Reduction of MPO-ANCA epitopes in SCG/Kj mice by 15-deoxyspergualin treatment restricted by IgG2b associated with crescentic glomerulonephritis

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Abstract

Objectives. The MPO-specific antineutrophil cytoplasmic antibodies (MPO-ANCA) are associated with renal failure. Epitopes of MPO-ANCA and immunoglobulin G (IgG) subclass and cytokine levels in the recovery phase were analysed by the administration of 15-deoxyspergualin (DSG) to SCG/Kj mice, which show spontaneous crescentic GN (CrGN).

Methods. We treated SCG/Kj mice by using DSG and MPO deletion mutants to investigate epitopes of MPO-ANCA associated with renal failure in SCG/Kj mice. After DSG treatment for 30 days, we observed histological changes in a crescentic formation and infiltration of neutrophils and lymphocytes into kidney, cytokines/chemokines and MPO-ANCA epitopes by deletion mutants.

Results. MPO-ANCA were reduced by the administration of DSG, and epitopes of MPO-ANCA, mainly H-6, decreased. Moreover, the IgG2b subclass of the H-6 epitope of MPO-ANCA was greatly decreased by DSG treatment. These observations correlated with a decrease in renal failure and proteinuria, infiltration of neutrophils and lymphocytes into glomeruli, and crescent formation. The CD4/CD8 ratio of spleenocytes ranged from 1.68 (0.24) in the non-treated group to 0.90 (0.12) at 100 µg/mouse/day in the DSG-treated group. In addition, elevated levels of IL-12p40, IL-10 and IL-13 in the active phase of CrGN clearly decreased with DSG treatment but not with TNF-α. In contrast, the IL-1α level increased, and IL-17 and IL-12p70 slightly increased with DSG treatment.

Conclusion. These results strongly suggest that DSG treatment of SCG/Kj mice leads to the reduction of risk antibodies in IgG2b and normalization of B-cell clones accompanied by recovery of the cytokine and chemokine balance.

Key words: Risk epitopes, MPO-ANCA, 15-deoxyspergualin, Rapidly progressive glomerulonephritis, ANCA-associated glomerulonephritis, SCG/Kj mouse.

Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are risk markers for primary systemic vasculitis, microscopic polyangiitis (MPA), WG and Churg–Strauss syndrome (CSS). Vasculitis and GN are strongly associated with ANCA, which react with major antigens such as MPO and proteinase 3 [1]. MPO-ANCA, in particular, are often associated with progression in MPA and are sometimes positive in the serum of patients with WG [2]. In WG and MPA, evidence suggests that an autoimmune inflammatory process involves active neutrophils as targets [3].
Anti-MPO antibodies from patients with MPA trigger the release of MPO from neutrophils and monocytes. Anti-MPO antibody could play a pathogenic role in vivo by triggering an oxidative burst leading to severe endothelial damage [4]. Moreover, MPO-ANCA are associated with injury of kidney glomeruli, showing haematuria and proteinuria [5–7]. Together with renal failure, elevation of inflammatory cytokines has been reported in patients with ANCA-positive small-vessel vasculitis, in particular the increase in TNF-α level in serum [8].

An animal model for vasculitis associated with the presence of MPO-ANCA in serum has been established, making possible examination of the mechanisms for development of the disease [5, 9, 10]. In model mice, renal crescent formation is also associated with increase in MPO-ANCA levels and neutrophil infiltration into glomeruli [11]. In addition, crescentic GN (CrGN) in model mice is suppressed by 15-deoxyspergualin (DSG) treatment [12], which is shown to have therapeutic efficacy in patients with proliferative CrGN [13–16]. However, the role of DSG in MPO-ANCA production in MPO-ANCA-associated vasculitis is still unknown.

On the other hand, MPO-ANCA recognize fragments of an MPO molecule showing different epitopes in humans [17–19], but controversy remains as to MPO epitope sites [20–22]. Therefore, the detailed mechanism of the MPO-ANCA risk epitope must be discussed further [21].

