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Chemical induction of HO-1 suppresses lupus nephritis by reducing local *i*NOS expression and synthesis of anti-dsDNA antibody

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SUMMARY

There is accumulating evidence that haem oxygenase (HO)-1 plays a protective role in various disorders. The beneficial efficacy of HO-1 induction therapy has been shown in renal diseases such as glomerulonephritis, interstitial nephritis and drug induced nephrotoxicity. However, involvement of HO-1 in the development of autoimmune renal diseases remains uncertain. To assess the clinical efficacy of HO-1 induction therapy for lupus glomerulonephritis, MRL/*lpr* mice were intraperitoneally injected with 100 μmol/kg hemin, a potent HO-1 inducer, or PBS as controls, once a week from 6 weeks of age to 21–24 weeks-old. We found that treatment with hemin led to a significant reduction of proteinuria and remarkable amelioration of glomerular lesions accompanied by decreased immune depositions. In addition, the circulating IgG anti-double-stranded DNA antibody level was significantly decreased in hemin treated mice when compared with controls. A single intraperitoneal injection with hemin resulted in reduction of inducible nitric oxide synthase expression in the kidney and spleen, and serum interferon-γ level. Our results suggest that HO-1 induction therapy ameliorates lupus nephritis by suppressing nitric oxide (NO) dependent inflammatory responses and attenuating production of pathogenic autoantibodies.

Keywords haem oxygenase-1 (HO-1) MRL/*lpr* inducible nitric oxide synthase (*i*NOS) interferon-gamma (IFN-γ)

INTRODUCTION

Haem oxygenase (HO) is the rate-limiting enzyme that catalyses haem into carbon monoxide (CO), Fe^{2+}, and biliverdin. The inducible form of HO-1, 32 kD heat shock protein, is expressed in response to various stimuli such as hydrogen peroxide, heat, heavy metal ions, hyperoxia, hypoxia, endotoxin and inflammatory cytokines, whereas HO-2, another isozyme of HO, is constitutively expressed [1]. Recent studies have shown that HO-1 plays a protective role in the development of various diseases including inflammatory diseases. The actions are mediated by haem degradation products and their metabolic derivatives [1–4]. Induction and forced expression of HO-1 suppress synthesis of inflammatory and proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumour necrosis factor (TNF), and stimulate that of anti-inflammatory cytokine, IL-10, in most of pathological conditions, though several exceptions have been documented in some disease models [5]. The modulating effects on cytokine production mainly rely on CO [6]. In addition, CO suppresses expressions of inducible nitric oxide synthase (*i*NOS) and cyclooxygenase (COX)-2, resulting in reduction of nitric oxide (NO) and prostaglandins (PGs), respectively, both of which are critical chemical mediators of inflammation [5]. Biliverdin, another haem degradation product, is subsequently converted to bilirubin by biliverdin reductase, whereas Fe^{2+} stimulates ferritin synthesis [7]. Both bilirubin and ferritin function as antioxidants [8]. Thus, multiple biochemical actions of haem degradation products and their metabolic derivatives contribute to the cytoprotective functions of HO.

We have previously reported that adenovirus vector mediated gene transfer of HO-1 cDNA suppressed lipopolysaccharide (LPS)-induced lung injury [9], influenza viral pneumonia [10], bleomycin-induced pulmonary fibrosis [11] and pseudomonas chronic respiratory infection [12] in murine models. Similarly, favourable outcomes of therapies using chemical inducers or gene HO-1 have been shown in various diseases including respiratory diseases, cardiovascular diseases, renal diseases, liver injuries, ocular diseases and organ transplantation of animal models [8,13,14].

In a patient with congenital HO-1 deficiency and HO-1-targeted mice mesangioproliferative glomerulonephritis is one of the most characteristic pathological features [13,15,16]. Accordingly,

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protective roles of HO-1 have been shown in ischemic renal injury, cisplatin induced nephrotoxicity, acute glomerulonephritis and rejection of renal transplantation [17–21]. Anti-glomerular basement membrane antibody-mediated glomerulonephritis was ameliorated by HO-1 induction therapy, in which *i*NOS was suggested as a major target [22]. Because NO is also involved in lupus nephritis of MRL/MP-*lpr/lpr* (MRL/*lpr*) mice [23], which spontaneously develop a systemic lupus erythematosus (SLE) like autoimmune disease characterized by polyclonal B cell activation associated with synthesis of various autoantibodies including nephritogenic IgG anti-double-stranded DNA (anti-dsDNA) antibody [24], we here examined effects of HO-1 induction therapy for the autoimmune mice.

