma proteins (pI=2.7–3.2). In addition, AGP is classified as one of the major acute-phase proteins in mammals and is known that its serum concentration increases two- to five-fold during inflammation (15). In patients with proteinuria, it has been observed that AGP leaks into urine (16,17). On the other hand, AGP has been reported to possesses unique biologic activities. For example, in vitro experiments have demonstrated that AGP modulates the activation of neutrophils, lymphocytes, and monocytes (–14,18–21). Daemen et al. (22) reported that exogenously administered AGP attenuates renal ischemia reperfusion injury in vivo, although its renoprotective mechanisms remain unclear. Importantly, we have previously found that AGP weakly activates the Toll-like receptor 4 (TLR4)/CD14 pathway and increases the expression of CD163, a cysteine-rich scavenger receptor in macrophages, particularly of the M2 phenotype (23,24). Interestingly, CD163-overexpressing macrophages inhibit LPS-induced inflammation in vitro (25). Furuhashi et al. (26) also found that CD163-expressing macrophages are associated with IL-10 production as an anti-inflammatory cytokine in anti-glomerular basement membrane GN rats. These findings have led to the idea that AGP may potentiate the suppression of renal inflammation and proteinuria by changing the macrophage phenotype to one with enhanced CD163 expression.

The purpose of this study is to clarify whether exogenously administered human AGP (hAGP) attenuates renal damage in ADR-induced nephropathy mice. In addition, possible mechanisms of this renoprotective effect are examined. Here, we provide evidence that administration of hAGP induces CD163-expressing macrophages in the kidney, which leads to reduced proteinuria and renal inflammation in ADR-induced nephropathy mice.

Materials and Methods

Purification of AGP

hAGP was purified from supernatant of human plasma fraction V donated by KM Biologics (Kumamoto, Japan). Supernatant was diluted with acetate buffer (10 mM) and injected onto an ion exchange column (Hitrap CM,Q column; GE Healthcare, Little Chalfont, UK) at a flow rate of 3 ml/min on an AKTAPrime Plus System (GE Healthcare). The bound materials were eluted with acetate buffer (10 mM) containing NaCl (0.5 mol/L). hAGP was eluted in the first peak. The eluted sample was dialyzed against distilled water at 4°C, freeze-dried, and stored at –20°C (27). The purity of hAGP was confirmed by SDS-PAGE. The secondary structure of hAGP was confirmed by circular dichroism.

Animal Studies

All animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University. All animals were housed in an environment with controlled temperature, a 12-hour light/dark cycle, and free access to food and water.

Six-week-old male BALB/c mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). After randomizing mice by body weight, mice were slowly injected intravenously with saline or ADR (15 mg/kg body weight). The first administration of saline or AGP (5 mg/mouse) was intra-peritoneally administered between 30 minutes and 1 hour after ADR injection, allowing for time for the ADR to disappear from the blood. Subsequently, saline or hAGP was administered at the same time for 4 consecutive days. No mice died under these experimental conditions. On day 7 and day 21 after ADR administration, urine was collected by metabolic cage for a total of 16 hours from 4:00 PM to 8:00 AM, and the urine volume measured. Mice were sacrificed at day 7 or day 21 after ADR administration. Kidneys and livers were harvested after perfusion with 0.9% NaCl. A part of the left kidney was used for quantitative RT-PCR and histologic analysis.

Renal Function

Urinary albumin was measured by ELISA (Shibayagi, Japan). BUN, creatinine, and total cholesterol were measured using FUJI DRI-CHEM (Fujifilm, Japan).

Histologic Analysis

Sections of renal tissue were stained with periodic acid-Schiff. Quantitative evaluation of glomerulosclerosis and damaged tubules was performed according to the report of Cao et al. (28). Imaging and analyses were performed using a Keyence BZ-X710 microscope (Keyence, Osaka, Japan). The images were randomly acquired, with 12–17 high-power fields collected for each mouse and then 21 glomeruli randomly traced within them. The percentage of glomerulosclerosis was computed from the area that was periodic acid-Schiff-positive in the total area of the same glomerulus. For analysis of damaged tubules, 10 high-power field images were randomly acquired of the renal cortex of each mouse. Three people counted damaged tubules, which revealed intraluminal casts, vacuolization, and droplets, and the percentage of damaged tubules was calculated using the number of damaged tubules divided by the total number of tubules. This analysis was performed in a blind manner.