Experimental animal data show that ANCA immunoglobulin G (IgG)s are directly involved in the pathogenesis of ANCA-associated vasculitis [23], and the pathogenic IgG subclass contributes to the development of MPO-ANCA-associated vasculitis [24]. IgG1 and IgG3 of subclasses of ANCA are especially potent in activating the neutrophil Fc receptor [25]. Xiao et al. [7] have demonstrated that anti-MPO IgG antibodies cause pauci-immune glomerular necrosis and crescent formation in the presence or absence of functional T or B lymphocytes. However, more precise pathogenic roles of MPO-ANCA in the development of GN and vasculitis in these murine models remain undetermined.

In the present study, we investigated epitopes of MPO-ANCA associated with renal failure in SCG/Kj mice using MPO deletion mutants. We also analysed the reduction in the IgG subclass restricted with the epitopes of MPO-ANCA when SCG/Kj mice were treated with an immunosuppressant DSG. Shifts of cytokine levels in blood and the ratio of T-cell CD4/CD8 of splenocytes by this treatment were also observed.

**Materials and methods**

**Materials**

DSG (Nippon Kayaku, Tokyo, Japan) was dissolved in endotoxin-free distilled water, which was sterilized through a Millipore filter.

**Animals**

Female SCG/Kj mice (Nippon Kayaku) were kept under the sterile pathogen-free conditions of the National Institute of Infectious Diseases (NIID, Tokyo, Japan) animal facilities. Animal care methods and experimental protocols were judged permissible by the NIID.

**Experimental design for DSG injection**

DSG was intraperitoneally injected into 8- to 10-week-old mice at dosages of 0 (distilled water), 20, 60 and 100 μg/day/mouse for 30 days. After 30 days, mice were sacrificed by CO2 gas for analysis. Blood samples were drawn from the heart into a tube for serum preparation or into a syringe for a blood test.

**Evaluation of renal lesion with histological examination of glomeruli**

After kidneys were removed, they were fixed with buffered formalin and embedded in paraffin. To assess the lesions in SCG/Kj mice, serial 3-μm sections were stained with haematoxylin and eosin (HE). The crescent score was evaluated by the modified method of Floege et al. [26] as follows: 22 glomerular cross-sections were graded by a relative area of the cellular crescent occupied in Bowman’s capsule as 0, negative; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%; the total grade in 22 glomeruli was defined as the crescent score. Moreover, each specimen was used to determine the crescent formation number. The number of neutrophils infiltrated into 22 glomeruli was based on nuclear morphology of HE staining. Neutrophil infiltration was scored in a range of 0–3 (0 = absent, 1 = mild, 2 = moderate and 3 = severe).

**Immunohistochemistry**

Kidney tissues were sectioned at an average thickness of 3 μm and fixed with cold acetone. The sections were blocked with 5% BSA (Wako, Tokyo, Japan) and incubated with FITC-labelled hamster anti-mouse CD3 (BD Biosciences, Heidelberg, Germany) and unlabelled rat anti-mouse CD45R/B220 mAbs (BD Biosciences) for 1 h at 37°C, followed by incubation with Alexa Fluor 488-conjugated goat antibody against rat IgG (x:1/200, Molecular Probes) for 1 h. After a thorough washing, the sections were mounted onto glass slides with PermaFluor Aqueous Mounting Medium (Dako, Tokyo, Japan). The samples were observed with an Eclipse TE2000 epifluorescence microscope (Nikon, Tokyo, Japan).

**Flow cytometry analysis**

Flow cytometry analysis by FACS for CD4/CD8 ratio was performed as follows. The CD3-gated CD4 and/or CD8 expression of T cells in spleen was determined by IF staining using directly conjugated antibodies. Isolated splenocytes were incubated for 2 h at 4°C with saturating amounts of conjugated mAbs directed against CD3, CD4 and CD8a (Bioscience, San Diego, CA, USA). The antibodies were conjugated with PE, APC or FITC, washed twice to remove unbound antibodies, and finally resuspended in 200 μl of Cell Wash (BD Biosciences). Analysis of all cells was performed on a FACSaria flow cytometer (BD Biosciences) using 20,000 events per sample. In addition, FACS analysis for population of...
CD3 and B220 was performed as follows. Expression on splenocyte subsets was determined by IF staining using directly conjugated antibodies. Splenocytes were incubated for 2 h at 4 °C with saturating amounts of conjugated mABs directed against CD3 and CD45R/B220 (BD Biosciences). The antibodies were conjugated to either FITC or PE, depending on the combination of specific antibodies used. The cells were analysed as described in CD3/B220 determined by a FACSCalibur flow cytometer (BD Biosciences) using 10,000 events per sample.