To study the effects of HO-1 induction on lupus nephritis in MRL/*lpr*, we monitored renal and immunological parameters in MRL/*lpr* receiving weekly intraperitoneal administration with hemin as an HO-1 inducer. The results showed that the induction of endogenous HO-1 successfully suppressed pathological injury of glomeruli and inhibited deposition of immune complexes. The therapeutic effects were associated with significant reduction of renal *i*NOS expression, and circulating levels of serum IgG anti-dsDNA antibody and interferon (IFN)-γ in hemin-treated mice. Thus, the data suggest that HO-1 induction therapy protects autoimmune glomerulonephritis through multiple mechanisms.

MATERIALS AND METHODS

Animal

Female MRL/*lpr* mice from SLC (Shizuoka, Japan) were intraperitoneally administered with 100 μmol/kg hemin (Sigma-Aldrich, St. Louis, MO, USA) once a week ($n = 16$) or PBS as controls ($n = 16$) from age of 6 week to 21–24 week or death. In some experiments, the mice were sacrificed at 24 or 48 h after a single injection with hemin or PBS.

Sera collection and isolation of organs

To examine circulating antibodies, sera were collected from the MRL/*lpr* mice during weekly treatment with hemin. For assessment of cytokines, sera were collected at 48 h after a single administration with hemin or PBS. Mice were sacrificed by cardiac punctures under anaesthesia with kethamin (Sigma) and xylazine (Sigma), and then the spleen and kidneys were surgically removed.

Cell culture

Spleen cells were suspended in RPMI1640 HEPES modification (Sigma) with 10% FCS (Equitech-Bio, Kerrville, TX, USA), 4·1 mM L-glutamine (Sigma), 100 U/ml penicillin and 0·1 mg/ml streptomycin (Sigma). Then, 1×10^7/ml of the cells were cultured in 12-well plates (Sumitomo, Osaka, Japan) with or without 100 μM hemin at 37°C and 5% CO_2.

Western analysis

Cellular proteins were extracted from freshly isolated spleen, kidney and cultured cells by adding lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl, 50 mM NaF, 1 mM EDTA, 1% Triton-X, and protease inhibitor (Sigma). Each lysate was resolved by 4%-20% of gradient polyacrylamide gel (Daiichi Kagaku, Tokyo, Japan) for electrophoresis and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% skim milk, the membrane was probed with rabbit anti-HO-1 polyclonal antibody (StressGen Biotechnologies, Victoria, BC, Canada) for 1 h at room temperature, followed by incubation with horse raddish peroxidase (HRP)-conjugated anti-rabbit Igs (Amersham Biosciences, Piscataway, NJ, USA) for 30 min. To detect *i*NOS, the membrane was serially incubated with rabbit anti-NOS2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h, biotin-labelled goat anti-rabbit IgG antibody (KPL, Gaithrsburg, MD, USA) for 30 min, and HRP-conjugated streptavidin for 30 min (Pierce, Rockford, IL, USA). Blots were developed using ECL chemiluminescent detection system (Amersham Life Sciences, Little Chalfont, UK) and exposed to Kodak Biomax film for 1–5 min (Kodak Imaging Systems, Rochester, NY, USA).

Measurement of urinary protein excretion

Urine was collected from individual mice for 24 h. Protein concentration in the urine was determined by using the Bio-Rad protein assay kit, according to the Bradford method (Biorad, Hercules, CA).

Assessment of kidney pathology

MRL/*lpr* mice, which received weekly hemin treatment until 21-week-old, were sacrificed to take out the kidneys. One kidney was fixed with 10% formalin, embedded in paraffin, sectioned, and stained with Periodic Acid Schiff (PAS), while the other was snap-frozen for immunofluorescent studies. Two renal immunopathologists independently read and interpreted the slides without prior knowledge of the treatment modality. Sixty glomeruli per mouse were evaluated by the score system as follows; score 0 represents no abnormality, whereas score 1, 2, 3 and 4 represent mild, moderate, moderately severe and severe abnormality with crescent formation and necrosis, respectively, as previously described [25].

For immunofluorescence study, the snap-frozen kidneys were sectioned by a cryostat and fixed in cold acetone for 20 min. After blocking with 10% normal goat serum (Nichirei, Tokyo, Japan) containing PBS for 30 min, the samples were incubated with alkaline phosphatase conjugated anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL, USA) for 1 h, and then with Alexa Flour 488 conjugated donkey anti-goat IgG (H + l) (Wako, Osaka, Japan) for another 1 h. The sections were subsequently analysed by laser fluorescence microscopy (LSM-GB200, Olympus, Tokyo, Japan).

