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Human Adipose Tissue-Derived Stem Cells Inhibit Coronary Artery Vasculitis in a Mouse Model of Kawasaki Disease

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Background: Adipose tissue-derived mesenchymal stem cells (ADSCs) are used for the treatment of various diseases because of their rapid proliferation and high anti-inflammatory and tissue repair properties. Kawasaki disease is a systemic vasculitis with coronary arteritis and aneurysms occurring in pediatric patients. In this study, we examined serologically and pathologically whether the administration of human ADSCs (hADSCs) to a mouse model of Kawasaki disease could suppress vasculitis.

Methods: *Candida albicans* water-soluble fractions were intraperitoneally injected into DBA/2 mice for 5 consecutive days to generate a mouse model of Kawasaki disease. The model mice were intravenously administered hADSCs or phosphate-buffered saline (PBS). Serum samples collected on days 15 and 29 were used to compare cytokine levels. Mouse hearts dissected on day 29 were subjected to hematoxylin and eosin and immunohistological staining using Galectin-1 (Gal-1), a protein involved in cardiovascular homeostasis, and CD44, a cell-surface marker of hADSCs.

Results: Comparison of inflammation-related cytokines showed a significant decrease in IL-1α expression at day 15 ($P<0.05$) and IL-6 expression at day 29 ($P<0.01$) in the hADSCs-treated group compared to the PBS group. Evaluation by hematoxylin and eosin staining showed decreased inflammatory cell infiltration and a tendency towards increased Gal-1 expression in the hADSCs group. CD44 expression was not observed in both the groups. The survival curve showed that the hADSCs group had a significantly longer survival time ($P<0.05$).

Conclusions: The present experimental results indicate that hADSCs have an early anti-inflammatory effect, and that Gal-1 may be involved in preventing inflammation and reducing tissue damage.

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Key words: Adipose tissue, mesenchymal stem cells, cytokines, Gal-1, Kawasaki disease

Introduction

Kawasaki disease (KD) is a vasculitis syndrome of unknown cause most often occurring in infancy[1]. Some patients with KD develop coronary aneurysms. The goal of acute treatment in KD is to end the inflammation at an early stage and suppress the occurrence of severe coronary vasculitis and aneurysms. Intravenous infusion of immunoglobulin preparations and moderate oral administration of aspirin are routinely used as first-line treatment. In cases where therapy is ineffective, various treatments, such as immunosuppressants and steroid pulses, are considered[2]. Despite these treatments, cases of severe coronary vasculitis and aneurysms with residual effects may occur, and various new treatment methods are being investigated.

Recently, the efficacy of cell therapy using bone marrow-derived mesenchymal stem cells has been demonstrated for the treatment of intestinal inflammatory diseases[3] and severe graft-versus-host disease associated with hematopoietic stem cell transplantation[4]. Adipose

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tissue-derived stem cells (ADSCs) contain a large amount of mesenchymal stem cells, show fast cell proliferation, and secrete large amounts of anti-inflammatory and tissue-regeneration promoting factors[4,5]. In addition, owing to their low immunogenicity, ADSCs can be administered widely, regardless of major histocompatibility complex (MHC) compatibility[3,5]. By collecting and banking human ADSC (hADSC)-containing tissues, which were previously considered medical waste, it may be possible to quickly provide cell therapy to critically ill patients.

In a previous study conducted at our institution[6], tail vein injection of mouse adipose tissue-derived stem cells predominantly suppressed coronary artery vasculitis in a mouse model of KD created using *Candida albicans* water-soluble fractions (CAWS)[7,8]. In the KD model mice, the coronary arteries and aortic root near the coronary artery orifice are the most affected, similar to what occurs in patients with KD[7].

In this study, we investigated whether hADSCs can suppress coronary artery inflammation in a mouse model of KD. The levels of serum cytokines and chemokines, such as IL-1 and IL-6, which are key cytokines in KD vasculitis and aneurysm formation, were examined. In addition, we investigated the relationship between hADSCs and Galectin-1 (Gal-1)[9,10], a family of galactoside-binding lectins that are widely expressed in a variety of cells and tissues and are key mediators of cardiovascular homeostasis[11]. Gal-1 promotes healing of acute and chronic inflammation by reconditioning innate and adaptive immune responses[12,13]. Moreover, Gal-1 has emerged as a promising therapeutic option for 3-D wound healing, as confirmed in ADSC 3-D medium[14]. In this study, we investigated the relationship between Gal-1 expression in cardiac tissues and serum Gal-1 levels following administration of hADSCs. This study aimed to demonstrate the efficacy and action mechanism of hADSCs in preventing the development of severe coronary artery vasculitis in a mouse model of KD, paving the way for potential clinical applications in the future.