**Determination of cytokines with Bio-Plex**

An aliquot of serum (12 μl) prepared from peripheral blood was measured for concentration of cytokines by the 23-Plex kit using Bio-Plex 200 according to the manufacturer’s protocol and analysed by the Bio-Plex Lumimex 100 XYP instrument (Bio-Rad, CA, USA). We assayed the following 23 cytokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, eotaxin, G-CSF, GM-CSF, INF-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES and TNF-α. Concentrations of cytokines and chemokines were calculated using Bio-Plex Manager 3.0 software (Bio-Rad) with a five-parameter curve-fitting algorithm applied for standard curve calculations.

**Measurement of mouse MPO-ANCA**

Sera were prepared from SCG/Kj mouse hearts. MPO-ANCA were measured as described previously [10]. Briefly, mouse MPO and human MPO III for positive control were coated onto an ELISA plate (TS Plate; Toyoshima, Tokyo, Japan). The coated ELISA plate was incubated overnight at 4 °C. The plate was blocked and mouse serum (1/50 dilution) was added for 1.5 h at room temperature. An AP-labelled anti-mouse IgG antibody (1/3000 dilution) or an AP-labelled anti-human IgG antibody (1/3000 dilution) was added and allowed to react for 2 h at room temperature. Afterwards, p-nitrophenylphosphate, an AP substrate, was added at a concentration of 1 mg/ml. After incubation at room temperature, absorbance at 405 and 650 nm was measured by a model LFA-096 automatic analyser. Concentrations of the sera of MPO-ANCA were determined with recombinant mouse MPO (rMPO) antigen for a standard curve.

**Measurement of MPO-ANCA epitopes**

Serum was plated onto a well of an ELISA plate (TS Plate; Toyoshima), which was coated with full-length rmMPO and deletion mutants of human MPO heavy chain H-4, H-5, H-6, H-7, H-8 and H-9 at a concentration of 0.1 μg/100 μl in each well, as described elsewhere [17, 18]. (AP)-conjugated anti-mouse IgG antibody (×1/3000 dilution) was used for the secondary antibody. The concentration of MPO-ANCA in the blood in mouse serum was determined as equivalent rabbit anti-MPO antibody (μg/ml), which was employed as a standard. Reactivity of MPO-ANCA in the serum of a SCG/Kj mouse was calculated from absorbance at 405 and 650 nm in ELISA analysed by the following formula:

\[
\% \text{ Reactivity} = \left( \frac{\text{Serum reacted to fragment} - \text{Negative control serum}}{\text{Polyclonal anti-MPO antibody} - \text{Negative control serum}} \right) \times 100
\]

**Determination of IgG subclass of MPO-ANCA with epitopes**

IgG subclasses of MPO-ANCA in serum were analysed by a plate coated with MPO deletion mutants. Sera from C57BL/6 mice were used as a negative control. The isotyping of the antibody response was performed by incubation with biotinylated anti-mouse IgG1, IgG2a, IgG2b and IgG3 (100 μl/well; PharMingen, San Diego, CA, USA and Becton Dickinson, Franklin Lakes, NJ, USA) for 2 h. Wells were incubated with AP-conjugated avidin (×1/1000 dilution, 100 μl/well; Sigma, St Louis, MO, USA) for 2 h, and the colour in the plates was read at optical density 405 and 650 nm.

**Statistical analysis**

Values were expressed as mean (s.d.) and analysed for statistical differences by Student’s *t*-test. *P* < 0.05 was considered statistically significant.

**Results**

**Reduction of renal failure by treatment with DSG**

We observed the effect of DSG on renal failure. Proteinuria in SCG/Kj mice treated with DSG (dosages of 0, 20, 60 and 100 μg/mouse/day) was significantly reduced at days 15 and 25 in a dose-dependent manner (Fig. 1A). In addition, haematuria was also decreased by treatment with DSG (data not shown).

