

Heme oxygenase 1 attenuates the development of atopic dermatitis-like lesions in mice: Implications for human disease

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Background: Oxidative stress has been implicated in the exacerbation of atopic dermatitis (AD).

Objective: We sought to investigate the pathophysiologic roles of inducible antioxidant heme oxygenase (HO) 1 in the development of AD.

Methods: Serum HO-1 levels of patients with AD (n = 100) and age-matched healthy control subjects (n = 72) were determined by means of ELISA. The relationships between serum HO-1 levels and clinical severities, laboratory parameters, and cytokines/chemokines were assessed. Skin lesions of patients with AD and psoriasis were analyzed by means of immunohistochemistry. A murine AD model, DS-Nh, was used to further investigate localization and function of HO-1. Evaluation of symptoms, serum IgE and IL-18 levels, immunoblotting results, and histologic analyses of skin were performed. The effect of intraperitoneally administered hemin, a potent HO-1 inducer, or zinc protoporphyrin IX, an inhibitor of HO, was monitored.

Results: Serum HO-1 levels were significantly increased in patients with AD compared with those seen in healthy control subjects and were associated with AD disease severity. Serum HO-1 levels correlated with serum IgE, lactate dehydrogenase, IL-18, and thymus and activation-regulated chemokine levels. HO-1-expressing cells were accumulated in skin lesions of patients with AD and DS-Nh mice. Immunofluorescence of mouse skin lesions revealed that HO-1-positive cells were

macrophages and dendritic cells. Treatment with hemin, but not with zinc protoporphyrin IX, attenuated the development of the skin lesions in DS-Nh mice and reduced serum IL-18 levels.

Conclusion: HO-1 levels were increased in sera and skin lesions of patients with AD. Enhancement of HO-1 attenuated the development of skin lesions in mice, suggesting that HO-1 induction offers a promising therapeutic strategy for AD. (J Allergy Clin Immunol 2008;122:1000-1008.)

Key words: Atopic dermatitis, heme oxygenase 1, allergy, inflammation

Atopic dermatitis (AD) is a common chronic inflammatory skin disease with various factors, including immunologic abnormalities and exposure to allergens, that contribute to the pathogenesis and development of skin lesions.¹ The frequency is estimated to be approximately 8% to 25% of the population worldwide, and the incidence is increasing, especially in developed countries.^{1,2} Oxidative stress has been implicated in cutaneous damage in various inflammatory skin diseases, including AD.³⁻⁵ Enhancement and attenuation of antioxidant defenses have been shown to be associated with the amelioration and exacerbation of AD, respectively.⁵

The present study focused on heme oxygenase (HO), one of the stress-response proteins, in the development of AD. HO is an enzyme that catalyzes the conversion of heme into carbon monoxide, Fe²⁺, and biliverdin.⁶ HO-1, an inducible form of HO, is a 32-kD heat shock protein expressed in response to various stressors.^{6,7} HO-1 has been reported to protect individual animals from a variety of pathologic conditions in murine models of lung inflammation^{8,9} and lupus nephritis.¹⁰ Lack of HO-1 expression causes severe chronic inflammation, as shown in studies of HO-1 knockout mice and a patient with HO-1 deficiency.^{11,12} Furthermore, pathologic roles of HO-1 have been shown in systemic inflammatory disorders, such as rheumatic diseases.¹³⁻¹⁵ Serum HO-1 levels are increased in patients with active hemophagocytic syndrome (HPS) and adult-onset Still's disease (ASD), both of which are accompanied by enormous macrophage activation.¹³ HO-1 expressed in synovial tissue counteracts inflammation and osteoclast-mediated bone destruction in patients with rheumatoid arthritis.^{14,16} Thus accumulating evidence reinforces the notion that HO-1 has anti-inflammatory and cytoprotective roles.

The role of HO-1 in allergic skin conditions, including AD, remains poorly understood. HO-1 expression can be modulated by a series of interactions among cytokines and chemokines in

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Abbreviations used

AD:	Atopic dermatitis
ASD:	Adult-onset Still's disease
DC:	Dendritic cell
HC:	Healthy control subject
HO:	Heme oxygenase
HPS:	Hemophagocytic syndrome
HRP:	Horseshoe peroxidase
iNOS:	Inducible nitric oxide synthase
LDH:	Lactate dehydrogenase
SPF:	Specific pathogen-free
TARC:	Thymus and activation-regulated chemokine
ZnPP:	Zinc protoporphyrin

patients with AD. T_H2 cytokines, such as IL-4, IL-10, and IL-13, have been shown to upregulate HO-1 expression.¹⁷ The anti-inflammatory functions of IL-10 are partly dependent on the HO-1 induction, whereas HO-1 augments the production of IL-10 and reduces the production of proinflammatory cytokines.^{8,18-20} On the other hand, the T_H1 cytokines TNF- α and IFN- γ repress HO-1 expression in general, thereby enhancing proinflammatory cytokines production.^{15,21,22} IL-18, which was originally identified as an IFN- γ -inducing factor, is implicated in not only T_H1 - but also T_H2 -mediated diseases.^{23,24} Interestingly, IL-18 is excessively produced in ASD and HPS, in both of which serum HO-1 levels are extremely increased.^{6,23}

The current study investigated the relationship of serum HO-1 levels with clinical severity and several parameters in patients with AD. Local expression of HO-1 in skin lesions of patients with AD and model mice was also studied. Moreover, the effects of the chemical induction or inhibition of HO-1 on symptomatic manifestations in AD model mice were examined. The findings suggested that HO-1 alleviates inflammation in patients with AD.

METHODS**Patients with AD and healthy control subjects**

All of the patients fulfilled the diagnostic criteria for AD proposed by Hanifin and Rajka.²⁵ Clinical characteristics of the patients are listed in Table I. The patients had been treated with the following reagents: topical corticosteroids, 36 patients; oral antihistamines and topical corticosteroids, 42 patients; oral antihistamines, 2 patients; and no treatment, 20 patients. None of the patients received oral corticosteroids, other immunosuppressants, or both. The clinical severity of AD was evaluated according to the criteria in the "Therapeutic guidelines for atopic dermatitis 2002."²⁶ The Global Clinical Severity is classified in the clinical condition as follows: very severe, inflamed skin lesions covering more than 30% of the body surface; severe, inflamed skin lesions covering more than 10% but less than 30% of the body surface; moderate, inflamed skin lesions covering less than 10% of the body surface; and mild, dry skin, scaling, and faint erythema only.²⁶ Of the total 100 patients studied, the number of patients affected with additional atopic diseases was as follows: allergic rhinitis, 36; conjunctivitis, 7; bronchial asthma, 33 (none had asthma attack more than a year); and pollinosis, 31. Peripheral blood was sampled from the patients to evaluate the peripheral WBC counts, serum total IgE levels, lactate dehydrogenase (LDH) levels, and thymus and activation-regulated chemokine (TARC) levels. An age-matched group of 72 individuals without any atopic diseases served as healthy control subjects (HCs). For immunohistochemistry, skin from 4 patients with AD (all male, mean global skin score of 3.6), 3 with pathologically proved psoriasis (1 female and 2 male patients), and 3 HCs (all male) were obtained. All the experiments were performed after obtaining written informed consent, which was approved by the local institutional review board.

TABLE I. Characteristics of the study subjects

Severity	n	Age (y)	Female/male	IgE (IU/mL)
AD	100	28.7 $\pm$ 9.2	41/59	6084 $\pm$ 10,557
1 (mild)	22	28.2 $\pm$ 7.1	14/8	1218 $\pm$ 2324
2 (moderate)	33	29.2 $\pm$ 8.5	17/16	2106 $\pm$ 3306
3 (severe)	18	32.2 $\pm$ 11.8	5/13	9943 $\pm$ 7636
4 (very severe)	27	30.6 $\pm$ 9.7	5/22	16,670 $\pm$ 17,903
HCs	72	31.3 $\pm$ 7.4	22/50	(<250)

The items in parentheses indicate the normal range of serum IgE levels in Yokohama City University Hospital.

