PEER-REVIEWED ORIGINAL ARTICLE

Administration of recombinant single chain fragment of variable region (hScFv) of IgG suppresses development of murine vasculitis induced with *Candida albicans* water-soluble fraction: An animal model of Kawasaki disease

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Abbreviations

ANCA: anti-neutrophilic cytoplasmic antibody, CADS: *Candida albicans* derived substances, CAWS: *Candida albicans* water-soluble fraction, HE: hematoxylin and eosin, hScFv: human single chain fragment of variable region, IgG: immunoglobulin G, IVIg; intravenous immunoglobulin, MPO: myeloperoxidase, SCG/Kj: spontaneous crescentic glomerulonephritis-forming/Kinjoh.

Abstract

***Background*: High-dose intravenous immunoglobulin (IVIg) treatment has been used for therapy of Kawasaki disease and other diseases. Due to the risks of immunoglobulin preparations such as undetectable infection included in donated blood and unknown mechanisms, recombinant immunoglobulins are required. *Candida albicans* water-soluble fraction (CAWS)-induced vasculitis, one of the murine model of Kawasaki disease vasculitis is thought to be suitable for examining the therapeutic effect of recombinant immunoglobulins, because IVIg treatment to CAWS-induced vasculitis by human immunoglobulin was effective. In the present study, we performed histological investigation of inhibitory effect of the recombinant single chain fragment of variable region (hScFv) of IgG on murine model of Kawasaki disease vasculitis.**

***Methods*: The incidence of panvasculitis and histological severity (i.e., the extent of the lesion and the degree of inflammation) of vasculitis were compared among each experimental group in CAWS-induced vasculitis in C57BL/6 mice. The following experimental groups were employed: No treatment (only CAWS injection), solvent, hScFv 2.25 mg/Kg/day, hScFv 4.5 mg/Kg/day, hScFv 9 mg/Kg/day, human native IgG 400 mg/Kg/day.**

***Results:* The incidence of panvasculitis showed in each group as follows. No treatment: 66.7% (4/6), solvent: 40% (2/5), hScFv 2.25 mg/Kg/day: 60% (3/5), hScFv 4.5 mg/Kg/day: 25% (1/4), hScFv 9 mg/Kg/day: 0% (0/1), and native human IgG 400 mg/Kg/day: 40% (2/5), respectively. Panvasculitis was developed in all treated groups other than hScFv 9 mg/kg/day, however the incidence of groups treated with hScFv 4.5 mg/Kg/day and native IgG 400 mg/Kg/day tended to be slightly lower than no treatment group. The extent of the lesion showed in each group as follows. No treatment: 2.33 ± 2.07, solvent: 1.80 ± 2.17, hScFv 2.25 mg/Kg/day: 1.20 ± 1.30, hScFv 4.5 mg/Kg/day: 1.00 ± 1.41, hScFv 9 mg/Kg/day: 1.00, and native IgG 400 mg/Kg/day: 1.80 ± 1.30, respectively. The degree of inflammation showed in each group as follows: No treatment: 6.17 ± 6.52, solvent: 4.80 ± 6.61, hScFv 2.25 mg/Kg/day: 3.60 ± 3.91, hScFv 4.5 mg/Kg/day: 2.00 ± 2.83, hScFv 9 mg/Kg/day: 1.00, and native IgG 400 mg/Kg/day: 4.00 ± 4.18, respectively. There was no significant inter-group variation, the extent of the lesion and degree of inflammation in each treatment group tended to be smaller and milder than those of no treatment group.**

***Conclusion*: The present study suggests that the hScFv has a slightly suppressive effect on development of vasculitis in animal model of Kawasaki disease vasculitis.**

Keywords: Kawasaki disease, hScFv of IgG, Vasculitis, Coronary arteritis, Candida

Introduction

Kawasaki disease is an acute febrile disease of children with unknown cause included in the vasculitis syndrome. Coronary arteries are frequently involved and the formation of coronary artery aneurysms caused by coronary arteritis is the leading cause of ischemic heart disease in children and has a significant impact on the prognosis of the affected child[1]. It is observed that severe inflammatory cell infiltration mainly consisting of macrophages and neutrophils associated with proliferative changes of fibroblasts and capillaries at the site of vasculitis[2-5]. For the first choice in the therapy, high-dose intravenous immunoglobulin (IVIg) treatment has been used[6,7]. Thus, the 24th National Survey of Kawasaki Disease in Japan revealed that 93.5% of patients have been treated with IVIg therapy, showing that efficacy is widely known[8]. However, the mechanism of the inhibitory effect of the IVIg therapy on the vasculitis is still unknown.