Materials and Methods

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. The study protocol was approved by the Animal Care and Use Committee of Nippon Medical School (Approval No. 2021-043).

Animals

Four-week-old DBA/2 mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions according to the Guidelines for Animal Care of the Tokyo National Institute of Infectious Diseases (NIID).

Preparation of CAWS

CAWS were prepared using the method previously described with modifications[7,8]. Briefly, *Candida albicans* water-soluble fractions were prepared from the *Candida albicans* NBRC 1358 strain, as previously described. Briefly, 5 L of C-restricted medium was incubated for 2 days at 27°C while supplying air at a rate of 5 L/min, and the mixture was swirled at 400 rpm. After incubation, an equal volume of ethanol was added and the mixture was left overnight. The precipitate was then dissolved in 250 mL of distilled water, ethanol was added, and the mixture was incubated overnight. The resultant precipitate was collected and dried by using acetone.

hADSCs

hADSCs were purchased from Lonza (Tokyo, Japan) hADSCs stored frozen at −80°C were thawed and sown into a flask. The cell culture was started at 37°C in a 5% CO_2 atmosphere using ReagentPack™ subculture reagent (Lonza, Tokyo, Japan) with ADSC-BulletKit™ (Lonza, Tokyo, Japan). The culture medium was changed every 2 days to grow the cells.

Induction of Vasculitis and Administration of hADSCs

To establish the KD mouse model, DBA/2 mice were injected with *Candida albicans* water-soluble fractions (0.5 mg/mouse/day) intraperitoneally on five consecutive days starting on day 0 of the experiment. hADSCs (2×10^5 cells) were injected via the tail vein into KD mice on day 8. Subsequently, the inhibitory effects on KD-associated vasculitis were compared between the non-treated phosphate-buffered saline (PBS; Wako, Osaka, Japan) group and the single hADSCs-administered group. Serum samples were collected on day 15. On day 29, the mice were positioned in the supine position with their extremities immobilized and anesthetized with 0.1 mg/100 g pentobarbital. Subsequently, thoracotomy was performed to remove the heart, and blood samples were collected on the same day.

Histological Evaluation

The heart tissue was fixed in 10% neutral buffered formalin solution for approximately 7 days. After fixation, they were embedded in paraffin. Serial sections were prepared with a thickness of 5 μm and stained with hematoxylin and eosin (HE). Sections showing both the aortic

root and the most severe inflammatory cell infiltration were used. The area (μm^2) of inflammatory cell infiltration was measured using a hybrid cell count system (KEYENCE, Osaka, Japan) and a KEYENCE BZX analyzer (KEYENCE, Osaka, Japan). The ratio of the inflammatory cell infiltration area to the total tissue area of the aortic root was calculated and the inflammatory cells causing the most severe infiltration were determined[15].

After HE staining was completed, immunostaining was performed using samples from four mice in the PBS group and five mice in the hADSCs group. hADSCs are highly positive for the mesenchymal stem cell surface marker CD44[16]. Thus, in our experiments, we used CD44 as a marker for hADSCs. Primary antibodies against CD44 (Abcam, ab51037, Cambridge, UK) and Gal-1 (BIOKE, #13888, Leiden, The Netherlands) were used. Human tonsil tissue was purchased from Japan Genetics Corporation (Tokyo, Japan) and used as a positive control for CD44.

Measurement of the Levels of Serum Cytokines and Chemokines

The levels of plasma cytokines and chemokines were measured using a Bio-Plex system (Bio-Rad, Hercules, CA, USA); 50-μL serum aliquots were collected from peripheral blood and diluted fourfold with dilution solution. The diluted samples were analyzed using the Bio-Plex Pro™ Mouse Cytokine 23-plex assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA, USA). We assayed the following 23 cytokines and chemokines: IL-1-alpha(α); IL-1-beta(β); IL-2; IL-3; IL-4; IL-5; IL-6; IL-9; IL-10; IL-12p40; IL-12p70; IL-13; IL-17; eotaxin; granulocyte-colony stimulating factor (G-CSF); granulocyte macrophage colony-stimulating factor (GM-CSF); interferon-gamma (IFN-γ); keratinocyte-derived cytokine (KC); monocyte chemotactic and activating factor (MCP-1); macrophage inflammatory protein-1α (MIP-1α); MIP-1β; regulated upon activation normal T-cell expressed and presumably secreted (RANTES); and TNF-α. The concentrations of cytokines and chemokines were calculated using Bio-Plex Manager software (version 3.0; Bio-Rad, Tokyo, Japan) with a five-parameter curve-fitting algorithm that was applied for standard curve calculations.