**Histopathological changes in glomeruli of SCG/Kj mice by treatment with DSG**

In addition, histological observations were performed. Figure 1B shows histological damage in crescentic glomeruli.
Fig. 1 Reduction of renal failure and histopathological changes in glomeruli of SCG/Kj mice by the administration of DSG. (A) Proteinuria in SCG/Kj mice treated with DSG at dosages of 0, 20, 60 or 100 μg/mouse/day was measured at Days 4, 10, 15 and 25 with a haemostat as described in the ‘Materials and methods’ section. (B) Histopathological changes in glomeruli: HE stain indicates crescentic formation (black arrow) and neutrophil infiltration (white arrow) in an SCG/Kj mouse with the administration of DSG at a dosage of 0 μg/mouse/day for 30 days (left) or 60 μg/mouse/day for 30 days (right), bar: 20 μm. Scores of crescentic formation (C) and of neutrophil infiltration (D) were determined as described in the ‘Materials and methods’ section. DSG: 0 μg/mouse/day for 30 days (open circle) or 60 μg/mouse/day for 30 days (closed circle); bars indicate mean scores. (E) Infiltrated T cells in renal perivascular region disappeared with DSG treatment (HE staining), bar: 50 μm. The IF image of CD3 (green, T cells) and B220 (red, B cells) of perivascular infiltrated cells revealed that these cells were mainly lymphocytes, bar: 50 μm. (F) The absolute number of splenic B cells in SCG/Kj mice was determined after flow cytometric analysis (percentage of B cells multiplied by total number of splenocytes). **P < 0.01.
formation in the glomeruli (black arrow), and neutrophil infiltration into glomeruli (white arrow) decreased with DSG administration. Crescentic formation significantly decreased with 60 μg/mouse/day DSG administration into SCG/Kj mice after 30 days [score 0.9 (0.7)] when compared with distilled-water-injected mice for control [score 1.3 (1.1); P = 0.01; Fig. 1C]. Moreover, neutrophil infiltration into glomeruli slightly decreased with 60 μg/mouse/day DSG administration into SCG/Kj mice after 30 days [score 1.9 (1.4)] when compared with distilled-water-injected mice for control [score 2.7 (1.1); Fig. 1D]. Profiles of infiltration of T and B cells into kidney was observed without treatment (Fig. 1Ea and b) but completely diminished with DSG treatment (Fig. 1Ec and d). In addition, B cells in spleen slightly decreased by treatment with DSG; 8.26 × 10^6 (3.93 × 10^6) in non-treated, 9.62 × 10^6 (0.87 × 10^6) in 20 μg/mouse/day and 6.17 × 10^6 (0.80 × 10^6) in 100 μg/mouse/day, respectively (Fig. 1F).

Changes in peripheral blood counts of white blood cells and platelets in SCG/Kj mice by treatment with DSG

Table 1 shows decreased white blood cell (WBC) counts, from 18 000 (6236) [mean (s.d.)] to 4467 (757)/μl; conversely, platelets increased from 42 × 10^4 (7 × 10^4) to 111 × 10^4 (21 × 10^4)/μl by treatment with DSG at a dose of 20 μg/mouse/day for 30 days. These data show recovery of the WBC counts to the level of C57BL/6 mice, whereas platelet numbers increased (Table 1). The mice have high WBC counts without treatment due to high counts of lymphocytes (Fig. 2). There was no difference in the profiles of lymphocytes, monocytes, neutrophils, eosinophils and basophils between DSG treatment and non-treatment, showing that progenitor cells of leucocytes were suppressed at the same rate.

Normalization effects of DSG on profiles of CD4 and CD8 cells and CD3+B220+ cells in the development of vasculitis