Immunoglobulin preparations for IVIg are used not only for Kawasaki disease but also for severe infections and various autoimmune diseases. Although domestic supply of immunoglobulin preparations is secured, the demand is increasing year by year. In addition, immunoglobulin preparations derived from donated blood of healthy volunteers may have a risk for unknown infections. Furthermore, the preparation of immunoglobulins is expensive. Under such circumstances, recombinant immunoglobulins are expected. Kameoka et al[9]. attempted recombinant immunoglobulins, in which the polyclonal mix batch of recombinant single chain fragment of variable region (hScFv) of IgG having VH-CH1 hinge composition was applied to a model mice SCG/Kj for spontaneous development of crescentic glomerulonephritis. The mouse shows an anti-neutrophilic cytoplasmic antibody (ANCA)-related vasculitis. It has been reported that hScFv has a suppressive effect on development and production of myeloperoxidase (MPO)-ANCA, on the other hand human native IgG has slightly suppressive effect on crescentic formation, production of MPO-ANCA[9,10]. However, inhibitory effect of hScFv on other vasculitis model have not been investigated.

A murine model of systemic vasculitis by using *Candida albicans (C. albicans)*-derived substances (CADS) has been established in 1979 by Murata *et al.*[11]. CADS was prepared by alkali extraction of *C. albicans* isolated from the stool of the patients with Kawasaki disease. Later, Ohno clarified that a *C. albicans* water-soluble fraction (CAWS) eluting in the culture supernatant of *C. albicans* grown in a fully synthetic medium also induces vasculitis similar to that in Murata's model[12].

In these models, the lesion distribution and histological images of the vasculitis are similar to those of the vasculitis in Kawasaki disease. The aortic root and the bifurcation of coronary artery are frequently involved by severe inflammatory infiltration mainly consist of neutrophils and macrophages[11-13]. Moreover, the model is thought to be suitable for examining the therapeutic effect of recombinant immunoglobulins, because IVIg treatment to CAWS-induced vasculitis by human immunoglobulin is effective[14].

In the present study, we performed histological investigation of inhibitory effect of hScFv which has been developed by Kameoka *et al*.[9] to the CAWS-induced vasculitis.

Materials and methods

***Animals*:** Mice, C57BL/6, male, 4 weeks of age purchased from Japan SLC, Inc. were used. All animal experiments were implemented following the guidelines from the University of Tokyo Pharmacy and Life Sciences (YAKU 10-47).

***Induction of vasculitis*:** CAWS were used for vasculitis inducer according to the previous report[12,13]. One mg of CAWS suspension in 0.2 mL of PBS and injected intraperitoneally into mouse in a day for 5 consecutive days. The mice were sacrificed 28 days after completion of the continuous inoculation of CAWS under dry ice.

***Preparation of hScFv*:** The polyclonal ScFv antibody mixture was prepared as described elsewhere[9].

***Administration of hScFv for treatment*:** hScFv was intraperitoneally administered for 5 consecutive days after the end of continuous inoculation of CAWS. The dosage of hScFv was 2.25, 4.5, and 9 mg/Kg/day, respectively. Because in SCG/Kj mice, an anti-inflammatory effect of hScFv at a concentration of 20-40 mg/Kg/day has been confirmed[9]. In order to examine the difference in therapeutic effect between hScFv and human immunoglobulin, human native IgG (Nihon Pharmacy Company, Osaka, Japan) was intraperitoneally administered at 400 mg/Kg/day for 5 consecutive days. This dose has been validated for the treatment of Kawasaki disease.

***Experimental group*:** Five groups shown below were set. 1) No treatment (n = 6), 2) Solvent (n = 5), 3) hScFv, 2.25 mg/Kg/day (n = 5), 4) hScFv 4.5 mg/Kg/day (n = 5) , 5) hScFv 9 mg/Kg/day (n = 5), 6) native IgG, 400 mg/Kg/day (n = 5).