ELISA

Gal-1 levels were analyzed using enzyme-linked immunosorbent assay (ELISA) (Boster Biological Technology, Pleasanton, CA, USA) in serial plasma samples from four mice in the hADSCs group and four mice in the PBS group, according to the manufacturer's instructions.

Comparison of Survival Rate

Mice were analyzed separately to compare the survival time between non-sacrificed animals in the hADSCs (n=5) and PBS (n=5) groups. Based on these results, Kaplan-Meier curves were generated and compared using Excel (BellCurve, SSRI, Tokyo, Japan).

Statistical Analysis

All values are presented are the medians (ranges). Comparisons of inflammatory areas and cytokine levels between the two groups were performed using the Mann-Whitney U test. Numerical values are presented as median and interquartile range (IQR).

Survival time was estimated using the Kaplan-Meier method, and survival estimates were compared using log-rank testing. Statistical analyses were performed using Excel statistical software (BellCurve, SSRI, Tokyo Japan). Statistical significance was set at $P<0.05$.

Results

We weighed the mice on days 0, 15 and 29. The mean body weight of the PBS group (N=20) was 19.3 (13.6-24.6) g on day 0; 21.8 (18.4-25.3) g on day 15; and 23.8 (21.5-27) g on day 29. The mean body weight of the hADSCs group (N=23) was 20.3 (13.6-27) g on day 0; 22.4 (19.2-28.4) g on day 15; and 23.5 (20.3-29.3) g on day 29.

Decreased Inflammatory Cell Invasion Area in the hADSCs Group

The tissue collected from the aortic origin in mice on day 29 was stained with HE (**Fig. 1a-f**) and the infiltration of inflammatory cells around the aortic valve was analyzed (**Fig. 2**). HE staining showed a prominent reduction in inflammatory cell infiltration in the hADSCs group (**Fig. 1a-c**) compared to the PBS group (**Fig. 1d and e**). In an analysis using the hybrid cell count system, the inflammatory area was smaller in the hADSCs group than in the PBS group (**Fig. 2a**), and the proportion of inflammatory cells was also predominantly reduced (**Fig. 2 b**) ($P<0.01$). Specifically, the inflammatory area in the hADSCs group (n=10) was 1,256 μm^2 (796-1,621 μm^2) and that in the PBS group (n=10) was 1,466 μm^2 (1,009-1,782 μm^2), with no statistically significant difference. The percentage of inflammatory cell infiltration was 24% (12.8-32%) in the hADSCs group and 52.5% (42.5-59.2%) in the PBS group, showing a significant difference ($P<0.01$).

High Gal-1 Expression in the hADSCs Group

Immunostaining showed that Gal-1 was highly expressed in the hADSCs group (**Fig. 3a**) compared to the PBS group (**Fig. 3b**). Specifically, the results showed that increased expression was localized at the origin of the