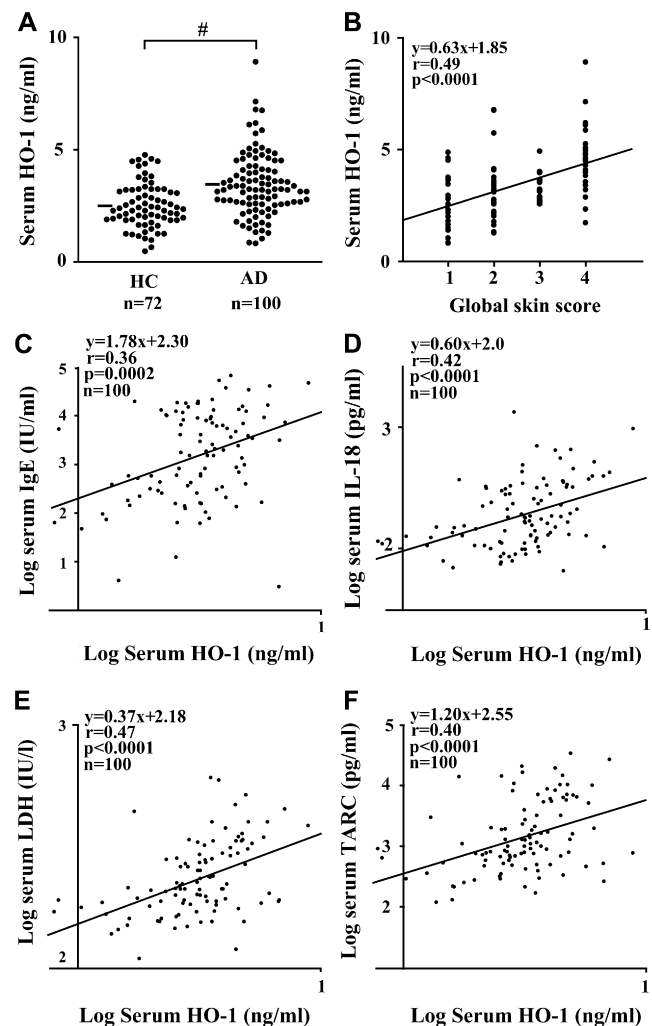


FIG 1. Serum levels of HO-1 in patients with AD ($n = 100$) and HCs ($n = 72$). **A:** Serum levels of HO-1 in patients with AD divided into 4 groups based on their disease severities (**B**). $**P < .01$ and $\#P < .0001$ versus HCs. Horizontal bars indicate mean serum HO-1 values. Relationships between serum HO-1 concentrations and laboratory parameters in patients with AD are shown in the log-log form. Correlations with serum IgE (**C**), IL-18 (**D**), LDH (**E**), and TARC (**F**) levels were analyzed by means of regression analysis.

Animals

Male DS-Nh mice (5 or 6 weeks old) raised under specific pathogen-free (SPF) conditions were provided from the Aburahi Laboratories (Shionogi and Co Ltd, Shiga, Japan). DS-Nh, mutant nonhair DS mice established in 1954,

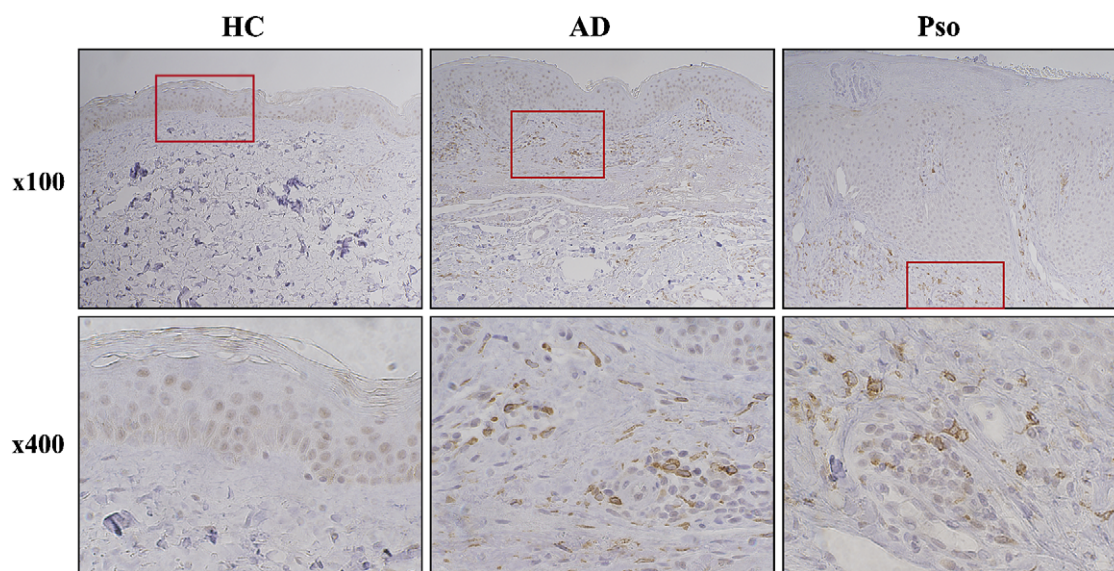


FIG 2. HO-1 immunohistochemistry of the skin ($\times 100$) from an HC, a patient with AD, and a patient with psoriasis (*Pso*). Red rectangles indicate enlarged areas ($\times 400$), which are shown in the lower part of the figure. HC, 44-year-old man; patient with AD, 68-year-old man, global skin score of 3; patient with psoriasis, 63-year-old man.

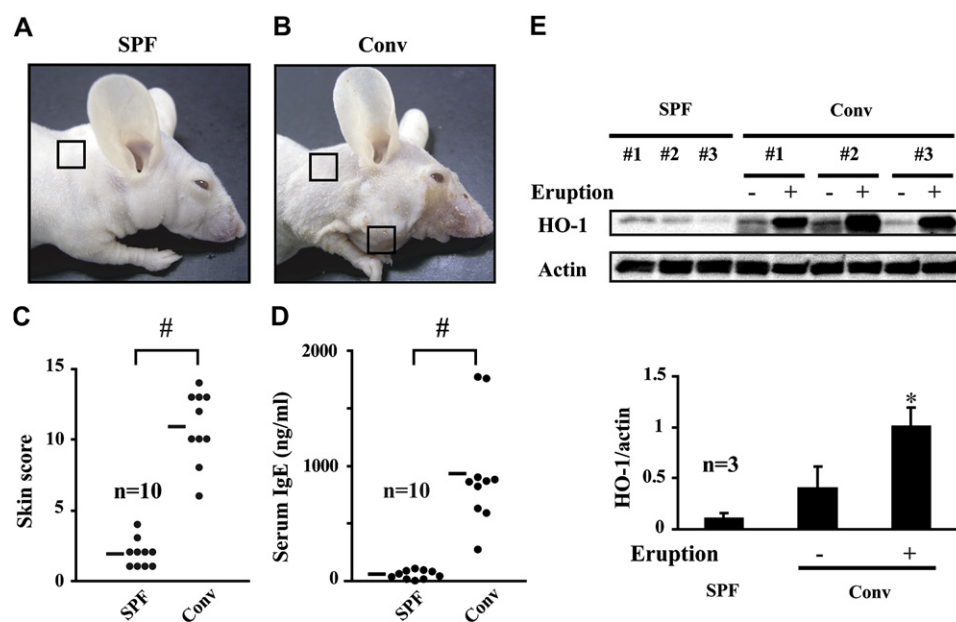


FIG 3. HO-1 expression of DS-Nh mice. Photographs of 18-week-old DS-Nh mice maintained under SPF (A), or conventional (*conv*; B) conditions. Solid squares indicate area with (bottom right) or without (upper left) eruption resected for immunoblottings (see Fig 3, E, *conv* no. 2). Skin scores (C); serum IgE levels (D). # $P < .001$ versus the SPF group. Horizontal bars represent mean levels. Immunoblotting of HO-1 and actin from DS-Nh mice skin with or without eruption (E). Densitometric analysis is shown below (means $\pm$ SEMs). * $P < .05$ versus the SPF group.

have considerable AD-like skin lesions, including accumulated eosinophils and mast cells with increased serum IgE levels, when they are housed under conventional conditions and display skin lesions of lesser severity and frequency under SPF conditions.²⁷ *Staphylococcus aureus* is a critical factor for the development of dermatitis in DS-Nh mice.²⁸ The DS-Nh mice used for the study were obtained as F1 (Nh/+) from male DS-Nh (Nh/Nh) $\times$ female DS (+/+).