Glomerular immunodeposits were also evaluated quantitatively by immunohistochemical technique. In brief, the formalin fixed sections were pretreated with proteinase K (Sigma) followed by incubation with alkaline phosphatase conjugated anti-mouse IgG for 1 h. The signals were visualized by HISTOFINE (Nichirei). Glomerular IgG deposits were graded from 0 to 3; 0: none, 1: minor, 2: moderate, and 3: severe deposition [26].

ELISA

Total IgG, M, and A were determined by using individual ELISA kits (Bethyl, Montogomery, TX, USA). ELISA kits for IgG anti-dsDNA antibody and IgG rheumatoid factor (RF) were purchased from Shibayagi, Gunma, Japan. Concentrations of IFN-γ, IL-4, IL-6, IL-10 and TNF were measured with specific ELISA kits, respectively (R & D Systems, Minneapolis, MI, USA).

Flow cytometry

Spleen cells (1×10^6) were incubated for 30 min at 4°C with the following fluorescein isothiocyanate or phycoerythrin conjugated

monoclonal anti-mouse antibodies; CD3, CD4, CD8 and CD19 (PharMingen, San Diego, CA, USA). We analysed cells located in the lymphocyte region based on FSC and SSC by FACS Calibur using the Cell Quest program (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis

Comparisons of two independent data sets were made by using Mann–Whitney U and χ^2 tests. A P-value < 0·05 was considered statistically significant.

RESULTS

HO-1 induction by intraperitoneal injection with hemin

We first examined HO-1 expression in the spleen from MRL/*lpr* mice by immunoblotting technique using specific anti-HO-1 antibody. Freshly isolated spleen cells from MRL/*lpr* mice expressed as little HO-1 as those from BALB/c mice, whereas substantial amounts of HO-1 were induced by *in vitro* treatment for 24 h with 100 μM hemin, a potent HO-1 inducer (Fig. 1a,b). To confirm HO-1 induction by *in vivo* hemin treatment, we examined HO-1 expression in the spleens and kidneys from mice at 24 h after a single intraperitoneal injection with 100 μmol/kg of hemin or PBS. The dose administered was selected based on the results of our previous study [9], which showed that this dosage successfully led to HO-1 dependent protection against LPS-induced acute lung injury in mice without adverse effects. As expected, a single intraperitoneal administration with hemin induced substantial amounts of HO-1 in the kidneys as well as the spleen (Fig. 1c,d).

Effects of weekly treatment with hemin on lupus nephritis

To examine the effects of HO-1 induction on lupus nephritis, MRL/*lpr* mice were intraperitoneally injected with 100 μmol/kg of hemin or PBS, as a control, once a week from 6 weeks to 24 weeks of age. Urine was collected from individual mice at 21 weeks of age. The urinary protein levels were quantitatively determined. The results showed that weekly hemin treatment significantly reduced proteinuria when compared with controls (P < 0·05, Fig. 2).

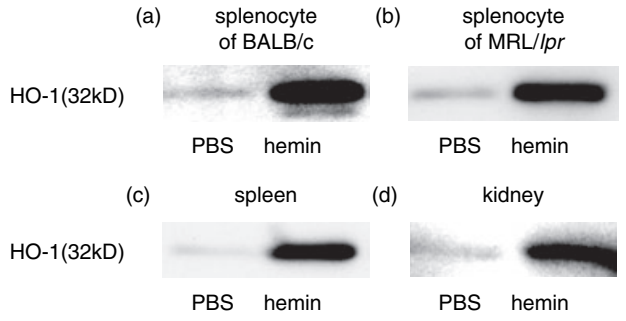

Fig. 1. Hemin-induced HO-1 expression in the spleen and kidney. HO-1 expression is determined by immunoblotting technique. Splenocytes from BALB/c (a) and MRL/*lpr* (b) were treated *in vitro* with or without 100 μM hemin for 24 h. The spleens (c) and kidneys (d) were recovered from MRL/*lpr* mice at 24 h after receiving a single intraperitoneal injection with hemin (100 μmol/kg) or PBS. All samples were obtained from mice at 16 weeks of age. Representative results of more than three individual experiments are shown. Both *in vitro* and *in vivo* treatment with hemin led to remarkable enhancement of HO-1 expression in the spleen and kidney.