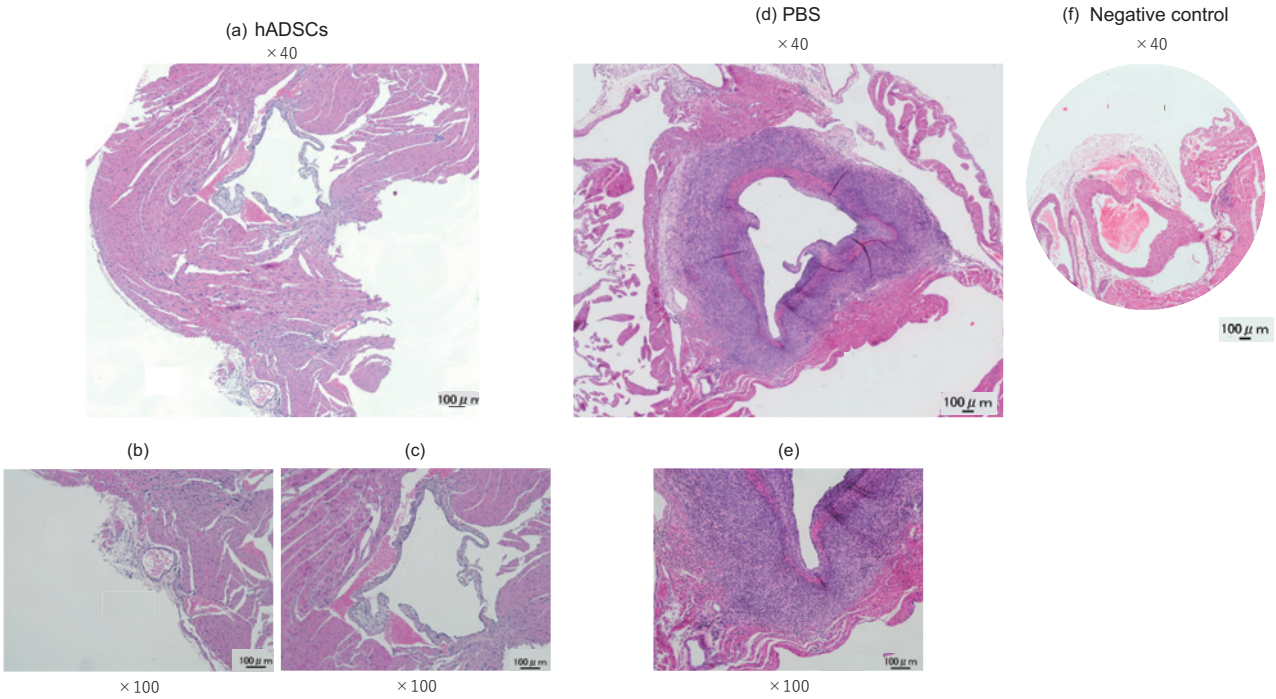

Fig. 1 Hearts of KD model and Negative control DBA/2 mice were dissected on day 29 and HE staining was performed to evaluate inflammation. hADSCs-treated at (a) 40 × and (b, c) 100 ×; PBS-treated at (d) 40 × and (e) 100 ×; and (f) Negative control DBA/2 in which CAWS were not injected at 40 ×.

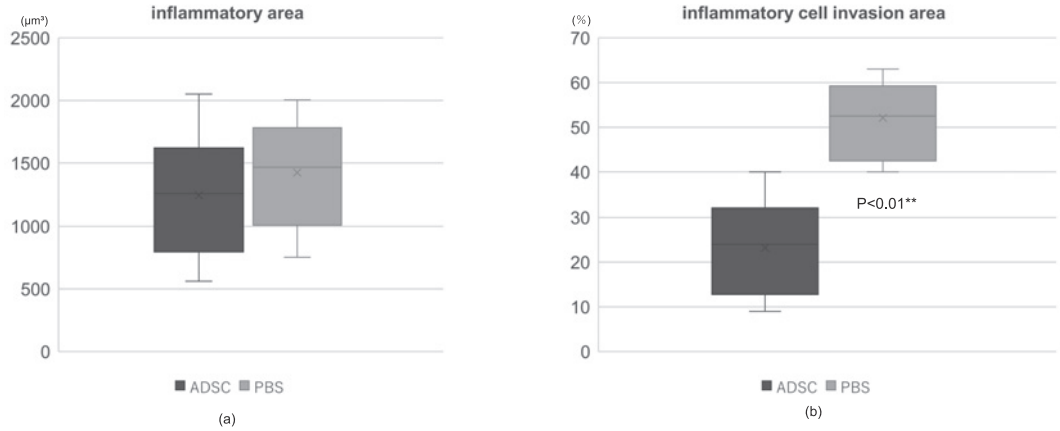

Fig. 2 The inflammatory area around the aortic valve of KD model mouse was compared between the hADSCs (n=10) and PBS groups (n=10) (a). The percentage of inflammatory cells in the inflammatory area was compared between the hADSC and PBS groups and the inflammatory area of the hADSC group was significantly smaller than that of the PBS group (P<0.01) (b).

aortic valve and pericoronary arteries in the KD model mice. The percentage of Gal-1 positive cells was 7.8% (4-11%) in the hADSCs group and 3.8% (3-6%) in the PBS group. Gal-1 tended to be upregulated in the hADSC group, but this difference was not significant(p=0.066).

CD44 was not expressed in the tissues of either the hADSCs (**Fig. 4a**) or the PBS group (**Fig. 4b**); human tonsil tissue showed uptake of CD44 for positive control (**Fig. 4c**).

No Significant Serological Differences in Serum Gal-1 between Groups

As immunostaining studies showed that Gal-1 was highly expressed in hADSCs, Gal-1 levels in mouse sera from both groups collected on days 15 and 29 were analyzed by ELISA. No significant differences in the Gal-1 values were obtained both on days 15 and 29.