Immunostaining Assay

Infiltration of macrophages was confirmed by immunostaining used anti-EMR1 antibody (F4/80; Dako, Santa Clara, CA). For each mouse, 10–12 high-power fields were randomly imaged and the number of F4/80+ cells was counted. The expression of nephrin and the renal localization of AGP were confirmed by immunofluorescence using nephrin and ORM1 antibodies. Deparaffinized sections were inactivated with Histo VT One (Nacalai Tesque, Kyoto, Japan) and incubated with nephrin (1:100; R&D Systems, Inc., Minneapolis, MN) or ORM1 antibody (1:50; Proteintech,
Figure 1. | Exogenous hAGP administration suppresses proteinuria and renal injury in ADR mice. (A) Schedule of human α1-acid glycoprotein (hAGP) treatment. BALB/c mice were administered with hAGP (5 mg/mouse) for 5 consecutive days after adriamycin (ADR) injection. Mice were sacrificed at day 21. (B) Urinary albumin to creatinine ratio at day 7 and day 21 after ADR injection. (C) Total plasma cholesterol at day 21 after ADR injection. (D) Representative periodic acid-Schiff-stained sections at day 21 after ADR injection, and quantitation of evaluation of glomerulosclerosis and tubule damage. Scale bars, 100 μm (control n=5; ADR and ADR+hAGP, n=6). (E) Representative
Kidney Cell Suspension

Kidney cell suspension for isolating renal macrophages was prepared according to the report of Cao et al. (29). Kidneys were harvested after perfusion with HBSS (without calcium) and DMEM containing 1 mg/ml collagenase IV and 100 μg/ml DNase I (Worthington Biochemical Corp, Lakewood, NJ). Kidney tissues were cut into a paste and shaken for 40 minutes at 37°C in DMEM containing collagenase IV and DNase I. Cell suspensions were passed through a 75-μm stainless mesh and centrifuged at 180 × g for 3 minutes. The pellets were suspended in red blood cell lysis buffer and centrifuged at 300 × g for 5 minutes. After washing with PBS(−), cell suspensions were incubated for 15 minutes at 4°C in blocking buffer (2% FBS and 2 mM EDTA) containing 10 μg/ml fragment crystallizable blocker. Cell suspensions were stained with anti-CD45.2, anti-F4/80, and antibodies to T cell, B cell, and natural killer cell lineages (TCRβ, TCRγδ, CD3e, CD19, and CD49b).

Isolation of Renal Macrophages

Kidney cell suspensions were gated on forward scatter and SSC to remove doublets, and exclude dead cells using propidium iodide. Then, cells were gated on leukocytes and SSC to remove doublets, and exclude dead cells using propidium iodide. After sorting, RNA isolation was performed using an NucleoSpin RNA XS column (Takara Bio, Otsu, Japan).

Quantitative RT-PCR

Total RNA from kidney and liver isolated by the RNAiso plus (Takara Bio) was reverse-transcribed with PrimeScript RT Master Mix (Takara Bio). Quantitative RT-PCR analysis was performed in an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA) using SYBR Premix Ex TaqII (Takara Bio). The quantitative RT-PCR method has been previously described (30) and the primers used are listed in the Supplemental Table. GAPDH was used as an internal control.

Statistical Analyses

The means for two group data were compared by the unpaired t test. The means for more than two groups were compared by one-way ANOVA followed by Tukey’s multiple comparison. Probability values of P<0.05 or P<0.01 were considered to be significant.

Results

Exogenous hAGP Administration Suppresses Proteinuria and Renal Injury in ADR Mice

After adriamycin injection (ADR mice), hAGP (5 mg/mouse) was intraperitoneally administrated for 5 consecutive days (Figure 1A, treatment schedule). The results show that the urinary albumin to creatinine ratio in ADR mice was significantly reduced from 7 to 21 days in the hAGP-treated group (Figure 1B). Furthermore, the increased plasma cholesterol level observed in the ADR group was significantly suppressed by hAGP administration (Figure 1C). The renal function of each mouse was measured (BUN and serum creatinine), but no significant differences were observed between the control, ADR, and ADR-treated with hAGP groups (Supplemental Figure 1B). Renal histologic analyses at day 21 showed that hAGP significantly suppressed glomerulosclerosis and tubular atrophy in ADR mice (Figure 1D). AGP distribution in the kidney was confirmed by immunofluorescence staining using anti-AGP antibody. Fluorescence was largely detected in the glomerulus in control mice, but was markedly decreased in the ADR-treated group. In contrast, similar levels of fluorescence in glomeruli were observed in control mice and ADR mice administered with hAGP (Figure 1E).