We next assessed histopathological findings in the kidney from 8 hemin treated mice and 9 PBS treated mice sacrificed at 21 week-old. Damage of individual glomeruli was graded from score 0–4. The mean pathological score in individual mice was significantly lower in hemin treated mice than controls (control *versus* hemin; P < 0·05 by Mann–Whitney U-test, Table 1, Fig. 3a,b). We compared frequency of each score in all glomeruli evaluated between individual groups (Table 1). In the hemin treated mice, most of glomeruli had mild injuries (score 1 and 2), but not intact (score 0), and frequency of severely damaged glomeruli classified into score 3 or score 4 was significantly fewer than controls (control *versus* hemin, P < 0·001, by χ^2 test, Table 1). While 18·3% of glomeruli were judged as score 4 showing advanced lesions such as glomerular hyalinization, crescent formation and necrosis in control mice, no glomerulus was categorized into score 4 in hemin treated mice.

Furthermore, immunofluorescent studies using anti-IgG antibodies demonstrated that glomerular immunodeposits were remarkably reduced by weekly hemin treatment (Fig. 3c,d). Glomerular immunodeposits were quantitatively evaluated by the immunohistochemical technique to minimize differences among every experiment (Fig. 3e,f). We found that glomerular immune deposition score was significantly lower in hemin treatment mice than controls (hemin 0·84 ± 0·30, control 1·70 ± 0·20, P < 0·05 by Mann–Whitney U-test). The results indicated that weekly hemin treatment suppressed immune complex mediated

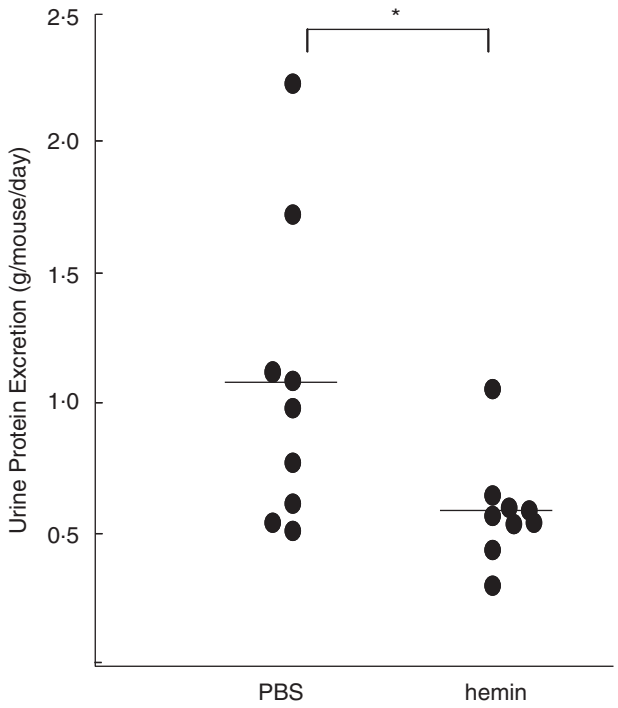

Fig. 2. Suppressive effects of weekly hemin treatment on proteinuria in MRL/*lpr* mice. Amounts of daily urinary protein excretion were determined in 21 week-old MRL/*lpr* mice which had received 100 μmol/kg of hemin (n = 9) or PBS (n = 9) weekly from 6 weeks of age. Dots indicate 24 h urinary protein excretion (g/mouse/day) of individual mice. Bars represent means of individual groups. Weekly hemin treatment significantly reduced amounts of urinary protein excretion (*P < 0·05 by Mann–Whitney U-test).

Table 1. Histopathological glomerular damage score in weekly hemin-treated and control MRL/*lpr* mice at 21 weeks of age

Treatment	No. of mice	No. of glomeruli	Mean score†*	Frequency of score (%)‡§				
				0	1	2	3	4
PBS	9	520	2·3 ± 0·3	0	27·1	36·0	18·6	18·3
Hemin	8	480	1·5 ± 0·1	0	57·9	38·8	3·3	0

Sixty glomeruli per mouse were studied, except for one PBS treated mouse in which 40 glomeruli were available. Damage of individual glomeruli was evaluated by the score system grading from score 0–4. † The pathological score was determined for every mouse by calculating the average of the glomerular scores examined. Mean score represents average ± SD of the pathological score in individual groups. The pathological score was significantly lower in hemin treated mice than controls (*$P < 0{\cdot}05$ by Mann–Whitney *U*-test). ‡ Frequency of each score was calculated in all glomeruli studied from individual groups. Glomerular damage was significantly milder in hemin treated mice than controls (§$P < 0{\cdot}001$, by χ^2 test).

glomerulonephritis in MRL/*lpr* mice, leading to clinical ameliorations.

Hemin treatment suppresses iNOS expression in the kidney

NO has been shown to play a critical role in the development of lupus nephritis [23]. Because *i*NOS is a possible therapeutic target in HO-1 induction therapy, we here examined its expression in the spleen and kidney from MRL/*lpr* mice (21 week-old) at 48 h after a single intraperitoneal injection with 100 μmol/kg of hemin. Immunoblotting analysis revealed that *i*NOS expressions in both the organs were remarkably decreased by hemin treatment (Fig. 4). These data are consistent with the hypothesis that reduction of *i*NOS expression was partly involved in favourable outcomes of HO-1 induction therapy for MRL/*lpr* mice.