They were divided into groups based on SPF and conventional conditions and then intraperitoneally administered either 4 $\mu\text{mol/L}$ per body hemin (Sigma-Aldrich, St Louis, Mo), 4 $\mu\text{mol/L}$ per body zinc protoporphyrin IX (ZnPPiX; Frontier Scientific, Logan, Utah), or PBS (Nissui, Tokyo, Japan) every week from 6 to 18 weeks of age. The dosages of hemin and ZnPPiX were determined on the basis of an experiment in which hemin and ZnPPiX efficiently induced or inhibited HO-1 activity.²⁹

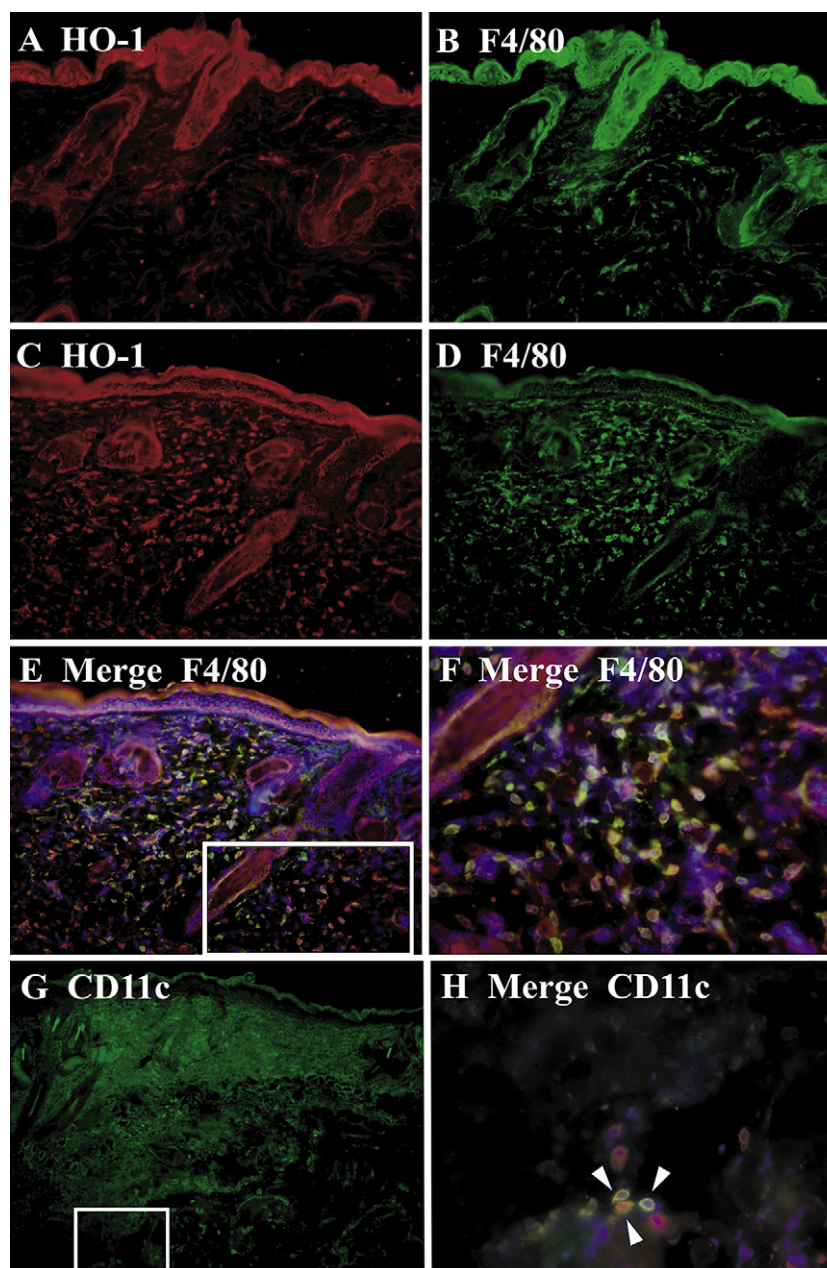


FIG 4. Immunofluorescent staining of intact skin from an SPF mouse (**A and B**) and skin lesion of a conventional DS-Nh mouse (**C-H**). **Fig 4, A**, HO-1; **Fig 4, B**, F4/80; **Fig 4, C**, HO-1; **Fig 4, D**, F4/80; **Fig 4, E**, merged image of HO-1, F4/80, and 4',6-diamidino-2-phenylindole ($\times 100$); **Fig 4, F**, enlarged view of **Fig 4, E**, in white square ($\times 200$); **Fig 4, G**, CD11c ($\times 100$); **Fig 4, H**, enlarged view of **Fig 4, G**, in white square. Merged image of HO-1, CD11c, and 4',6-diamidino-2-phenylindole ($\times 400$). Arrowheads indicate HO-1- and CD11c-positive DCs.

Twenty-four hours after a single injection or a week after the last injection, the mice were killed, and serum, spleen, dorsal nuchal skin, and lymph nodes (cervical, axillary, and cubital) were sampled. The study was conducted in accordance with the Guidelines for Animal Experiments of the Yokohama City University Graduate School of Medicine.

Evaluation of the clinical skin conditions of AD model animals

Symptoms of conventional and SPF DS-Nh mice were assessed every week. Clinical skin conditions defined as erythema, edema, excoriation/

erosion, dry skin/desquamation, and scratch were respectively graded from 0 to 3.²⁷ The "skin score" is defined as the sum of the individual scores.²⁷

Antibodies and reagents

Reagents were obtained as follows: normal rat serum (Sigma-Aldrich); normal rabbit serum (DAKO, Glostrup, Denmark); anti-human HO-1 mAb and polyclonal antibody (Stressgen, Victoria, Canada); anti-actin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif); anti-mouse CD11c mAb (eBioscience, San Diego, Calif); anti-mouse F4/80 mAb (Serotech, Oxford, United Kingdom); horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Amersham Life Sciences, Piscataway, NJ); HRP-conjugated

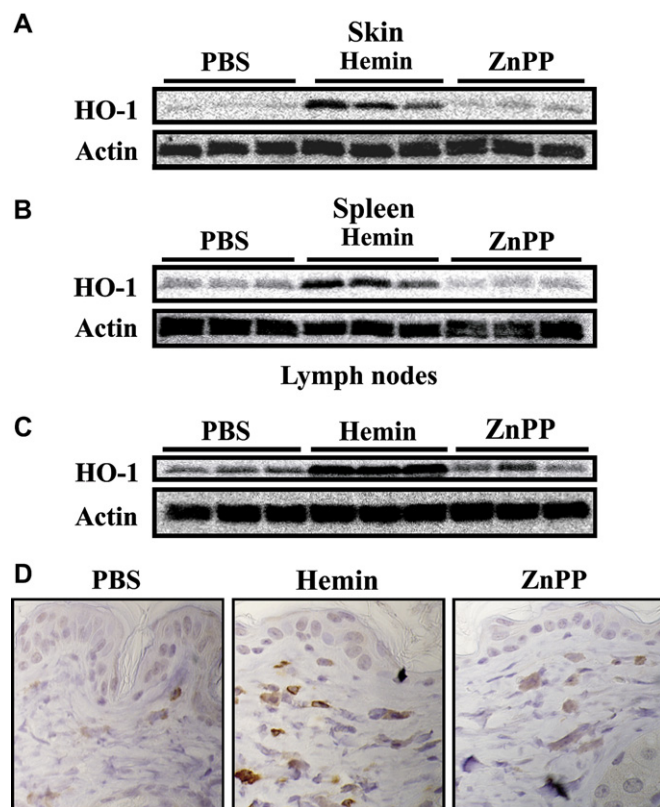


FIG 5. Influence of single intraperitoneal administration of PBS, hemin, or ZnPIX on HO-1 and actin expression in DS-Nh mice. Immunoblotting analysis of skin (A), spleen (B), and lymph nodes (C) of DS-Nh mice ($n = 3$) treated with PBS, hemin, or ZnPIX for 24 hours. Immunohistochemistry of HO-1 in DS-Nh mouse skin treated with PBS, hemin, and ZnPIX ($\times 400$; D).