Specifically, the level of Gal-1 in the PBS group was 11 ng/mL (7-13 ng/mL) on day 15 and 12 ng/mL (11-12

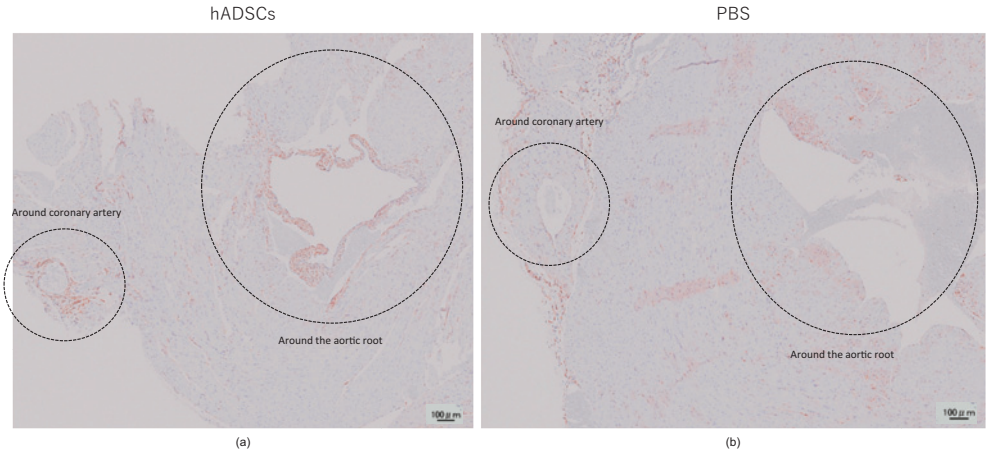

Fig. 3 Gal-1 expression was compared between the hADSCs group (n=5) (a) and the PBS group (n=4) (b) in the pericoronary artery and aortic root areas.

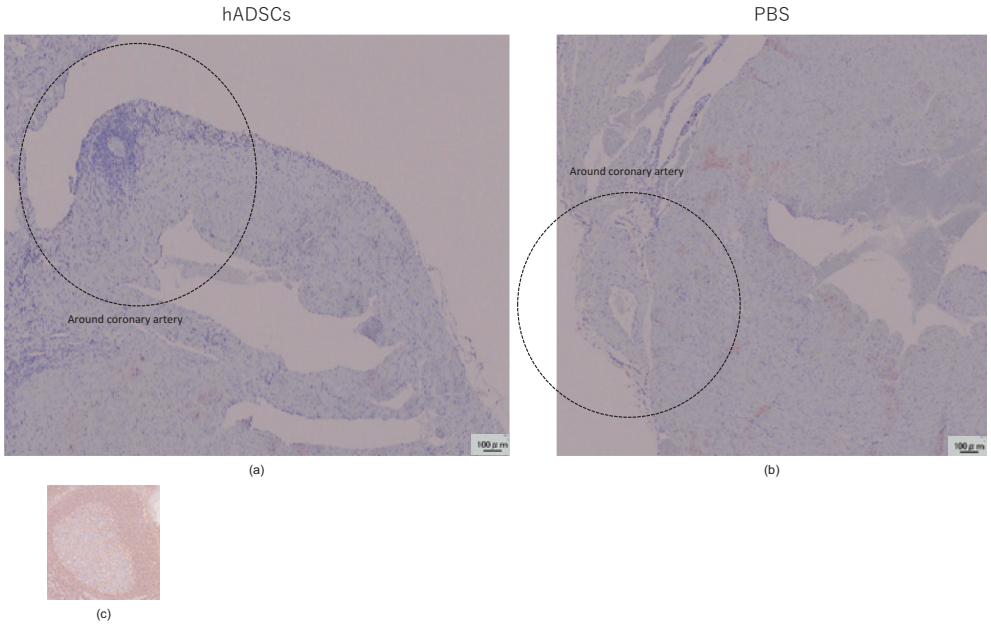

Fig. 4 CD44 expression was compared between the hADSC (n=5) (a) and PBS (n=4) groups (b). Human tonsil tissue was used as a positive control (c).

ng/mL) on day 29; the level of Gal-1 in the hADSCs group was 9 ng/mL (8-11 ng/mL) ng/mL on day 15 and 8 ng/mL (6-12 ng/mL) on day 29.

Decreased Expression of IL-1α (Day 15) and IL-6 (Day 29) in hADSCs Group

Cytokine measurements performed using blood samples collected on days 15 and 29 showed that IL-1α levels were predominantly reduced in the hADSCs group compared to the PBS group on day 15 ($P<0.05$). IL-6 increased between day 15 and day 29 in the PBS group, and there was a significant difference in IL-6 levels between the hADSCs and PBS groups on day 29 ($P<0.01$). There was no significant difference in IL-1β levels between the two groups.

Specifically, the blood concentration of IL-1α in the hADSCs group was 38.95 pg/mL (32.72-44.05 pg/mL) on day 15 and 12.88 pg/mL (2.8-19.93 pg/mL) on day 29; IL-1α concentration in the PBS group was 56.02 pg/mL (19.6-92.35 pg/mL) on day 15 and 19.19 pg/mL (7.24-46.1 pg/mL) on day 29.