***Histological evaluation of vasculitis*:** Histological assessments were carried out in accordance with the previously described methods[13,14]. After the mice were sacrificed, serial sections of the coronary arteries and the aortic root were stained by the hematoxylin and eosin (HE), elastica van Gieson (EvG), and Azan Mallory (AM) staining methods. The stained specimens were carefully examined for inflammatory lesions of the vessel wall under a light microscope. The same site was anatomically divided into 5 segments, i.e., left coronary artery, right coronary artery, left coronary sinus, right coronary sinus, and non-coronary sinus. The degree of inflammation in each segment was assessed using four scores: 0=no inflammation, 1=inflammation in the intima (i.e., endoarteritis), 2=inflammation in the intima and adventitia, and 3=inflammation in all layers of the vascular wall (i.e., panvasculitis) in Table 1. The total number of segments with score 1 or greater was defined as the extent of the lesion, while the total score for all 5 segments was defined as the degree of inflammation for one mouse. Panvasculitis was defined as a positive finding for vasculitis. Comparative investigation was performed regarding the incidence of vasculitis at the coronary artery and aortic root. In order to evaluate the histological severity in the coronary arteries and aortic root, the extent of the lesions and the degree of inflammation were compared among experimental groups.

Table 1. The degree of inflammation in each segment

Score	Evidences	Remarks
0	no inflammation	
1	inflammation in the intima	Endoarteritis
2	inflammation in the intima and adventitia	
3	inflammation in all layers of the vascular wall	Panvasculitis

***Statistical Analysis*:** The incidence of panvasculitis in each group was examined using the chi-square test. The extent of the lesion and the degree of inflammation in each group were analyzed with the Kruskal-Wallis test. In the case that the Kruscal-Wallis test found a significant intergroup variation, the Steel Dwas test was used to perform multiple comparison between experimental groups. However it was impossible to compare between hScFv 9 mg/Kg/day and other groups, because only one mouse was able to survive with hScFv 9 mg/Kg/day. For all tests, $p<0.05$ was defined as representing a significant difference.

Results

***Mouse mortality rate during experiment*:** The mouse mortality rate of each group was hScFv 9 mg/Kg/day: 80% (4/5), and hScFv 4.5 mg/Kg/day: 20% (1/5). However it was difficult to determine the cause of death because no histological analysis was performed on the dead mice in this study.

***Incidence of vasculitis*:** The incidence of panvasculitis in each group was 66.7% (4/6) in no treatment, 40% (2/5) in solvent, 60% (3/5) in treatment with hScFv 2.25 mg/Kg/day, 25% (1/4) in treatment with hScFv 4.5 mg, 0% (0/1) in treatment with hScFv 9 mg/Kg/day, and 40% (2/5) in treatment with native IgG 400 mg/Kg/day (Figure 1). Only one mouse was able to survive with hScFv 9 mg/Kg/day and could not be compared with another mouse. Groups in hScFv 4.5 mg/Kg/day and native IgG 400 mg/Kg/day showed lower incidence than no treatment (p=0.26 and p=0.39, respectively) group. In addition, hScFv 4.5 mg/Kg/day group showed lower incidence than native IgG 400 mg/Kg/day (p=0.16).

Histology of vascular lesions:

-Panvasculitis: Figure 2 shows histological observations of vascular lesions. In no treatment group, panvasculitis was observed in four mice and endoarteritis in the aortic root was observed in other two mice. In two out of four mice with panvasculitis, large lesions that encompassed the aortic root and bilateral coronary arteries were conspicuous (Figure 2 a-d). The other two mice had panvasculitis in only non-coronary sinus. As inflammatory cells neutrophils and macrophages mainly located in the vascular lesion and associated with proliferation of fibroblasts and capillaries. In addition, internal and/or external elastic lamina were destroyed by inflammation and resulting in dilation of blood vessel. Mouse treated with hScFv at 9 mg/Kg/day, minute focus of endoarteritis was observed in aortic root, but panvasculitis defined as inflammation involving all layers of vascular wall was not observed (Figure 2 q, r). Mouse treated with hScFv at 2.25 mg/Kg/day and 4.5 mg/Kg/day, and native IgG 400 mg/Kg/day, panvasculitis occurred but was confined to the aortic root, showing no lesion was found in the coronary artery (Figure 2 i-l, s-v). In addition to this, minute endoarteritis was observed in several places.