Since we observed reduction of renal failure in urine, histology and lymph node, we analysed the profile of the spleen cell by FACS in SCG/Kj mice (Fig. 3A). Double-negative CD4−CD8− cells were 81.6 (1.3)% in the non-treated group, 65.0 (2.6)% in 20 μg/mouse/day in treatment with DSG and 77.5 (23.5)% in 100 μg/mouse/day, respectively. In contrast, 10.9 (0.3)% of CD4+CD8+ cells were in the non-treated group, whereas 16.6 (2.3)% in 20 μg/mouse/day and 11.1 (2.2)% in 100 μg/mouse/day. DSG was shown to affect predominance of CD8+ cells more than of CD4+ cells by FACS analysis, since results in a ratio of CD4/CD8 decreased with DSG treatment in a dose-dependent manner: 1.68 (0.24) in 0 μg/mouse/day, 0.99 (0.17) in 20 μg/mouse/day and 0.90 (0.12) in 100 μg/mouse/day, respectively (Fig. 3B). Furthermore, CD3+B220+ cells in spleen significantly decreased with DSG treatment; 64.4 (7.7) in the non-treated group, 36.4 (13.4) in 20 μg/mouse/day and 20.6 (3.2) in 100 μg/mouse/day (Fig. 3C, D).

Changes in cytokine levels in serum of SCG/Kj mice by the administration of DSG

Since DSG caused a decrease in both CD4/CD8 ratio and CD3+B220+ in spleen, we analysed the profiles of cytokines/chemokines by DSG treatment (Fig. 4). As shown in Fig. 4, IL-12p40, IL-10 and IL-13 in these cytokines decreased with DSG treatment, but not with TNF-α which is well known to be important in B-cell activation and a key factor in ANCA-associated vasculitis. TNF-α concentrations in sera of patients with rapidly progressive GN (RPGN) are high [8]; this is particularly true in the early phase in SCG/Kj mice [10]. We confirmed that the TNF-α level was high in the serum of SCG/Kj mice without the administration of DSG. In addition, other inflammatory cytokine levels in serum, such as IL-12p40, IL-10 and IL-13, decreased by treatment with DSG treatment in a dose-dependent manner (Fig. 4). In contrast, the cytokine level of IL-1α increased, and IL-17 and IL-12p70 were slightly increased by DSG treatment.
Fig. 3 Normalization effects of DSG on profiles of CD4 and CD8 cells in the development of vasculitis. CD4/CD8 ratio analysis by FACS using APC-conjugated anti-mouse CD4 and FITC-conjugated CD8. (A) 0, 20 and 100 μg/mouse/day for 30 days. (B) CD4/CD8 ratio in splenocytes of mice treated with 0, 20 and 100 μg/mouse/day for 30 days. (C and D) Splenocytes were stained for anti-mouse CD3 and anti-mouse B220, and analysed by FACS. The population of CD3\(^+\)B220\(^+\) cells was decreased by DSG treatment. *\(P < 0.05\), **\(P < 0.01\).

![CD4/CD8 ratio](image)

![CD4/CD8 ratio in splenocytes](image)

![Splenocytes stained for anti-mouse CD3 and anti-mouse B220](image)

Fig. 4 Cytokine and chemokine levels of sera of SCG/Kj mice by the administration of DSG. Cytokine and chemokine levels in 12 μl of serum of SCG/Kj mice administered DSG were simultaneously measured with Bio-Plex 200 (Bio-Rad), as described in the ‘Materials and methods’ section. *\(P < 0.05\), **\(P < 0.01\).

![Cytokine and chemokine levels](image)
Reduction of MPO-ANCA level in serum of SCG/Kj mice by treatment with DSG

The autoantibody MPO-ANCA level in serum is a risk factor in SCG/Kj mice; therefore, we measured the reduction of the MPO-ANCA level by DSG treatment. MPO-ANCA levels in serum without the administration of DSG were higher than those of normal C57BL/6 mice even before over-proteinuria (data not shown). DSG reduced the MPO-ANCA level in a dose-dependent manner (Fig. 5A).

Reduction of MPO-ANCA epitopes by treatment with DSG

Since MPO-ANCA recognizes fragments of the MPO molecule in patients showing different epitopes in its heavy chain but not light chain [17, 18], we focused on epitope specificity in the heavy chain of MPO of MPO-ANCA in SCG/Kj mice. MPO-ANCA reactivity was observed mainly in the H-6 fragment and to a lesser extent in the H-9 fragment (Fig. 5B). The percentage reactivity in H-6 was significantly reduced by the administration of DSG, whereas