Effects of hemin treatment on immunological parameters

Significant reduction of glomerular immunodeposits in the hemin treated mice raised the possibility that HO-1 induction suppressed synthesis of nephritogenic antibody, IgG anti-dsDNA antibody. We compared serum levels of specific and nonspecific antibodies between MRL/*lpr* mice receiving weekly hemin and those receiving PBS by using ELISA systems. The results demonstrated that titres of IgG anti-dsDNA antibody were significantly lower in 16 week-old mice receiving weekly hemin than in controls (Fig. 5a,b). While titres of the pathogenic antibodies increased with age in control mice, those in hemin treated mice remained as low as those in young mice (Fig. 5b). Nevertheless, serum IgG RF levels were not different between the two groups at 16 weeks of age (control group 54·4 ± 24·3 U/ml, hemin group 27·0 ± 7·8 U/ml, control *versus* hemin; NS), or at 20 weeks of age (control group 78·7 ± 32·9 U/ml, hemin group 52·3 ± 25·6 U/ml, control *versus* hemin; NS). In addition, serum levels of IgG, IgM and IgA were comparable between both the groups at any time (data not shown). Significant differences were not found in serum levels of IgG subclasses either (data not shown). These findings suggested that HO-1 induction selectively inhibited IgG anti-dsDNA antibody synthesis, resulting in reduction of glomerular immunodeposits and subsequent glomerular injuries.

We also investigated effects of HO-1 induction on other immunological parameters in MRL/*lpr*. Abnormal accumulation of double negative T cells, so-called *lpr* cells, is characteristic of MRL/*lpr* mice and elimination of the population leads to clinical remission [27]. To examine whether HO-1 induction affects lymphocyte populations in MRL/*lpr* mice, we determined frequency of B cells and *lpr* cells in the spleen from the mice treated weekly with hemin or PBS at 24 weeks of age by using flowcytometric analysis. Frequencies of the lymphocyte subsets examined including *lpr* cells, did not differ between the two groups (Table 2).

Table 2. Effects of weekly hemin treatment on lymphocyte subsets in the spleen

Treatment	CD3+ CD4-CD8-	CD19+
PBS ($n = 7$)	48·6 ± 3·8	7·7 ± 2·6
Hemin ($n = 7$)	47·9 ± 3·9	10·7 ± 2·1

Frequency of B cells and *lpr* cells in the spleen lymphocytes from mice treated weekly with hemin or PBS at 24-week-old was determined by the flowcytometric analysis using anti-CD3, CD4, CD8 and CD19 mAbs. Results are expressed as mean ± SEM.

Dysregulation of cytokine profiles is another feature of MRL/*lpr* mice [28–30]. Relative predominance of Th1 to Th2 is associated with autoimmune diseases in MRL/*lpr* mice [29,31]. To assess the effects of hemin administration on Th1/Th2 balance in MRL/*lpr* mice, serum levels of IL-4 and IFN-γ were measured by ELISA at 48 h after hemin (100 μmol/kg) or PBS was intraperitoneally given to 21-week-old mice. We found that hemin treatment reduced serum concentration of IFN-γ and that IL-4 was undetectable in all samples studied (Fig. 6). We also measured serum IL-6, IL-10, and TNF, all of which have been reported to be involved in manifestations of MRL/*lpr* and can be modulated by HO-1 induction [5,6,32,33]. However, there were no differences in these cytokines between the two groups. These data suggest that a relative Th1 shift is partly corrected by chemical induction of HO-1 in MRL/*lpr* mice. The immunomodulatory effects of HO-1 induction may partly contribute to reduction of the nephritogenic antibody and glomerular injuries in MRL/*lpr* mice.