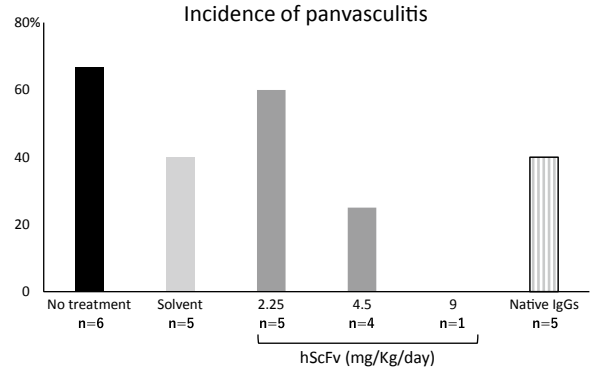

Figure 1. The incidence of panvasculitis
The bar graph shows the incidence of panvasculitis. The black bar: No treatment, light gray bar: solvent, dark gray bars: treatment with hScFv, and stripe bar: treatment with native IgG 400 mg/Kg/day.

-Extent of the lesion: The extent of the lesion (i.e., the total number of involved segments) showed in each group as follows. No treatment: 2.33 ± 2.07, solvent: 1.80 ± 2.17, hScFv 2.25 mg/Kg/day: 1.20 ± 1.30, hScFv 4.5 mg/Kg/day: 1.00 ± 1.41, hScFv 9 mg/Kg/day: 1.00, and native IgG 400 mg/Kg/day: 1.80 ± 1.30, respectively. There was no significant inter-group variation (p=0.71), but the extent of the leion in each treatment group tended to be smaller than no treatment. Also, the extent of the lesion in the hScFv treatment group tended to be smaller than native IgG 400 mg/Kg/day (Figure 3a).

-Degree of inflammation: The degree of inflammation (i.e., the total score for all 5 segments) showed in each group as follows: No treatment: 6.17 ± 6.52, solvent: 4.80 ± 6.61, hScFv 2.25 mg/Kg/day: 3.60 ± 3.91, hScFv 4.5 mg/Kg/day: 2.00 ± 2.83, hScFv 9 mg/Kg/day: 1.00, and native IgG 400 mg/Kg/day: 4.00 ± 4.18 (Figure 3b). There was no significant inter-group variation (p=0.66), and the degree of inflammation for each treatment group tended to be lower than no treatment group. Also, the degree of inflammation in the group of hScFv 4.5 mg/Kg/day tended to be lower than native IgG 400 mg/Kg/day (Figure 3b).

Discussion

In the present study, the inhibitory effects of hScFv on the Kawasaki disease vasculitis model was analyzed histologically. As shown in Figure 1 and 3, the incidence and severity of vasculitis in hScFv 4.5 mg/Kg/day tended to be lower and milder than those in no treatment. hScFv protein derived from a mixture of 204 clones was slightly effective against CAWS-induced vasculitis in murine model of Kawasaki disease vasculitis, suggesting that selected single or a few clone(s) from the 204 clone library may have a higher suppression of the vasculitis. If single or several clones will be selected from 204 clones, it may show high therapeutic effect. Also, hScFv treatment slightly inhibited the development of severity in the CAWS-induced vasculitis in a dose dependent manner, and that was not inferior to native IgG 400 mg/Kg/day. This suggests that hScFv may contain proteins in the clones binding to several key molecules to develop vasculitis, which is considered to be the reason why it is effective at a smaller dose than native human IgG. In the present study, no coronary arteritis was observed in the groups with hScFv. However it could not be clarified that inhibitory effect on coronary arteritis was higher than that of vasculitis in aortic root because the incidence of CAWS-induced coronary arteritis is slightly lower than that of vasculitis in aortic root in both non-treated and treated groups.