**Fig. 5** Reduction of MPO-ANCA and epitopes of MPO-ANCA by the administration of DSG. (A) MPO-ANCA levels in sera of SCG/Kj mice treated with DSG were measured by ELISA. (B) Reactivity to MPO fragments (H-4, H-5, H-6, H-7, H-8 and H-9) was determined using deletion mutants (C) as described in the ‘Materials and methods’ section. DSG was administered at dosages of 0, 20, 60 or 100 μg/mouse/day for 30 days. *P < 0.05, **P < 0.01.
sera in SCG/Kj mice without the administration of DSG still contained high reactivity against MPO fragments (Fig. 5B). DSG reduced reactivity against the H-6 fragment compared with full-length MPO heavy chain and other MPO fragments. Also, reactivity against H-7 and H-9 was decreased by DSG treatment. However, reactivity to H-4 and H-5 was low, and H-8 was changed only to a small degree.

Significant reduction in IgG2b subclass of the epitope H-6 of MPO-ANCA by treatment with DSG

Surprisingly, MPO-ANCA with the epitope H-6 were greatly reduced by 87.2 (35.3)% in IgG2b, whereas IgG1 [20.6 (8.3)%], IgG2a [4.2 (1.8)%] and IgG3 [17.4 (7.6)%] were slightly reduced (Fig. 6). In addition, other epitopes such as H-7 were also reduced in IgG1 [4.9 (2.1)%], IgG2a [1.1 (0.4)%], IgG2b [51.3 (20.8)%] and IgG3 [8.3 (3.4)%] and H-8 was reduced in IgG1 [9.1 (3.7)%], IgG2a [1.9 (1.0)%], IgG2b [37.1 (15.0)%] and IgG3 [4.5 (1.2)%]. Moreover, epitopes H-4 [17.8 (7.2)%] and H-5 [23.3 (9.4)%] were observed to be restricted with IgG2b.

Discussion

In the present study, DSG-treated mice developed renal injury, as demonstrated by abnormal proteinuria, and serum cytokine levels were significantly different from control mice. Also, glomerular crescentic formation significantly decreased in DSG-treated SCG/Kj mice after 30-day treatment when compared with control SCG/Kj mice. This crescentic formation may be caused by the IC, which develops into vasculitis [5]. Moreover, crescentic formation in glomeruli correlates with neutrophil infiltration, damage of glomeruli and IC deposit without treatment [5]. Although control mice accumulated neutrophils in glomeruli, DSG-injected SCG/Kj mice reduced neutrophil accumulation by DSG treatment, which is immunosuppressant for graft rejection [13–16]. Our results indicate that the increase in glomerular crescentic formation is associated with neutrophil infiltration. In the present study, decrease in WBC counts confirmed the findings from a previous study that showed that DSG exerts no nephro- or hepatotoxicity, but reversibly induces leukopenia [14]. In contrast, the increase in platelet counts in the present study and other reports of DSG in NZW × BXSB F1 mice [27] suggest that the decrease in platelets in these autoimmune mice may be recovered in the recovery phase. Infiltration of T and B cells into kidney diminished greatly with DSG treatment, suggesting that DSG suppresses infiltration of inflammatory cells due to a decrease in migration and proliferation of the number of T and B cells in spleen.

In addition to the contribution of neutrophil infiltration to the development of vasculitis, other immune responses such as CD4/CD8 ratio and cytokines and chemokines have been observed. Crescent formation is mediated by infiltrating CD4+ T cells but is independent of CD8+ T cells [28] and autologous antibody [29]. T-cell infiltration into glomeruli was suppressed by DSG treatment, although the number of CD4+ cells was relatively lower than that of CD8+ cells. Our results confirmed that CD4+ T cells decreased by DSG treatment, whereas CD8+ T cells increased. The determination of the ratio of CD4+ to CD8+ cells is widely used to monitor alterations within the T-cell compartment. As illustrated, the ratio by