Because plasma AGP levels are upregulated during inflammation, we examined the change in levels of endogenous mouse AGP (mAGP) in plasma of ADR mice by western blotting. Although ADR injection increased the expression of AGP mRNA (Orm1, Orm2, and Orm3) in liver separated by 10% SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA) by wet electroblotting. The membranes were incubated in ORM1 antibody (1:5000; Proteintech). Antibody signals were detected with LAS-4000 minutes (GE Healthcare).

Kidney Cell Suspension

Kidney cell suspension for isolating renal macrophages was prepared according to the report of Cao et al. (29). Kidneys were harvested after perfusion with HBSS (without calcium) and DMEM containing 1 mg/ml collagenase IV and 100 μg/ml DNase I (Worthington Biochemical Corp, Lakewood, NJ). Kidney tissues were cut into a paste and shaken for 40 minutes at 37°C in DMEM containing collagenase IV and DNase I. Cell suspensions were passed through a 75-μm stainless mesh and centrifuged at 180 × g for 3 minutes. The pellets were suspended in red blood cell lysis buffer and centrifuged at 300 × g for 5 minutes. After washing with PBS(−), cell suspensions were incubated for 15 minutes at 4°C in blocking buffer (2% FBS and 2 mM EDTA) containing 10 μg/ml fragment crystallizable blocker. Cell suspensions were stained with anti-CD45.2, anti-F4/80, and antibodies to T cell, B cell, and natural killer cell lineages (TCRβ, TCRγδ, CD3e, CD19, and CD49b).

Isolation of Renal Macrophages

Kidney cell suspensions were gated on forward scatter and SSC to remove doublets, and exclude dead cells using propidium iodide. Then, cells were gated on leukocytes using an anti-CD45.2 antibody and T cell, B cell, and natural killer cell lineages excluded. Macrophages were isolated using anti-F4/80 antibody. After sorting, RNA isolation was performed using an NucleoSpin RNA XS column (Takara Bio, Otsu, Japan).

Cell Culture

Human THP-1 cells were grown in RPMI 1640 (Gibco, Grand Island, NY) containing 10% FBS, and 1% penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA) for 90 minutes at 4°C, followed by incubation with Alexa 488 donkey anti-goat IgG(H+L) (1:500; Abcam plc, Cambridge, UK) or Alexa 4,6-diamidino-2-phenylindole (H+L) (1:200; Thermo Fisher Scientific, Waltham, MA) for 40 minutes at room temperature. Images were obtained on a Keyence BZ-X710 microscope (Keyence).
Figure 1F, plasma mAGP tended to decrease between 7 and 21 days. In contrast to plasma levels, urinary mAGP was increased in ADR mice (Figure 1G). These data suggest that although mAGP production is increased in renal inflammation induced by ADR injection, plasma mAGP levels do not increase due to its higher levels of urinary excretion.

Exogenous hAGP Administration Exerts Anti-inflammatory Effects in Kidneys of ADR Mice

To evaluate the inflammatory condition, kidney sections were stained with anti-F4/80 antibody, a representative macrophage marker. The results show that hAGP administration significantly decreases macrophage infiltration.
It has been reported that the expression of TGF-β increases in damaged glomerular cells including podocytes, whereas IL-1β is produced by macrophages infiltrating into the glomerulus (31,32). Therefore, levels of mRNA for TGF-β and IL-1β were measured. As shown in Figure 2, B and C, TGF-β and IL-1β were significantly decreased in ADR mice treated with hAGP. Interestingly, hAGP administration significantly increased CD163 mRNA levels but did not increased CD206 mRNA levels in kidney. Both of these molecules are representative M2 macrophage markers (Figure 2, D and E), whereas the expression of inducible nitric oxide synthase (iNOS), which is used as an M1
macrophage marker, significantly decrease in the hAGP-administered group (Figure 2F). Moreover, in the hAGP-administered group, there was significantly increased expression of IL-10 compared with control mice (Figure 2G).