Some mice treated with hScFv 9 mg/Kg/day and hScFv 4.5 mg/Kg/day died before the sacrifice. It has been reported that anaphylactic shock, thromboembolism due to an increasing of blood viscosity, and liver injury, and renal disorder are an adverse effect of IVIg therapy. It is very important to determine the cause of death, however it was difficult to determine the cause of death because no histological analysis was performed on the dead mice in this study. Also, for IVIg, high-dose administration of gammaglobulin and its influence on the function of the kidney is not small, therefore the lowest dose showing therapeutic efficacy is important. If it is possible to reduce the dose by using a specific clone with higher vasculitis-suppressing effect, it will also lead to the avoidance of serious adverse events.

This model has been demonstrated that some molecules such as MPO[16] and tumor necrosis factor-α[17] associated with the onset of vasculitis in this model. In addition, mannan, one of the major components of CAWS is essential for development of CAWS-induced vasculitis[18]. Moreover, it may be possible to elucidate the etiology or pathophysiology of vasculitis if an epitope site and target molecule of hScFv is determined. The selected hScFv is expected not only as a therapeutic agent but also as a useful tool for elucidating the etiology and pathophysiology of CAWS-induced vasculitis.

In view of past nation-wide epidemics, it has been suspected that some kind of microorganism is involved in the onset of Kawasaki disease[19], however there is still no consensus regarding the etiology of Kawasaki disease. Regarding the pathophysiology, it was supported that innate immunity affected the onset of Kawasaki disease[20,21]. Recently, we reported that dectin 2, one of the innate immune receptor, was essential for onset of CAWS-induced vasculitis[22]. In this manner, CAWS-induced vasculitis shares many similarities such as not only distribution and histological features of vasculitis but also mechanism of development of vasculitis, in which involvement of microorganisms and the innate immune system is suspected. If the proteins derived from selected clones with high therapeutic effect can be identified, hScFv will be useful for the treatment of Kawasaki disease vasculitis as well as CAWS-induced vasculitis.

Conclusion

The present study suggests that the mix batch of recombinant hScFv proteins derived from 204 clones having VH-CH1 hinge composition suppressed development of vasculitis in animal model of Kawasaki disease vasculitis.

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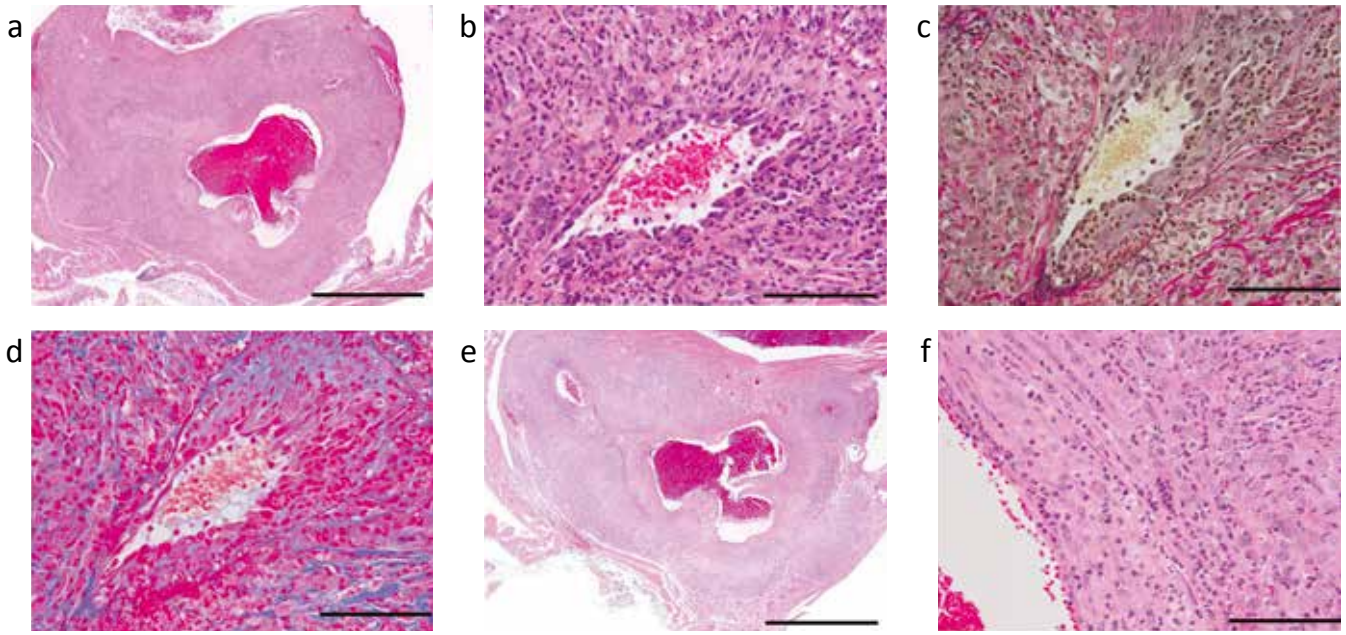
Conflicts of interest