anti-goat IgG (Zymed, South San Francisco, Calif); and Alexa Fluor 594 goat anti-rabbit antibody and Alexa Fluor 488 goat anti-hamster or anti-rat Ab (Molecular Probes, Eugene, Ore).

ELISA

Serum protein levels were determined by using ELISA kits as follows: human HO-1 ELISA kit (Stressgen)^{9,13}; human and mouse IL-4, IL-10, IFN- γ , and TARC ELISA kits (R&D Systems, Minneapolis, Minn); mouse IgE ELISA kit (Shibayagi, Gunma, Japan); and human and mouse IL-18 ELISA kits (MBL, Nagoya, Japan).

Real-time PCR

Real-time PCR was performed as described in the Methods section of the Online Repository at www.jacionline.org.

Immunoblotting analysis

Immunoblotting analysis was performed as described in the Methods section of the Online Repository.

Immunohistochemistry and fluorescent staining

Immunohistochemistry and fluorescent staining were performed as described in the Methods section of the Online Repository.

Statistical analysis

The Mann-Whitney U test, Kruskal-Wallis test, Wilcoxon signed-rank test, regression analysis, and 2-way ANOVA repeated measures with the Dunnett

post-hoc test were used to evaluate statistical significance of the results. P values of less than .05 were considered statistically significant.

RESULTS

Increased serum HO-1 levels in patients with AD

Serum HO-1 levels in patients with AD and HCs were measured by means of ELISA. As shown in Fig 1, A, serum HO-1 levels were increased in the patients with AD compared with those in the HCs. Moreover, the levels were closely correlated with the severity of cutaneous manifestations (Fig 1, B). Serum HO-1 levels of 10 patients with AD were monitored for 269.8 ± 89.0 days (range, 131–416 days). Although global skin scores were reduced after treatment with topical corticosteroids and oral antihistamines (pretreatment, 3.4 ± 0.8 ; posttreatment, 2.5 ± 0.8 ; $P < .05$), serum HO-1 levels also significantly decreased among these patients (pretreatment, 4.6 ± 1.0 ng/mL; posttreatment, 3.7 ± 0.7 ng/mL; $P < .05$). Although other allergic complications can exert an influence on serum HO-1 levels, differences were insignificant between patients with or without allergic rhinitis, conjunctivitis, asthma, and/or pollinosis (see Fig E1 in the Online Repository at www.jacionline.org).

Relationships between serum HO-1 levels and laboratory parameters of AD

The relationships of the serum HO-1 levels to laboratory parameters of AD, such as serum IgE and LDH levels and peripheral eosinophil counts, were examined.^{30,31} Regression analysis revealed that serum HO-1 levels positively correlated with serum IgE and LDH levels (Fig 1, C). In peripheral leukocyte fractions serum HO-1 levels correlated with monocyte counts ($P < .01$) and with neutrophil counts ($P < .05$), although not with eosinophil, lymphocyte, and basophil counts (see Fig E2 in the Online Repository at www.jacionline.org). The circulating cytokines and chemokines IL-4, IL-10, IFN- γ , IL-18, and TARC are implicated in the pathogenesis of AD.^{1,24,32} Of them, the serum levels of IL-18 and TARC, but not IL-4, IL-10, or IFN- γ , were statistically correlated with the serum HO-1 level (Fig 2 and see Fig E3 in the Online Repository at www.jacionline.org). The severity of cutaneous manifestations in the patients with AD was well correlated with serum IL-18 levels, further supporting the importance of the cytokine in AD (see Fig E4 in the Online Repository at www.jacionline.org). Thus increased serum HO-1 levels are associated with conventional disease parameters in patients with AD.

HO-1-expressing cells accumulated in skin lesions of patients with AD

HO-1 expression in skin lesions of patients with AD was evaluated (Fig 2). Immunohistochemistry demonstrated that HO-1-expressing cells were accumulated in the dermis of patients with AD but scarcely in HCs (Fig 2). Consistent with previous reports,^{33,34} patients with psoriasis also showed increased HO-1 expression in their dermis.

HO-1-positive macrophages and dendritic cells accumulated in the skin lesions of DS-Nh mice

HO-1 expression in AD-like skin lesions was validated by using DS-Nh mice. The mice maintained under conventional

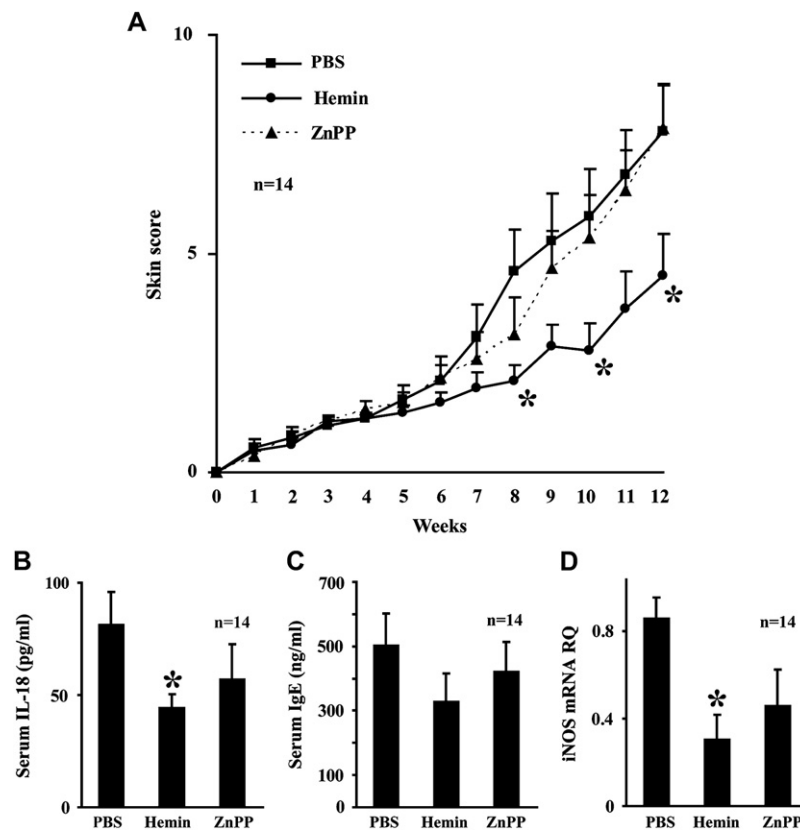


FIG 6. Effects of HO-1 inducer or inhibitor on the development of AD-like skin lesions in DS-Nh mice ($n = 14$). PBS, hemin ($4 \mu\text{mol/L}$), or ZnPPIX ($4 \mu\text{mol/L}$) was intraperitoneally injected every week from 6 to 18 weeks of age. **A**, Skin scores. **B**, Serum IL-18 levels. **C**, Serum IgE levels of PBS-, hemin-, and ZnPPIX-treated mice. **D**, Real-time PCR analysis of iNOS mRNA expressions of spleens. Data are presented as means $\pm$ SEMs. RQ, Relative quantity. * $P < .05$ versus the PBS-treated group.

conditions had more skin lesions (Fig 3, A-C) and a higher titer of serum IgE (Fig 3, D) than SPF-maintained mice, as previously shown.²⁷ Immunoblotting analysis revealed abundant HO-1 protein expression in the skin eruption of DS-Nh mice maintained under conventional conditions (see Fig 3E). HO-1 was increased even in the skin, without any evident eruption in the conventional mice, whereas it was scarcely expressed in the intact skin of SPF-maintained mice (see Fig 3E). Immunohistochemical studies demonstrated that a majority of the HO-1-positive cells in atopic skin coexpressed F4/80, a macrophage marker, and a minority were positive for CD11c, a dendritic cell (DC) marker (Fig 4). Staining in the epidermis was judged as a nonspecific finding because it was also noted when treated with control rat or rabbit serum (see Fig E5 in the Online Repository at www.jacionline.org).