DISCUSSION

This study is the first to demonstrate that chemical induction of HO-1 results in amelioration of lupus nephritis in MRL/*lpr* mice and is associated with suppression of *i*NOS expression in the kidney and reduction of circulating levels of IgG anti-dsDNA antibody and IFN-γ. These data suggest that not only anti-

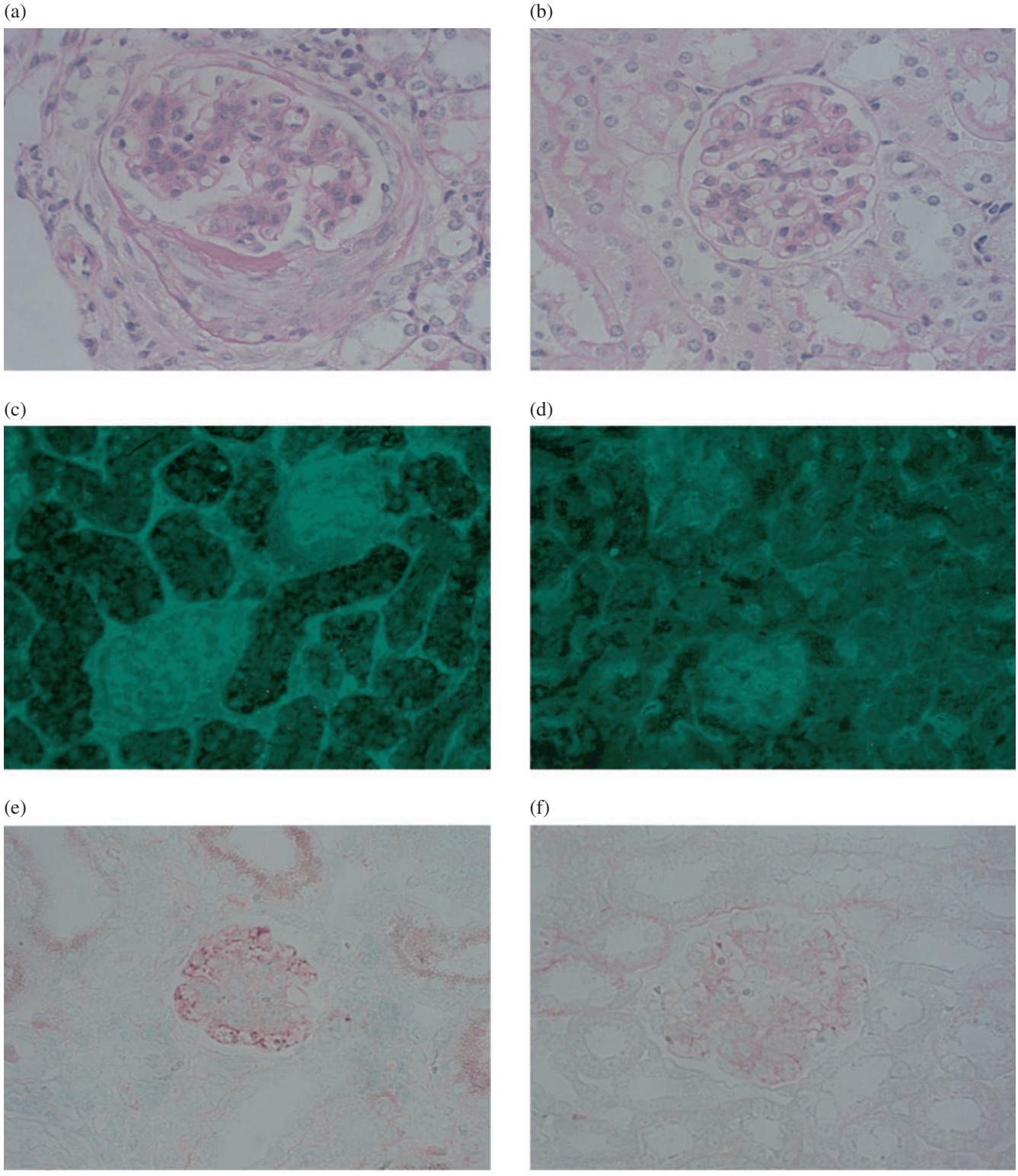

Fig. 3. Amelioration of renal histological and immunopathological findings by hemin treatment in MRL/*lpr* mice. Representative PAS staining renal sections from (a) control mice ($n = 9$) and (b) hemin treated mice ($n = 8$) are shown. Histopathological glomerular damage of (a) was judged as score 4, whereas that of (b) was score 1. Representative immunofluorescent sections using anti-mouse IgG antibodies are shown in control (c) and hemin treated mouse (d). Glomerular immunodepositions were massive in a control mouse (c), but scant in hemin treated mouse (d). Representative immunohistochemical sections are shown in control (e) and hemin treated mouse (f). Glomerular IgG deposition of (e) was judged as score 3, whereas that of (f) was score 1. Original magnifications of (a–f) are × 400.

Fig. 4. Reduction of *i*NOS expression in the spleen and kidneys by hemin. Hemin (100 μmol/kg) or PBS was intraperitoneally given to 21-week-old MRL/*lpr* mice. 48 h later, *i*NOS expression in the spleen and kidneys were examined by immunoblotting technique. Representative results of more than three individual experiments are shown. Hemin treatment led to substantial reduction of *i*NOS expressions in the both organs.