The authors have no conflicts of interest regarding the data reported herein.



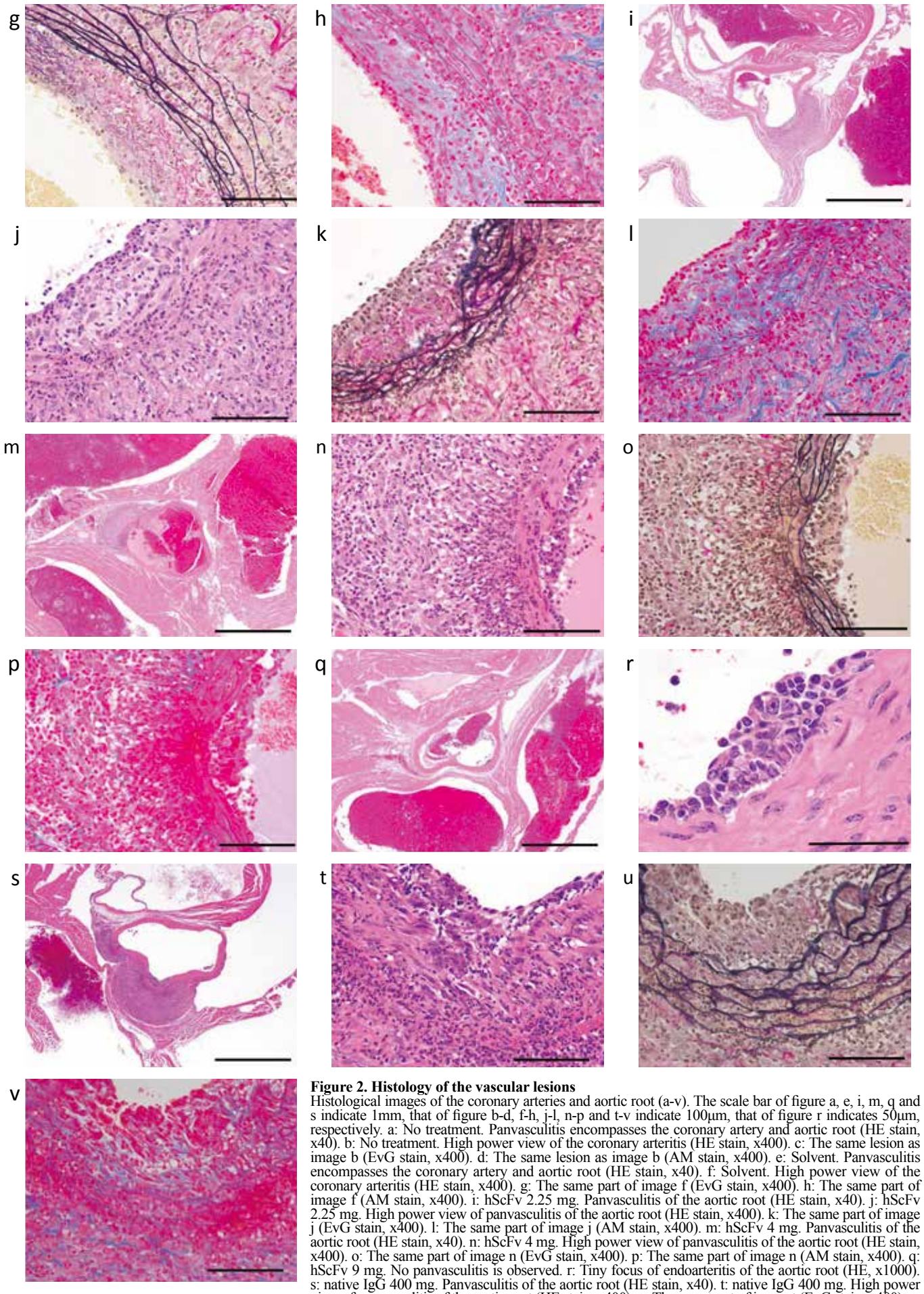


Figure 2. Histology of the vascular lesions
Histological images of the coronary arteries and aortic root (a-v). The scale bar of figure a, e, i, m, q and s indicate 1mm, that of figure b-d, f-h, j-l, n-p and t-v indicate 100μm, that of figure r indicates 50μm, respectively. a: No treatment. Panvasculitis encompasses the coronary artery and aortic root (HE stain, x40). b: No treatment. High power view of the coronary arteritis (HE stain, x400). c: The same lesion as image b (EvG stain, x400). d: The same lesion as image b (AM stain, x400). e: Solvent. Panvasculitis encompasses the coronary artery and aortic root (HE stain, x40). f: Solvent. High power view of the coronary arteritis (HE stain, x400). g: The same part of image f (EvG stain, x400). h: The same part of image f (AM stain, x400). i: hScFv 2.25 mg. Panvasculitis of the aortic root (HE stain, x40). j: hScFv 2.25 mg. High power view of panvasculitis of the aortic root (HE stain, x400). k: The same part of image j (EvG stain, x400). l: The same part of image j (AM stain, x400). m: hScFv 4 mg. Panvasculitis of the aortic root (HE stain, x40). n: hScFv 4 mg. High power view of panvasculitis of the aortic root (HE stain, x400). o: The same part of image n (EvG stain, x400). p: The same part of image n (AM stain, x400). q: hScFv 9 mg. No panvasculitis is observed. r: Tiny focus of endoarteritis of the aortic root (HE, x1000). s: native IgG 400 mg. Panvasculitis of the aortic root (HE stain, x40). t: native IgG 400 mg. High power view of panvasculitis of the aortic root (HE stain, x400). u: The same part of image t (EvG stain, x400). v: The same part of image t (AM stain, x400).

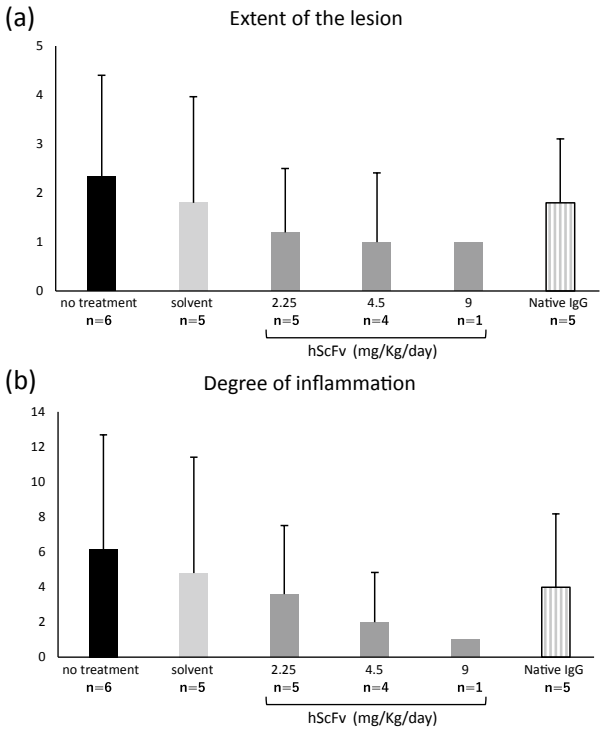


Figure 3. Histological severity of vasculitis
The extent of the lesion (i.e., the total number of involved segments) and (i.e., the total score for all 5 segments) are shown in figure 3a and 3b, respectively. Although there was no intergroup variation, the extent of the lesion and the degree of inflammation all treated groups tended to be smaller and milder than those of no treatment group. The extent of the lesion of all hScFv groups tended to be lower than that of native IgG group. The degree of inflammation of hScFv 4.5 mg/Kg/day tended to be lower than that of native IgG 400 mg/Kg/day.

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