The blood concentration of IL-6 in the hADSCs group was 11.90 pg/mL (7.61-20.58 pg/mL) on day 15 and was 14.36 pg/mL (10.72-20.89 pg/mL) on day 29; IL-6 concentration in the PBS group was 17.08 pg/mL (12.43-27.07 pg/mL) on day 15 and 37.64 pg/mL (13.15-50.38 pg/mL) on day 29. The blood concentration of IL-1β in the hADSCs group was 2.05 pg/mL (1.63-2.78 pg/mL) on day 15 and 2.12 pg/mL (2.05-2.35 pg/mL) on day 29; IL-

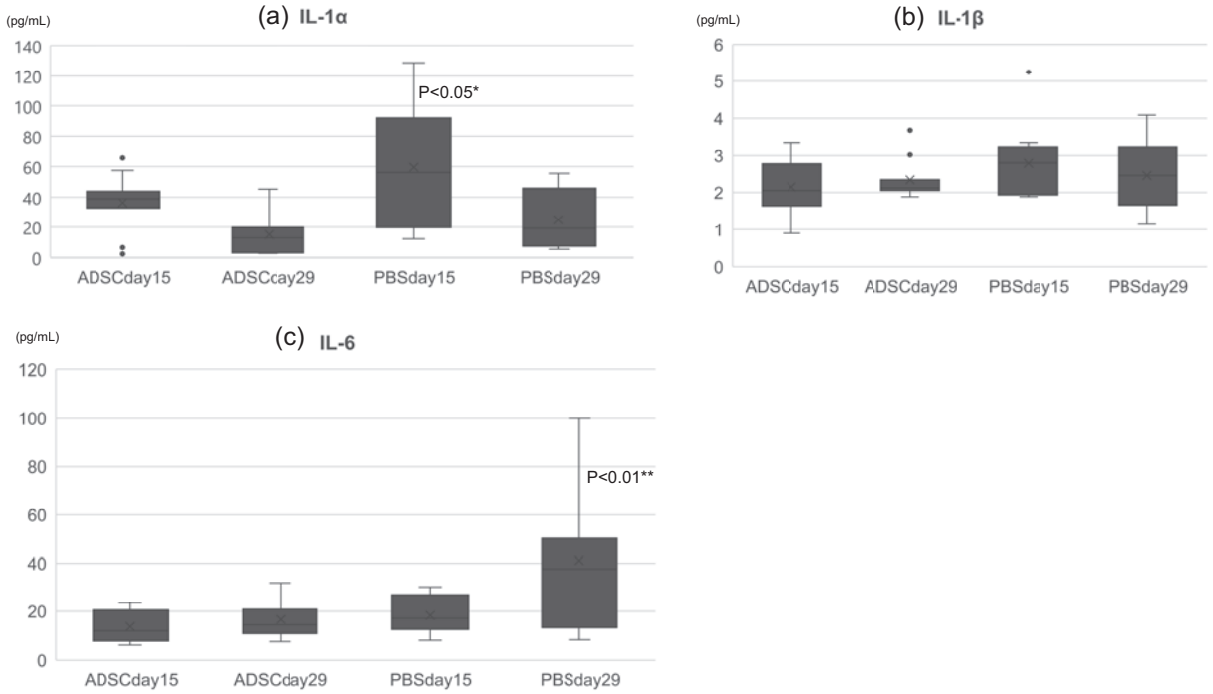

Fig. 5 Blood samples were collected from the tail vein of KD model mice on days 15 and day 29. The levels of IL-1α, IL-1β, and IL-6 on days 15 and day 29 in both groups (n=11 and n=11) are shown.
(a): IL-1α levels were significantly higher in the PBS group than in the hADSC group at day 15 (P<0.05).
(b): IL-1β levels were not significantly different between the groups.
(c): IL-6 levels were significantly higher in the PBS group than in the hADSC group at day 29 (P<0.01).

1βconcentration in the PBS group was 2.8 pg/mL (1.93-3.24 pg/mL) on day 15 and 2.46 pg/mL (1.65-3.24 pg/mL) on day 29 (**Fig. 5**).

Increased Survival Rate in hADSCs Group

The survival curve showed that the hADSCs group had a predominantly higher survival rate than the PBS group (P<0.01). At day 70, 80% of the mice in the PBS group had died, whereas 80% of the mice in the hADSCs group were alive (**Fig. 6**).

Discussion

In a previous study conducted at our institution[6], mouse ADSCs were shown to suppress CAWS-induced vasculitis. ADSCs not only reduced inflammation around the aortic valve but also decreased the expression of inflammatory cytokines and prolonged survival. Previous studies using mouse models have reported pharmacological effects when hADSCs were administered, owing to the low immunogenicity of hADSCs[3,5]. It is expected that low immunogenicity contributes to low production of immune sources and that hADSCs are viable when injected into mice. In this study, we investigated whether hADSCs suppress the development of coronary artery vasculitis in a mouse model of KD.