Effect of hemin and ZnPPIX on the development of AD-like skin lesions in DS-Nh mice

HO-1 expression was examined in the skin, lymph nodes, and spleens from 7-week-old SPF DS-Nh mice at 24 hours after a single injection with hemin, an HO-1 inducer, or ZnPPIX, an inhibitor. Immunoblotting revealed abundant HO-1 expression in the skin, which macroscopically appeared intact, under treatment with hemin but not with ZnPPIX. Similarly, hemin enhanced HO-1 expression in the spleen and lymph nodes (Fig 5, A-C).

Immunohistochemical studies showed that hemin-induced HO-1-positive cells coexpressed F4/80 in the dermis (Fig 5, A and D, and see Fig E6 in the Online Repository at www.jacionline.org).

Hemin, $4 \mu\text{mol/L}$, was intraperitoneally injected every week from 6 to 18 weeks of age to further examine the effects of hemin-induced HO-1 on the AD-like skin lesions in DS-Nh mice. As shown in Fig 6, A, treatment with hemin significantly attenuated development of the AD-like skin lesions in the DS-Nh mice maintained under conventional conditions. On the other hand, weekly administration of ZnPPIX, which inhibited HO-1 enzymatic activity, had no influence on the skin lesions (Fig 6, A). In terms of humoral factors, serum IL-18 levels were reduced in hemin-treated mice (Fig 6, B). Interestingly, there was a strong positive correlation between skin scores and serum IL-18 levels (see Fig E7 in the Online Repository at www.jacionline.org), as shown in patients with AD. IgE levels were relatively low in hemin-treated mice compared with those in the PBS-treated groups, although they did not reach statistical significance (Fig 6, C). In concordance with clinical manifestations, no significant differences were found in serum IL-18 or IgE levels between ZnPPIX-treated mice and control animals. Quantification of spleen mRNA revealed that inducible nitric oxide synthase (iNOS) expression was significantly reduced in the hemin-treated group (Fig 6, D). These findings suggest that HO-1 plays a certain role in suppressing the development of AD.

DISCUSSION

The present study demonstrates that serum HO-1 levels are increased in patients with AD and that the levels correlate with the severity of clinical manifestations and conventional disease markers. In an AD mouse model HO-1 protein was abundantly expressed in resident macrophages and DCs in the AD-like skin lesions. Interestingly, chemical induction of HO-1 by hemin suppressed the development of these AD-like skin lesions, suggesting a protective role of HO-1 against skin inflammation.

The precise mechanism of HO-1 induction in patients with AD remains to be elucidated. T_H2 cytokines, including IL-4 and IL-10, can contribute to an increase in HO-1 levels^{35,36}; however, circulating levels of these cytokines did not correlate with serum HO-1 levels. Rather, the levels showed significant correlation with IL-18. Moreover, our data confirmed that serum IL-18 levels were correlated with the severity of skin manifestations in the patients and in the murine model (see Figs E2 and E7). Although IL-18 was originally identified as an IFN- γ -inducing factor, the cytokine is also implicated in allergic conditions, especially infection-associated AD models,²⁴ such as DS-Nh. A close link between HO-1 and IL-18 is supported by the evidence that both the proteins are mainly produced by macrophages,^{6,15,23} and in fact, HO-1 and IL-18 levels are immensely increased in macrophage activation syndromes, such as ASD and HPS.^{13,37} Attenuating IL-18 by hemin might account for the therapeutic effect, although an interaction between IL-18 and HO-1 in AD remains to be determined.

Abnormal circulating HO-1 levels have been shown to be characteristic of certain pathologic conditions, although the sources and mechanisms remain undetermined. Schipper et al³⁸ first observed reduced plasma HO-1 concentrations in patients with Alzheimer's disease. We have previously reported that serum HO-1 levels were increased in patients with ASD,¹³ HPS,¹³ and silicosis⁹ and were closely correlated with the clinical activities of each disease. The current study demonstrates that serum HO-1 levels were significantly increased in patients with AD, although no study, to our knowledge, has ever shown abnormal serum HO-1 levels in allergic disorders. Because HO-1 is highly expressed in skin lesions of patients with AD, circulating HO-1 is considered to be derived from the affected skin, at least in large part.

The results show the suppressive effects of hemin on the development of AD-like dermatitis, suggesting a regulatory role of HO-1 in patients with AD. Bronchial asthma is another representative atopic disorder, in which airway macrophages expressing a high level of HO-1³⁹ play a suppressive role in the progression of airway inflammation.^{40,41} As for skin, a protective role of HO-1 has been reported in experimental wounding,⁴² ultraviolet radiation,⁴³ and psoriasis.³³ The functions of HO-1 in skin have been attributed to attenuating antigen presentation by DCs^{34,44} and TARC production by keratinocytes.⁴⁵ Our data suggest that local inflammatory responses were suppressed by an accumulation of HO-1-overexpressing hemin-primed macrophages in the skin lesions. Similar findings have been demonstrated in a pancreatitis model, in which HO-1-overexpressing hemin-primed macrophages home to the pancreas and protect the host from pancreatitis.⁴⁶ Thus modulating macrophage function by means of HO-1 is a potential therapeutic target for AD.

The reason why ZnPPiX had a null effect on the development of the AD-like skin lesions is unclear. Although the dosage of ZnPPiX in our experiments achieved a sufficient level to inhibit HO-1 activity,^{9,29} the dosage might not be enough to suppress

functions of abundantly expressed HO-1 in the AD-like skin lesions of this study.

As demonstrated in Fig 6, C, treatment with hemin led to a reduced expression of iNOS mRNA, which is involved in the exacerbation of AD.^{47,48} Because HO-1 induction has been reported to suppress iNOS expression,¹⁰ it is consistent that a suppression of iNOS would be associated with the amelioration of dermatitis.

In conclusion, this study provides the first evidence that HO-1 regulates AD development. HO-1 induction offers a promising therapeutic strategy.

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Clinical implications: Enhancement of HO-1 offers a promising therapeutic approach to AD.