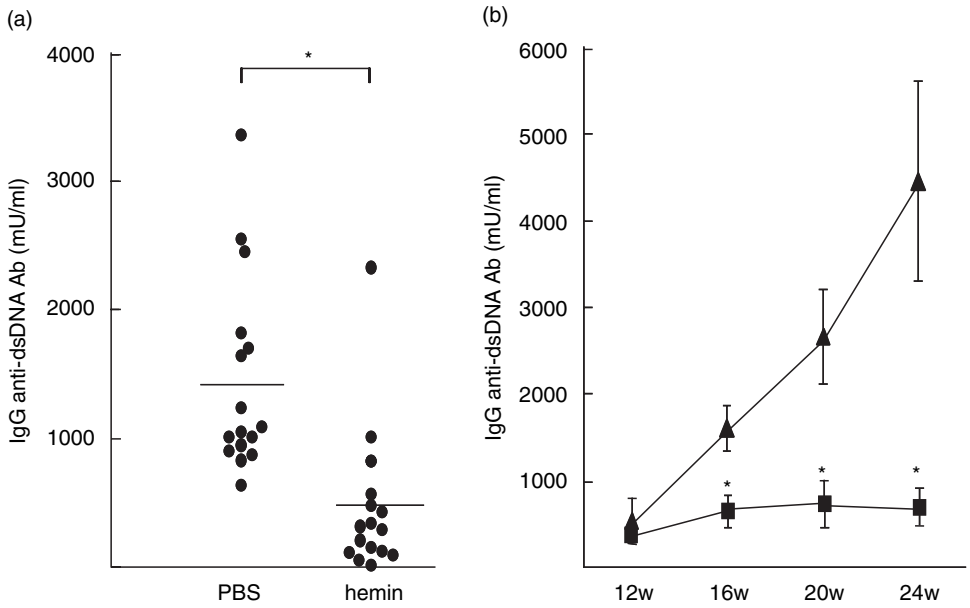

Fig. 5. Suppressive effects of hemin treatment on autoantibody synthesis in MRL/*lpr* mice. Serum IgG anti-dsDNA antibody levels in MRL/*lpr* mice treated with hemin (100 μmol/kg) or PBS every week from 6-week-old were determined by ELISA. (a) Serum IgG anti-dsDNA antibody levels at 16 weeks of age were significantly lower in MRL/*lpr* mice treated with hemin ($n = 16$) than those with PBS ($n = 16$) (*$P < 0{\cdot}05$ by Mann–Whitney *U*-test). (b) A significant reduction was also observed at 20 and 24 weeks of age (▲) PBS ($n = 11$); (■) hemin ($n = 11$), *$P < 0{\cdot}05$ by Mann–Whitney *U*-test). The data shown are the mean ± SEM.

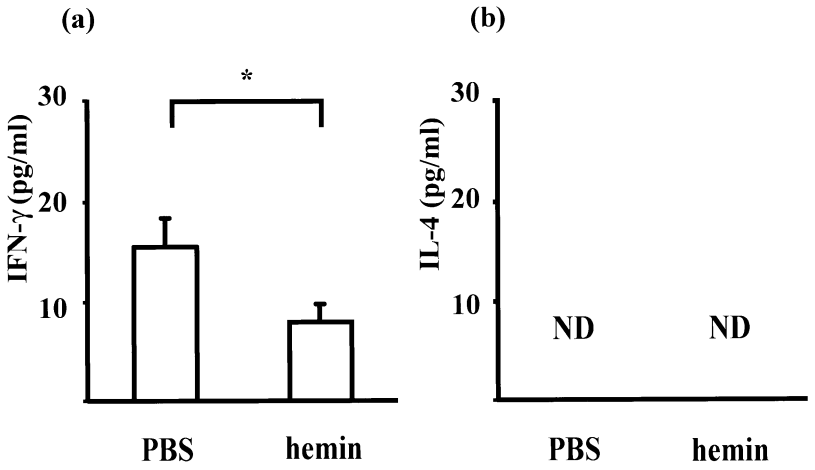

Fig. 6. Modulation of hemin treatment on cytokine profiles in MRL/*lpr* mice. Concentrations of IFN-$\gamma$and IL-4 were determined by ELISA of sera from MRL/*lpr* mice (21-week-old) at 48 h after a single intraperitoneal injection with 100 μmol/kg hemin ($n = 7$) or PBS ($n = 7$). Results are expressed as mean ± SEM (*$P < 0{\cdot}05$ by Mann–Whitney *U*-test). The serum levels of IFN-γ were significantly decreased by hemin treatment (a), whereas no difference was found in IL-4 (b).

inflammatory but also immunomodulatory effects of HO-1 induction prevent the development of autoimmune nephritis.