Pathological analysis showed that hADSCs suppressed inflammation in a mouse model of severe coronary artery vasculitis, especially by reducing the inflammatory cell invasion rate at day 29. As will be discussed later, this is supported pathologically by the fact that suppression of the expression of IL-1α, the upstream cytokine of inflammation[17], was achieved in the hADSCs-treated group on day 15, with suppression of the increase in IL-6 expression on day 29. Thus, treatment with hADSCs reduced the extent and degree of inflammation. CD44, which was used as a marker of hADSCs in this study, was not expressed around the aortic valve in both the hADSCs-treated group and the PBS group. Mesenchymal stem cells are mobilized to inflammatory sites by a process called homing[18]. As CD44 expression was not observed in the tissues collected on day 29, we opined that further investigation in the early inflammatory tissues was necessary. In contrast other studies indicate that the administered hADSCs did not directly reach the inflammatory tissues, rather exerting their effect through remote signals. Previous studies have shown that ADSCs-derived exosomes attenuate myocardial infarction injury or exert their anti-inflammatory effects by promoting miRNA-205-mediated cardiac angiogenesis[16]. Moreover, ADSCs-derived exosomes have been shown to have a cardioprotective effect by activating the Wnt/β-catenin signaling

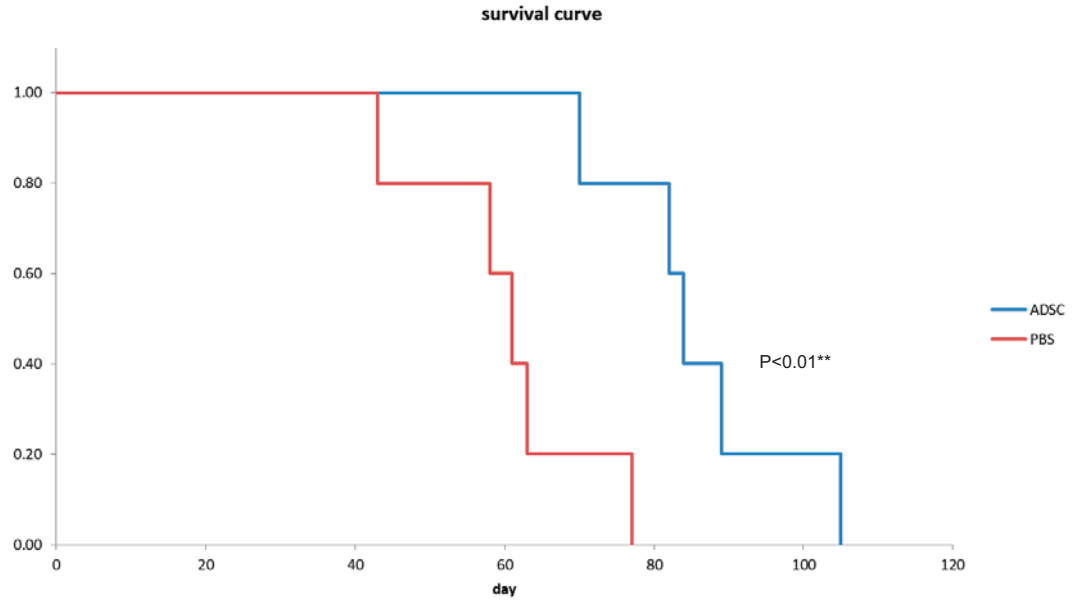

Fig. 6 Kaplan-Meier curves comparing the survival rates of KD model hADSCs-treated (n=5) and PBS-treated (n=5) mice. The hADSC group achieved longer survival than the PBS group (P<0.05).

pathway when ADSCs are administered[19]. We aim to conduct a more in-depth investigation to ascertain whether hADSCs exhibit homing to inflammatory tissues, thereby exerting direct effects, or if they promote tissue repair through signal transduction mechanisms in the future.