**Exogenous hAGP Administration Induces a CD163-Expressing Macrophage Phenotype at Day 7 in ADR Mice**

Although hAGP administration significantly decreased the urinary albumin to creatinine ratio at day 7 (Figure 1B), neither renal histologic damage nor infiltration of macrophages were found in any of the three groups (control, ADR, and ADR + hAGP) (Figure 3, A and C). Compared with control mice, nephrin expression as an indicator of podocyte injury was decreased in ADR mice treated with or without hAGP at day 7 (Figure 3B). On the other hand, the localization of AGP to the glomerulus was increased by hAGP administration in ADR mice at day 7 (Figure 3B). Under the same experimental conditions, hAGP administration suppressed adriamycin-induced TGF-β mRNA expression (Figure 3E), whereas no significant change in IL-1β mRNA expression was found in any of the three groups (Figure 3E). Because the infiltration of macrophages was not changed between the ADR and hAGP-treated ADR groups (Figure 3C), we isolated macrophages from kidney to evaluate the macrophage phenotype. As shown in Figure 4A, the mRNA expression of CD163 was decreased in macrophages from ADR mice, whereas hAGP administration tended to increase the mRNA expression of CD163 (Figure 4A). Next, we administered hAGP to control mice and measured the mRNA expression of CD163 and CD206 in the isolated macrophages. Although the glomerular fluorescence intensities of AGP were not significantly changed between control and hAGP-treated control mice, hAGP administration significantly increased CD163-expressing renal macrophages, even in the control mice (Figure 4B).

**hAGP Treatment Switches PMA-Differentiated THP-1 Cells into CD163-Expressing Macrophages**

To further study the effect of hAGP on the plasticity of macrophage phenotypes, PMA-differentiated THP-1 cells, a macrophage-like cell type, were treated with hAGP. Because hAGP circulates at 0.5 mg/ml in the plasma of healthy humans and levels increase to 1.0–2.5 mg/ml during inflammation, in vitro experiments were performed using these plasma concentrations of hAGP. Expression of CD163 mRNA was increased by hAGP in a dose-dependent manner, consistent with our previous results (23). In addition, we found that hAGP dose-dependently decreased the expression of CD206 and iNOS mRNA.
These in vitro data are consistent with the changes in renal expression of CD163 and iNOS mRNA shown in Figures 2 and 4. Interestingly, hAGP also dose-dependently increased the expression of IL-10 mRNA (Figure 5D). The changes of CD163 and CD206 expression in macrophages treated with hAGP were compared with those in IL-4/IL-13- or IL-4-treated macrophages, a representative M2 macrophage phenotype. IL-4/IL-13 or IL-4 treatment did not result in an increase in CD163 expression in macrophages, whereas CD206 expression was significantly increased (Figure 5, E and F).

The AGP-Induced CD163-Expressing Macrophage Phenotype has Anti-Inflammatory Properties

Next, we compared the phenotypic and anti-inflammatory properties of macrophages treated with AGP or IL-4/IL-13 under inflammatory conditions. As shown in Figure 6, hAGP- or IL-4/IL-13-induced macrophages maintained their phenotypes even after LPS challenge for 2 hours. The expression of IL-10 mRNA was measured after stimulation with LPS for 2 hours after treatment with either AGP or IL-4/IL-13. Interestingly, AGP-treated macrophages had a greater ability to induce IL-10 expression compared with IL-4/IL-13-treated macrophages (Figure 6D).

Discussion

In this study, we found that AGP potentiates the change of macrophage phenotype to CD163-expressing macrophages with anti-inflammatory functions in vivo, thereby reducing proteinuria, inflammation, and renal damage.

Although AGP-induced CD163-expressing macrophages have M2-like properties, they differ from IL-4/IL-13-induced M2 macrophages (Figure 5). Both CD163 and CD206 are normally used as markers of M2 macrophages (33,34), but the changes in expression of each marker are not always consistent during macrophage activation switching. For example, Porcheray et al. showed that IL-4 induced CD206 but not CD163 in human monocyte-derived macrophages. In contrast, IL-10 induced CD163 but not CD206 under the same conditions (33). These results are similar to the characteristics of hAGP-treated macrophages, as shown in Figure 5. Moreover, Furuhashi et al. (26) previously reported that adipose-derived stromal cells induce both CD163⁺ macrophages and CD206⁺ macrophages in crescentic GN rats, and that CD163⁺ macrophages had higher levels of IL-10 expression compared with CD206⁺ macrophages. This observation is in agreement with the our results, which show that hAGP-induced CD163⁺ macrophages have increased IL-10 production ability compared with IL-4/IL-13-induced CD206⁺ macrophages (Figure 6). Previously, we reported that hAGP increases CD163 and
IL-10 via weak stimulation of the TLR4/CD14 pathway (23). Subsequently, Nakamura et al. reported that ORM1, one of the AGP variants, induces M2b monocytes in vitro (35) and produces IL-10, which is known to be induced by TLR4 signals. Therefore, the AGP-induced CD163-expressing macrophages found in this study may correspond to M2b macrophages, which are also known as regulatory macrophages. It is recognized that regulatory macrophages produce high levels of IL-10, which limits inflammation by suppressing the production and activation of various proinflammatory cytokines. Although IL-4/IL-13-induced macrophages are also effective against inflammatory disease, they have the potential to induce renal fibrosis. This is supported by our recent study in which exogenously administered hAGP suppressed unilateral ureter obstruction-induced renal fibrosis in a mouse model (27).