REFERENCES

- Boguniewicz M, Schmid-Grendelmeier P, Leung DY. Atopic dermatitis. *J Allergy Clin Immunol* 2006;118:40-3.
- Weston WL, Howe W. Epidemiology, clinical manifestations, and diagnosis of atopic dermatitis (eczema). UpToDate 2007. Available at: <http://www.uptodate.com>. Accessed June 16, 2008.
- Bowler RP, Crapo JD. Oxidative stress in allergic respiratory diseases. *J Allergy Clin Immunol* 2002;110:349-56.
- Fuchs J, Zollner TM, Kaufmann R, Podda M. Redox-modulated pathways in inflammatory skin diseases. *Free Radic Biol Med* 2001;30:337-53.
- Tsukahara H, Shibata R, Ohshima Y, Todoroki Y, Sato S, Ohta N, et al. Oxidative stress and altered antioxidant defenses in children with acute exacerbation of atopic dermatitis. *Life Sci* 2003;72:2509-16.
- Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 2003;24:449-55.
- Morse D, Choi AM. Heme oxygenase-1: the "emerging molecule" has arrived. *Am J Respir Cell Mol Biol* 2002;27:8-16.
- Inoue S, Suzuki M, Nagashima Y, Suzuki S, Hashiba T, Tsuburai T, et al. Transfer of heme oxygenase 1 cDNA by a replication-deficient adenovirus enhances interleukin 10 production from alveolar macrophages that attenuates lipopolysaccharide-induced acute lung injury in mice. *Hum Gene Ther* 2001;12:967-79.
- Sato T, Takeno M, Honma K, Yamauchi H, Saito Y, Sasaki T, et al. Heme Oxygenase-1, a Potential Biomarker of Chronic Silicosis, Attenuates Silica-induced Lung Injury. *Am J Respir Crit Care Med* 2006;174:906-14.
- Takeda Y, Takeno M, Iwasaki M, Kobayashi H, Kirino Y, Ueda A, et al. Chemical induction of HO-1 suppresses lupus nephritis by reducing local iNOS expression and synthesis of anti-dsDNA antibody. *Clin Exp Immunol* 2004;138:237-44.
- Poss KD, Tonegawa S. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci U S A* 1997;94:10925-30.
- Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, et al. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* 1999;103:129-35.
- Kirino Y, Takeno M, Iwasaki M, Ueda A, Ohno S, Shirai A, et al. Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia. *Arthritis Res Ther* 2005;7:R616-24.
- Kobayashi H, Takeno M, Saito T, Takeda Y, Kirino Y, Noyori K, et al. Regulatory role of heme oxygenase 1 in inflammation of rheumatoid arthritis. *Arthritis Rheum* 2006;54:1132-42.
- Kirino Y, Takeno M, Murakami S, Kobayashi M, Kobayashi H, Miura K, et al. Tumor necrosis factor alpha acceleration of inflammatory responses by down-regulating heme oxygenase 1 in human peripheral monocytes. *Arthritis Rheum* 2007;56:464-75.
- Zwerina J, Tzima S, Hayer S, Redlich K, Hoffmann O, Hanslik-Schnabel B, et al. Heme oxygenase 1 (HO-1) regulates osteoclastogenesis and bone resorption. *FASEB J* 2005;19:2011-3.

17. Chaitidis P, O'Donnell V, Kuban RJ, Bermudez-Fajardo A, Ungethuen U, Kuhn H. Gene expression alterations of human peripheral blood monocytes induced by medium-term treatment with the TH2-cytokines interleukin-4 and -13. *Cytokine* 2005; 30:366-77.
18. Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nat Med* 2002;8:240-6.
19. Ricchetti GA, Williams LM, Foxwell BM. Heme oxygenase 1 expression induced by IL-10 requires STAT-3 and phosphoinositol-3 kinase and is inhibited by lipopolysaccharide. *J Leukoc Biol* 2004;76:719-26.
20. Jung M, Sabat R, Kratzschmar J, Seidel H, Wolk K, Schonbein C, et al. Expression profiling of IL-10-regulated genes in human monocytes and peripheral blood mononuclear cells from psoriatic patients during IL-10 therapy. *Eur J Immunol* 2004; 34:481-93.
21. Fernandez P, Guillen MI, Gomar F, Alcaraz MJ. Expression of heme oxygenase-1 and regulation by cytokines in human osteoarthritic chondrocytes. *Biochem Pharmacol* 2003;66:2049-52.
22. Udono-Fujimori R, Takahashi K, Takeda K, Furuyama K, Kaneko K, Takahashi S, et al. Expression of heme oxygenase-1 is repressed by interferon-gamma and induced by hypoxia in human retinal pigment epithelial cells. *Eur J Biochem* 2004;271:3076-84.
23. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. Interleukin-18 regulates both Th1 and Th2 responses. *Annu Rev Immunol* 2001;19:423-74.
24. Tsutsui H, Yoshimoto T, Hayashi N, Mizutani H, Nakanishi K. Induction of allergic inflammation by interleukin-18 in experimental animal models. *Immunol Rev* 2004;202:115-38.
25. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 1980;92:44-7.
26. Masutaka F, Yoichi K, Shoso Y, Toshiyuki A, Kazuo A, Shuhei I, et al. Therapeutic guidelines for atopic dermatitis 2002. *Allergol Int* 2005;54:45-9.
27. Hikita I, Yoshioka T, Mizoguchi T, Tsukahara K, Tsuru K, Nagai H, et al. Characterization of dermatitis arising spontaneously in DS-Nh mice maintained under conventional conditions: another possible model for atopic dermatitis. *J Dermatol Sci* 2002;30:142-53.
28. Haraguchi M, Hino M, Tanaka H, Maru M. Naturally occurring dermatitis associated with *Staphylococcus aureus* in DS-Nh mice. *Exp Anim* 1997;46: 225-9.
29. Ndisang JF, Wu L, Zhao W, Wang R. Induction of heme oxygenase-1 and stimulation of cGMP production by hemin in aortic tissues from hypertensive rats. *Blood* 2003;101:3893-900.
30. Johnson EE, Irons JS, Patterson R, Roberts M. Serum IgE concentration in atopic dermatitis. Relationship to severity of disease and presence of atopic respiratory disease. *J Allergy Clin Immunol* 1974;54:94-9.
31. Mukai H, Noguchi T, Kamimura K, Nishioka K, Nishiyama S. Significance of elevated serum LDH (lactate dehydrogenase) activity in atopic dermatitis. *J Dermatol* 1990;17:477-81.
32. Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, et al. Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. *J Allergy Clin Immunol* 2001;107:535-41.
33. Wojas-Pelc A, Marcinkiewicz J. What is a role of haeme oxygenase-1 in psoriasis? Current concepts of pathogenesis. *Int J Exp Pathol* 2007;88:95-102.
34. Listopad J, Asadullah K, Sievers C, Ritter T, Meisel C, Sabat R, et al. Heme oxygenase-1 inhibits T cell-dependent skin inflammation and differentiation and function of antigen-presenting cells. *Exp Dermatol* 2007;16:661-70.
35. Ohmen JD, Hanifin JM, Nickoloff BJ, Rea TH, Wyzykowski R, Kim J, et al. Overexpression of IL-10 in atopic dermatitis. Contrasting cytokine patterns with delayed-type hypersensitivity reactions. *J Immunol* 1995;154:1956-63.
36. Lester MR, Hofer MF, Gately M, Trumble A, Leung DY. Down-regulating effects of IL-4 and IL-10 on the IFN-gamma response in atopic dermatitis. *J Immunol* 1995;154:6174-81.
37. Kawashima M, Yamamura M, Taniai M, Yamauchi H, Tanimoto T, Kurimoto M, et al. Levels of interleukin-18 and its binding inhibitors in the blood circulation of patients with adult-onset Still's disease. *Arthritis Rheum* 2001;44:550-60.
38. Schipper HM, Chertkow H, Mehndate K, Frankel D, Melmed C, Bergman H. Evaluation of heme oxygenase-1 as a systemic biological marker of sporadic AD. *Neurology* 2000;54:1297-304.
39. Horvath I, Donnelly LE, Kiss A, Paredi P, Kharitonov SA, Barnes PJ. Raised levels of exhaled carbon monoxide are associated with an increased expression of heme oxygenase-1 in airway macrophages in asthma: a new marker of oxidative stress. *Thorax* 1998;53:668-72.
40. Xia ZW, Zhong WW, Xu LQ, Sun JL, Shen QX, Wang JG, et al. Heme oxygenase-1-mediated CD4+CD25high regulatory T cells suppress allergic airway inflammation. *J Immunol* 2006;177:5936-45.
41. Almolki A, Taille C, Martin GF, Jose PJ, Zedda C, Conti M, et al. Heme oxygenase attenuates allergen-induced airway inflammation and hyperreactivity in guinea pigs. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L26-34.
42. Wagener FA, van Beurden HE, von den Hoff JW, Adema GJ, Figdor CG. The heme-heme oxygenase system: a molecular switch in wound healing. *Blood* 2003;102:521-8.
43. Allanson M, Reeve VE. Immunoprotective UVA (320-400 nm) irradiation upregulates heme oxygenase-1 in the dermis and epidermis of hairless mouse skin. *J Invest Dermatol* 2004;122:1030-6.
44. Chauveau C, Remy S, Royer PJ, Hill M, Tanguy-Royer S, Hubert FX, et al. Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. *Blood* 2005;106:1694-702.
45. Lee BS, Shim SM, Heo J, Pae HO, Seo BY, Han SY, et al. Wogonin suppresses TARC expression induced by mite antigen via heme oxygenase 1 in human keratinocytes. Suppressive effect of wogonin on mite antigen-induced TARC expression. *J Dermatol Sci* 2007;46:31-40.
46. Nakamichi I, Habtezion A, Zhong B, Contag CH, Butcher EC, Omary MB. Hemin-activated macrophages home to the pancreas and protect from acute pancreatitis via heme oxygenase-1 induction. *J Clin Invest* 2005;115:3007-14.
47. Rowe A, Farrell AM, Bunker CB. Constitutive endothelial and inducible nitric oxide synthase in inflammatory dermatoses. *Br J Dermatol* 1997;136:18-23.
48. Kubo M, Kambayashi Y, Takemoto K, Okuda J, Muto M, Ogino K. Reactive nitrogen species formation in eosinophils and imbalance in nitric oxide metabolism are involved in atopic dermatitis-like skin lesions in NC/Nga mice. *Free Radic Res* 2005;39:719-27.