NO generation system is considered as one of the major targets of HO-1 induction therapy, especially for glomerular diseases, because excessive NO output is involved in glomerular injuries [22]. This is the case for lupus nephritis in MRL/*lpr* mice. Beneficial effects of NOS inhibitors have been demonstrated [23]. Both L-NMMA, a nonspecific NOS inhibitor, and L-NIL, a specific *i*NOS inhibitor, ameliorated the renal diseases without affecting deposition of immune complexes on glomeruli. Rather, NOS inhibitors suppressed NO dependent inflammatory reactions subsequent to immunodeposits as shown in kidney, skin and lung diseases of animal models [23,34,35]. For example, NOS inhibitors remarkably suppressed macrophage accumulation and activation at the sites of immune deposits [34,35].

In this study, unlike NOS inhibitors, hemin treatment significantly reduced not only serum IgG anti-dsDNA antibody level but also the glomerular immunodeposits in MRL/*lpr* mice. Reilly *et al*. [23] reported that L-NMMA, a nonspecific NOS inhibitor, showed moderately suppressive effects on serum level of anti-dsDNA antibody, but not on antiglomerular basement membrane antibody, whereas L-NIL, a specific *i*NOS inhibitor, did not affect levels of either autoantibodies. Because CO selectively suppresses *i*NOS [7], it is likely that alternative mechanisms operate in the reduction of anti-dsDNA antibody synthesis in hemin treated mice. Therefore, we investigated additional immunological effects of HO-1 induction.

Besides polyclonal B cell activation with synthesis of various autoantibodies, relative Th1 cytokine predominance is one of the characteristic features in MRL/*lpr* mice [31]. Serum IFN-γ level and its mRNA expression in the spleen and other organs are increased in MRL/*lpr* mice [29,36]. IFN-γ or IFN-γ receptor deficient MRL/*lpr* mice develop much less serious disease manifestations [37]. All of these findings indicate that IFN-γ plays a critical role in this disease. In the present study, treatment with hemin led to a significant decrease in serum IFN-γ concentrations of MRL/*lpr* mice, suggesting that suppressive effects on IFN-γ production are partly involved in the reduction of IgG anti-dsDNA antibody level and directly contribute to improvement of renal lesions in HO-1 induction therapy. Although the relationship between HO-1 expression and IFN-γ synthesis has been controversial, up-regulation of HO-1 led to reduction of IFN-γ synthesis in some experimental systems [38–41]. Treatment with cobalt protoporphyrin, an HO-1 inducer, decreases production of IL-10, IFN-γ and TNF in mouse allogeneic mixed lymphocyte reaction [38]. The blockade of selectin-P-selectin glycoprotein ligand-1 (PSGl–1) interactions prolongs survival of cardiac allografts by suppressing Th1 type cytokines associated with increased HO-1 expression [39]. In addition, HO-1 inducers modulate intracellular signal transduction through IFN-γ receptors [40]. Biliverdin, which is converted into bilirubin, one of the haem degradation products, also interferes with T cell signalling [41]. The molecular targets are nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB), both of which are involved in transcription of Th1 cytokines such as IL-2 and IFN-γ[41]. These findings indicate that HO-1 induction therapy counteracts functions of IFN-γ by inhibiting the synthesis as well as the postreceptor intracellular signalling.

Possible implications of superoxides in SLE have been demonstrated [42–44]. Rokutan *et al*. [42] showed increased oxygen intermediates in the organs from MRL/*lpr* mice, whereas reduced serum levels of antioxidants and radical scavengers have been reported in human SLE [43]. Treatment with antioxidants, such as vitamin E, suppressed circulating anti-dsDNA antibody levels and the development of renal disease [44,45]. Therefore, it is plausible that antioxidant effects are implicated in therapeutic outcomes of HO-1 induction for MRL/*lpr* mice, because haem degradation products eventually lead to synthesis of biliverdin and ferritin as potent antioxidants [8]. Besides our findings in this study, more complex immunomodulatory and anti-inflammatory effects of HO-1 induction may contribute to amelioration of renal disease of MRL/*lpr*.

Collectively, our data demonstrate that HO-1 induction therapy ameliorates lupus nephritis, possibly through multiple mechanisms, including suppression of NO synthesis, inhibition of antibody synthesis and modulation of cytokine production. Thus, pharmacological induction of HO-1 as well as genetic overexpression of HO-1 may be novel therapeutic strategies for lupus nephritis and other autoimmune diseases.

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