Gal-1 is a glycolipid involved in the inhibition of arachidonic acid release, and high expression of Gal-1 contributes to the suppression of inflammation[20]. Recently, it has been suggested that Gal-1 may act as a ligand for leukocyte binding and migration in vascular endothelial cells[18]. Thus, Gal-1 exerts immune regulatory effects in animal models of acute/chronic inflammation and plays a role in repairing injured tissue[9]. Several studies have demonstrated the immunomodulatory functions of Gal-1, and because of its inhibitory effects on neutrophil and T cell trafficking and inducing effect on T cell apoptosis, it may have anti-inflammatory effects[9]. In a mouse model of cardiac restraint, Gal-1 contributes significantly to the maintenance of pericardial homeostasis after inflammation[11]. Based on the above findings, we hypothesized that Gal-1 contributes to the suppression of inflammation around the aortic valve in CAWS mice following intravenous injection of hADSCs. In the current study, we showed that in CAWS mice, after intravenous hADSCs infusion, there was a trend towards increased Gal-1 expression in the aortic valve and pericoronary arteries. The high expression of Gal-1 at inflammatory sites in CAWS mice suggests that tissue repair is functional after hADSC administration.

As immunostaining showed that Gal-1 was highly expressed in the aortic root and pericoronary arteries, we examined whether serum Gal-1 on days 15 and 29 was significantly different between the hADSCs-treated group and the PBS group, but no significant difference was found. Studies that have serologically evaluated the relationship between serum Gal-1 levels and inflammation include one study that evaluated inflammation after surgery[21] and one study of inflammation in the acute phase of myocardial infarction[22]. A previous study evaluated the increase in serum Gal-1 levels up to 24 h after surgery, and a latter study evaluated the increase in serum Gal-1 levels up to 24 h after myocardial infarction. No study has measured serum Gal-1 levels for more than a week after anti-inflammatory treatment, as in the present study. Based on the present results, serum Gal-1 may not be used to evaluate the treatment after administration of hADSCs for more than a week. The possibility that hADSCs and Gal-1-mediated tissue repair may be important in the mouse model of Kawasaki disease will be further investigated.

The Involvement of innate immunity in the pathogenesis of KD, which causes an excessive immune response, has attracted attention, and it is known that neutrophil- and macrophage-based inflammation is induced in the coronary arteries. IL-1, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α are the most important cytokines identified as being involved in the onset of KD and aneurysm formation[23]. IL-1 has a major inflammatory effect on the coro-

nary arteries in KD[17,24,25]. IL-6 plays a major role in the pathophysiology of KD via megakaryocyte maturation[23], leading to thrombocytosis. In our study, hADSCs were used to predominantly suppress IL-1α and IL-6, which are the mainstream inflammatory cytokines in KD. In particular, IL-1α could be suppressed at day 15, thus an early therapeutic effect may be expected. IL-1α suppression is considered to contribute to the suppression of inflammation at an early stage, which is the purpose of KD treatments. On the other hand, studies of Il-1β inhibitors in KD model mice have shown that suppression of Il-1β achieved suppression of inflammation[26]; however, no changes in the level of Il-1β were observed in this study. Another study reported that etanercept, a TNF-αinhibitor, suppresses arteritis in KD model mice[27]. In this study, TNF-α levels were also measured, but many of the values were out of range and could not be accurately assessed. We assume that the achievement of early suppression of IL-1α, the upstream of inflammation, contributed to the suppression of IL-6 in the hADSCs group on day 29.

Since the medium-term lethality rate of the KD model mice used in this study is almost 100%[4], it was also possible to evaluate the efficacy of vasculitis treatment by observing a prolonged survival rate. Thus, the survival curves showed a significantly higher survival rate in the hADSCs group, suggesting that hADSCs are effective in the treatment of KD. It is possible that hADSCs contributed to the prolongation of survival by suppressing severe inflammation around the aortic valve and coronary arteries in pathologically confirmed KD model mice.

One limitation is that we did not assess safety issues, adverse events, or causes of death in this study. It is important to determine whether the causes of death from hADSCs therapy are associated with coronary or adverse events (i.e., thromboembolic events). Secondly, a detailed examination of histopathological differences between the two groups was not feasible. Specifically, the type of inflammatory cells and the site of infiltration warrant further study. Finally, serum Gal-1 was measured on days 15 and 29, after inflammation had occurred, and it is possible that serum Gal-1 levels may change when evaluation is performed early in the inflammatory process.

In conclusion, administration of hADSCs suppressed CAWS-induced vasculitis. hADSCs inhibited inflammatory cell infiltration in KD model mice, resulting in early suppression of inflammatory cytokines and improved survival. As in other cardiovascular diseases, Gal-1 may be involved in the suppression of inflammation following hADSC administration. hADSCs are easily generated, well-tolerated, and have great potential for future therapies in patients with severe KD.

Author contributions: R.F. performed the experiments and analyzed the data. T.U. designed the study. R.F. and T.U. wrote the manuscript. T.I. and R.M. contributed to data analysis. R.F. and Y.I. critically reviewed the manuscript and supervised the study. N.M. provided CAWS. All the authors have read and approved the final manuscript.

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Conflict of Interest: The authors declare no conflict of interest.

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