Administration of hAGP inhibited urinary albumin in ADR mice between days 7 and 21. However, renal histologic damage and infiltration of macrophages in ADR mice were markedly weaker at day 7 than at day 21. Reflecting these data, the expression of IL-1β mRNA was not changed in ADR mice because IL-1β is produced by infiltrating glomerular macrophages. These results imply that other mechanisms may be involved in the decrease in urinary albumin in hAGP-treated mice at day 7. The glomerular barrier has both size and negative charge selectivity. Negative charge selectivity is a result of the endothelial cell surface layer (ESL), which functions to suppress albumin leakage into urine (39–41). Interestingly, AGP is localized at the ESL and contributes to the negative charge barrier in glomeruli because AGP is a highly negative charged protein resulting from the presence of sialylated N-glycans (pI of 2.8–3.8) (13).

Hjalmarsson et al. (42) reported that the administration of hAGP increased negative charges at the ESL and improved podocyte morphology in rats with puromycin aminonucleoside-induced nephrosis. Our study found that localization of AGP in the glomerulus was increased by hAGP treatment at day 7 (Figure 3B). Because it has been reported that damaged glomerular cells such as podocytes produced TGF-β, the increased glomerular localization of AGP might contribute to the inhibition of TGF-β mRNA expression in ADR mice (Figure 3D). In addition, these data suggest that administered hAGP might be localized for at least 3 days in the glomerulus and hence might contribute to the suppression of proteinuria at day 7. On the other hand, increased glomerular localization of hAGP was not found in hAGP-treated control mice (Figure 4B). This result indicates that the accumulation of AGP may require a decreasing negative charge in the glomerulus. Further investigation would be needed to elucidate the role of exogenously administered hAGP on glomerular negative charge selectivity in the future. In addition, the anti-AGP (ORM1) antibody used in this study has cross reactivity with hAGP and mAGP. Therefore, we could not clearly distinguish whether the localization of AGP in the glomerulus came from endogenous or exogenous AGP under our experimental conditions. Further study may be needed using hAGP- or mAGP-specific antibodies if such antibodies are commercially available.

In conclusion, this study indicates that AGP has a unique mechanism in regulating inflammation and has the potential to suppress urinary protein levels by targeting the ESL. The results showed that endogenous AGP could work to protect against glomerular disease. Because proteinuria is an independent risk factor for CKD progression and CVD.

Figure 6. The hAGP-induced macrophage phenotype demonstrates anti-inflammatory properties. (A–D) The expression of CD163, CD206, iNOS, and IL-10 mRNAs in PMA-differentiated THP-1 cells stimulated with LPS (100 ng/ml) for 2 hours after treatment with AGP (1.0 mg/ml), or IL-4 (10 ng/ml) and IL-13 (10 ng/ml), for 24 hours (n=4 per group). All data are mean ± SE; *P<0.05, **P<0.01; by one-way ANOVA and post hoc Tukey’s multiple comparison.
onset (2,3), it is speculated that alleviation of proteinuria and protection against renal tissue damage by AGP could be a new therapeutic strategy for CKD such as nephrosis, and diabetic nephropathy and CVD. Future investigation is needed to evaluate if AGP is also protective if given in a later phase of the disease.

Author Contributions

R. Fujimura, H. Watanabe, and T. Maruyama conceptualized the study; J. Bi, R. Fujimura, Y. Fujiwara, T. Imafuku, K. Kobayashi, T. Koga, H. Komori, M. Miyahisa, K. Nishida, and H. Maeda were responsible for investigation; R. Fujimura was responsible for formal analysis, visualization, and the original draft of the manuscript; H. Watanabe and T. Maruyama were responsible for funding acquisition, project administration, and supervision; R. Fujimura, Y. Fujiwara, T. Koga, H. Watanabe, and T. Maruyama were responsible for methodology; R. Fujimura, H. Watanabe, and T. Maruyama were responsible for data curation; R. Fujimura, Y. Fujiwara, T. Koga, H. Watanabe, and T. Maruyama were responsible for resources; and R. Fujimura, H. Watanabe, Y. Fujiwara, T. Koga, H. Maeda, M. Tanaka, K. Matsuishi, T. Wada, M. Fukagawa, and T. Maruyama reviewed and edited the manuscript.

Disclosures


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