**Fig. 6** Reduction of IgG subclasses of the epitope of MPO-ANCA by the administration of DSG. IgG subclasses IgG1, IgG2a, IgG2b and IgG3 in sera were determined with several MPO heavy chain deletion fragments (Full: full length, H-4, H-5, H-6, H-7, H-8 and H-9) reactive to MPO-ANCA in sera of SCG/Kj mice.
histogram plotting in the present study of the DSG treatment group (20 and 100 µg/mouse/day) was 0.87, whereas the ratio of the control group was 1.16 (Fig. 2B). The decrease in the ratio of CD4/CD8 to a normal level by treatment with DSG showed a shift in the T-cell population in spleen associated with histological recovery, suggesting that T-cell populations, such as CD4 and CD8 cells, may be related to the development of vasculitis described elsewhere [30]. However, different background T cells from SCG/Kj mouse cannot be tested because they will be rejected when transferred into a disparate background. Double-negative CD4+CD8− cells were decreased by treatment with DSG in the present study, in addition to the report of double-negative T cells in the SCG mouse [9], but the role of double-negative T cells in RPN1 inductuction is still not clear. In addition, the slight decrease in B cells in spleen by treatment with DSG may be related to the decrease in MPO-ANCA levels and its epitopes in serum, resulting in recovery from renal failure. We demonstrated that B220-positive cells in spleen were significantly decreased by DSG treatment at 100 µg/mouse/day, whereas no difference was shown between 0 and 20 µg/mouse/day. However, B-cell-specific reduction was observed in the MRL/lpr mouse, the background of the SCG/Kj mouse, autoimmune disease is dependent on CD4+ but not CD8+ T cells, and many CD4+CD8− B220+ T cells traverse a CD8 developmental pathway [31].

On the other hand, the serum levels of IL-12p40, IL-10 and IL-13, elevated in the active phase of CrGN, were clearly decreased in a dose-dependent manner by DSG administered to SCG/Kj mice, while improving the prognosis of GN in SCG/Kj mice treated with DSG. However, the serum levels of IL-12p70, IL-17 and IL-16p70 slightly increased. These results correlated with the decrease in proteinuria, haematuria and MPO-ANCA concentrations in sera as an indicator of the severity of MPO-ANCA vasculitis. Furthermore, DSG administration reduced the specific cytokines for which DSG enhances IL-12p40 secretion and thus modulates the Th1/Th2 cytokine balance [32]. Our results suggest that DSG suppresses pathophysiological changes in SCG/Kj mice. Vasculitis develops with the increase in MPO-ANCA and TNF-α levels in serum of patients with RPGN [33]. Several groups have demonstrated that TNF-α induces crescentic injury in experimental models [34–38]. Timoshanko et al. [39] have reported that intrinsic renal cells are the major cellular source of TNF-α contributing to inflammatory injury in CrGN. Indeed, treatment of patients with RPON with intravenous immunoglobulin reduces the TNF-α level in serum [8]. In the present study, the TNF-α level of mice administered DSG at 100 µg/day decreased, showing that DSG improved the TNF-α level correlated with recovery from renal failure. However, the level increased in serum of mice administered DSG at 60 µg/day. Ishida-Oka wara et al. [10] reported that the TNF-α level in plasma in the early phase differed from the level at the intermediate to late stage of renal failure in SCG/Kj mice. This is confirmed by neutrophils primed with TNF-α suppressed in

\[ \frac{O_2}{O_4} \] production by neutrophils [40]. In the present study, a decrease in TNF-α level depending on the DSG dosage was not observed, suggesting that DSG may affect other cascades rather than production of TNF-α.