METHODS

Real-time PCR

Total RNA was isolated from the spleen cells by using RNAiso (Takara, Shiga, Japan).^{E1-E4} After treatment with DNAase I (Invitrogen, Carlsbad, Calif), 1 µg of total RNA served as the template for single-stranded cDNA synthesis in a reaction with a Random Hexamer (Invitrogen) and a SuperScript II (Invitrogen). Panels of the primers and probes of mouse iNOS and glyceraldehyde-3-phosphate dehydrogenase for real-time PCR were purchased from Applied Biosystems (Foster City, Calif). Real-time PCR was performed with a TaqMan Universal Master mix (Applied Biosystems), and the data were analyzed with the ABI prism 7500 sequence detection system (Applied Biosystems). Briefly, 1/50 of cDNA derived from 1 µg of total RNA, 200 nmol/L probe, and 800 nmol/L primers was incubated in 25 µL at 50°C for 2 minutes and 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. The analysis system determined the number of cycles at which the amplified DNA in the sample exceeded the threshold during the PCR. The data were standardized to the expression of glyceraldehyde-3-phosphate dehydrogenase. The $\Delta\Delta C_T$ method was used to semiquantify mRNA levels, according to the manufacturer's protocol (Applied Biosystems).

Immunoblotting analysis

Expression of HO-1 protein was determined by immunoblotting, as previously reported.^{E3,E4} Briefly, cells were treated with the lysis buffer (137 mmol/L NaCl, 20 mmol/L Tris-HCl, 50 mmol/L NaF, 1 mmol/L EDTA, and 0.1% Triton-X) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) for 30 minutes on ice, and the supernatants were recovered by means of centrifugation at 15,000 rpm for 30 minutes. The samples were then electrophoretically separated on an 8% to 16% gradient of polyacrylamide gel (Daiichi Kagaku, Tokyo, Japan) and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, Mass). After blocking with 5% skim milk-Tris-buffered saline for an hour at room temperature, the membrane was incubated with anti-HO-1 ($\times 1000$) mAb or anti-actin goat antibody ($\times 2000$) for an hour at room temperature and subsequently for 45 minutes with HRP-conjugated secondary antibody. The signals were developed by using the enhanced chemiluminescence detection system (Amersham Life Sciences). The amount of blotted protein was densitometrically measured by

using Scion image analysis software and image processing software (NIH Image Engineering, Bethesda, Md).

Immunohistochemistry and fluorescent staining

For immunohistochemistry, tissue specimens were fixed with 20% formalin and embedded in paraffin. Four-micrometer-thick tissue sections were subjected to histopathologic analyses. For staining, the sections were dewaxed, rehydrated, and autoclaved in 10 mmol/L citrate buffer, pH 6.0 (121°C for 15 minutes), for antigen retrieval. Then intrinsic peroxidase activity was quenched with 0.3% hydrogen peroxide. Immunostaining was performed overnight at 4°C with optimally diluted anti-HO-1 mAb by using the Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingame, Calif). The labeled antigens were visualized with the Histofine-PO kit (Nichirei Pharmaceutical, Tokyo, Japan), followed by 3,3'-diaminobenzidine reaction. The sections were counterstained with hematoxylin.

For fluorescent staining, specimens were embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan). After blocking for an hour with Image-iT FX signal enhancer (Molecular Probes), the sections were incubated with the mixture of primary antibodies at 4°C overnight. Labeled antigens were visualized by means of incubation with Alexa Fluor 488- and 594-conjugated secondary antibodies. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Amersham Life Sciences).

REFERENCES

- E1. Kirino Y, Takeno M, Iwasaki M, Ueda A, Ohno S, Shirai A, et al. Increased serum HO-1 in hemophagocytic syndrome and adult-onset Still's disease: use in the differential diagnosis of hyperferritinemia. *Arthritis Res Ther* 2005;7:R616-24.
- E2. Takeda Y, Takeno M, Iwasaki M, Kobayashi H, Kirino Y, Ueda A, et al. Chemical induction of HO-1 suppresses lupus nephritis by reducing local iNOS expression and synthesis of anti-dsDNA antibody. *Clin Exp Immunol* 2004;138:237-44.
- E3. Kobayashi H, Takeno M, Saito T, Takeda Y, Kirino Y, Noyori K, et al. Regulatory role of heme oxygenase 1 in inflammation of rheumatoid arthritis. *Arthritis Rheum* 2006;54:1132-42.
- E4. Kirino Y, Takeno M, Murakami S, Kobayashi M, Kobayashi H, Miura K, et al. Tumor necrosis factor alpha acceleration of inflammatory responses by down-regulating heme oxygenase 1 in human peripheral monocytes. *Arthritis Rheum* 2007;56:464-75.

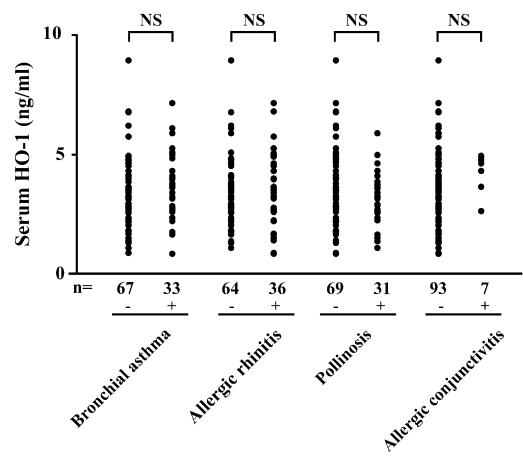


FIG E1. Serum levels of HO-1 in patients with AD (n = 100) with or without bronchial asthma, allergic rhinitis, pollinosis, and allergic conjunctivitis. NS, Not significant.