Renal crescent formation is associated with an increase in MPO-ANCA level and neutrophil infiltration into glomeruli [11]. In this study, during GN, proteinuria was decreased in DSG-treated SCG/Kj mice, whereas it was enhanced in non-treated SCG/Kj mice. Also, significant dose-dependent reduction of MPO-ANCA levels in the blood in SCG/Kj mice by DSG treatment may be mainly due to immunosuppression. It is true that MPO-ANCA levels in the serum increase accompanied by crescent formation in glomeruli with CrGN from Day 20 in SCG/Kj mice [10]. We have demonstrated that high-titre MPO-ANCA appears before crescentic formation in the glomeruli of SCG/Kj mice [10]. On the other hand, MPO-ANCA are major ANCA-associated vasculitis pathogens [41, 42] and recognize different binding sites (epitopes) on their corresponding antigens. Differences in binding specificity may influence the pathogenic potential of the antibodies. We previously reported relevant data between the MPO-ANCA risk epitope(s) and high-titre sera of MPO-ANCA patients [17, 18], but controversy remains as to MPO epitope sites [19, 21]. The immunodominant epitopes have not been precisely defined. In the present study, we showed that the strongest reactivity against the H-6 fragment in SCG/Kj mice was significantly decreased by DSG treatment in a dose-dependent manner. Erdbrügger et al. [20] reported that PR3-ANCA and MPO-ANCA do not interfere with the enzymatic activity of MPO, suggesting that a major epitope may be close to the last half of the heavy chain, the reactive sites in our results. However, the correlation between epitopes and vasculitis is not understood. In the present article, the mapping of the antigenic epitopes is relevant to MPO-ANCA associated with vasculitis of initiation and regulation of autoimmune responses. Here, we used human MPO deletion fragments, because the homology of amino acid sequence of MPO in human and mouse is \( \sim 85–90\% \). Therefore, mouse MPO-ANCA could recognize human MPO. It has been reported that mouse MPO-ANCA reacts against human MPO. The differences in binding specificity may influence the pathogenic potential of the antibodies [43]. Furthermore, we have reported a human MPO epitope using 3D human MPO surface data (Protein Data Bank 1CXP) [44], because mouse MPO protein data are not available. Van der Geld et al. [21] showed that non-contiguous amino acids are important to the structure of risk epitopes. In the present study, MPO-ANCA reacted against the non-contiguous amino acid region, showing an involvement of \( \sim 85–90\% \) homology in the region of the conserved amino acid sequence between human and mouse molecules. Therefore, we used human deletion fragments for mouse serum to react with regions that preserved 3D configuration. The mouse serum strongly reacted with full-length human MPO, and fragments in those restricted regions may be important as risk epitope(s) in SCG/Kj mice.
According to the correlation between MPO-ANCA epitopes and renal failure in the present and other studies, the association of IgG subclasses with the epitopes is interesting. The IgG subclass of ANCA has been demonstrated in clinical trials, and experimental animal data show that ANCA IgGs are directly involved in the pathogenesis of ANCA-associated vasculitis [25]. Previously, another group reported that the IgG subclass in MPO-ANCA IgG4 subclass might play a role in the development of WG [45]. We here determined that the pathogenic IgG subclass reacts against the MPO antigen and contributes to the development of MPO-ANCA associated with vasculitis [46]. In the present study, MPO-ANCA reactivity against the H-6 fragment, predominantly of the IgG2b subclasses, is particularly represented. The reactivity of IgG2b against H-6 of MPO fragments was significantly stronger than that of other fragments under non-treated conditions. According to our data, DSG may inhibit production of IgG2b against the risk epitopes H-6 and H-9, especially H-6. This result correlated with the profiles of IL-12p70 (Th1-type cytokine) and the IgG2b switch demonstrated in rat [47]. However, MPO-ANCA associated with vasculitis in SCG/Kj mice has not yet been studied. A similar result reported that PR3 and MPO promote proliferation of CD4+ T cells from patients with ANCA-associated vasculitis, but also cross-stimulate T cells from healthy individuals [48]. DSG has been used at a dose of 0.5 mg/kg/day for 2–3 weeks until the WBC count dropped to 3000/μl, followed by a rest until a WBC of at least 4000/μl was reached; however, no immunosuppressants other than steroids were allowed during the study [15]. In addition, prolonged treatment of refractory WG with DSG was performed, using a similar protocol [16].

Our results may show supportive data for clinical usage due to the fact that biological consequences of DSG affect a broad range of cytokines and chemokines and MPO-ANCA-specific IgG subclass production in SCG/Kj mice. The DSG treatment may lead to make the Th1/Th2 balance normal. These results strongly suggest that epitopes of MPO-ANCA may be restricted by their IgG subclasses.

Rheumatology key messages

- DSG treatment of SCG/Kj mice lead to a reduction in risk epitopes of MPO-ANCA.
- DSG treatment affected reduction of IgG2b accompanied by a shift from Th2 to Th1 in the cytokine balance.

Acknowledgements

We thank our colleagues at the Chiba University Graduate School of Medicine and the National Institute of Infectious Diseases, Japan, for valuable discussions.

Funding: This study was supported in part by grants from the Health Science Foundation and Ministry of Health, Labour and Welfare of Japan.

Disclosure statement: The authors have declared no conflicts of interest.

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