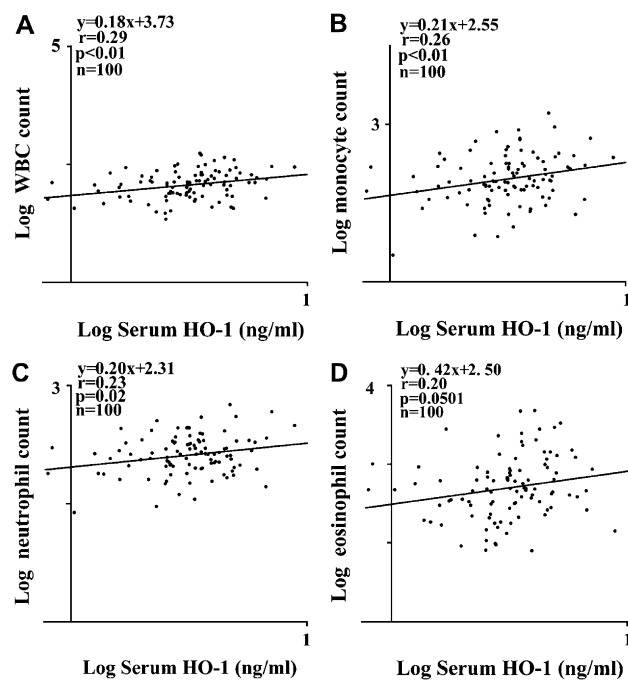


FIG E2. Comparison between serum HO-1 concentrations and laboratory parameters in patients with AD ($n = 100$) are shown in log-log form. Correlations with serum WBC (**A**), monocyte count (**B**), neutrophil count (**C**), and eosinophil count (**D**) were analyzed by means of regression analysis.

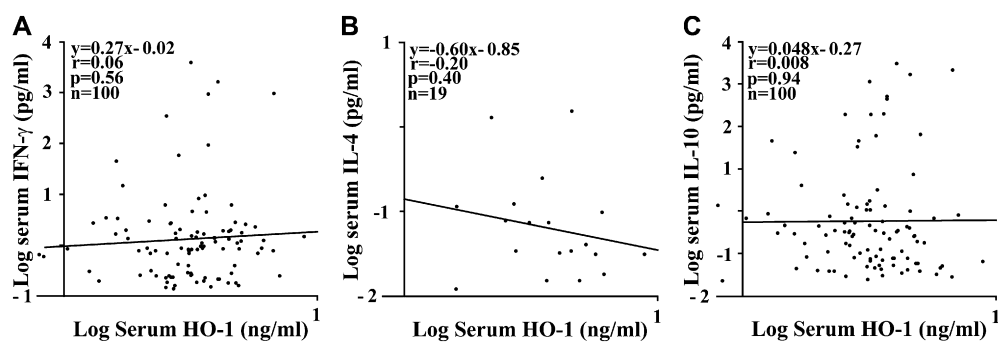


FIG E3. Comparison between serum HO-1 concentrations and cytokine levels in patients with AD are shown in log-log form. Correlations with serum IFN- γ (**A**), IL-4 (**B**), and IL-10 (**C**) were analyzed by means of regression analysis.

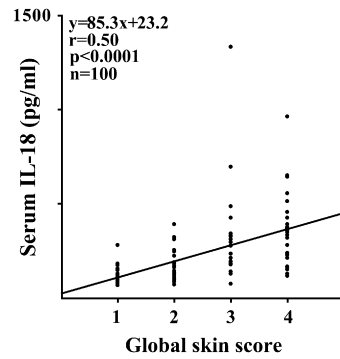


FIG E4. Comparison between serum levels of IL-18 and global skin scores in patients with AD divided into 4 groups based on their disease severities.

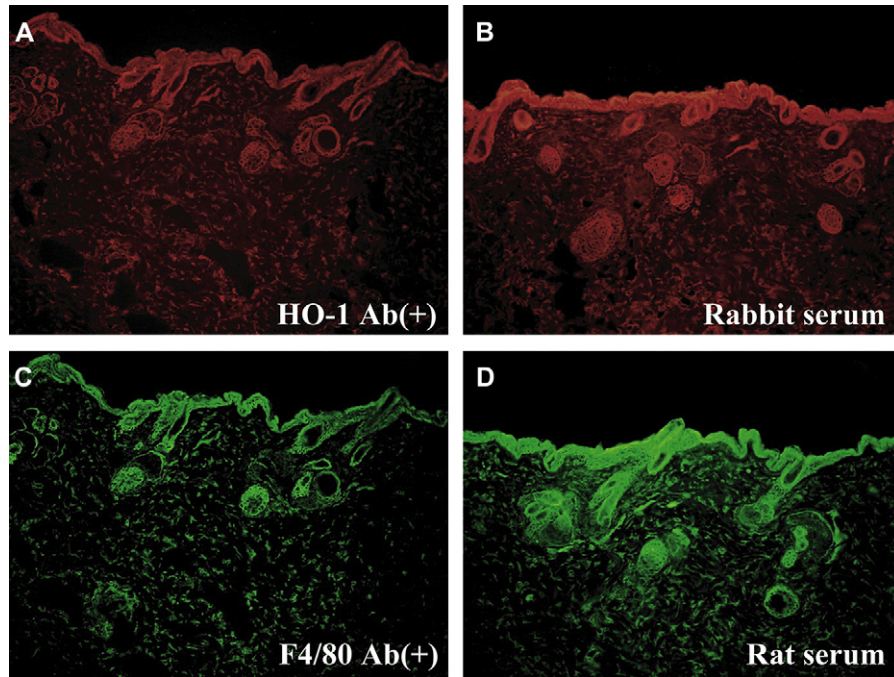


FIG E5. Immunofluorescent staining of intact skin from 6-week-old SPF DS-Nh mice. **A**, HO-1. **B**, Normal rabbit serum. **C**, F4/80. **D**, Normal rat serum ($\times 200$). *DAPI*, 4',6-Diamidino-2-phenylindole.

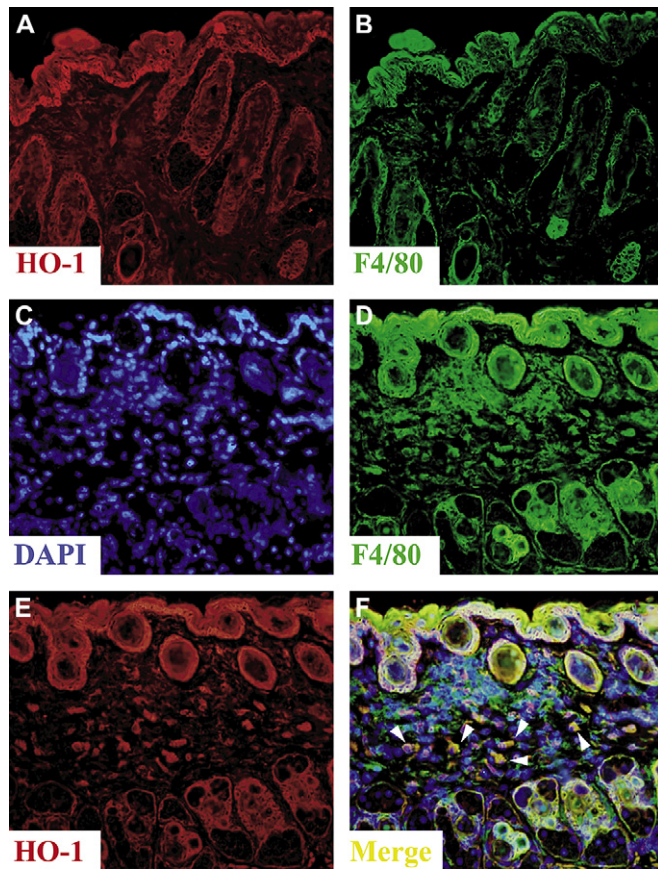


FIG E6. Immunofluorescent staining of skin from 7-week-old SPF DS-Nh mice treated with PBS (**A and B**) or hemin (**C-F**) for 24 hours. Fig E6, **A**, HO-1; Fig E6, **B**, F4/80; Fig E6, **C**, 4',6-diamidino-2-phenylindole; Fig E6, **D**, F4/80; Fig E6, **E**, HO-1; and Fig E6, **F**, merged image of HO-1, F4/80, and 4',6-diamidino-2-phenylindole ($\times 200$). *Arrowheads* indicate HO-1- and F4/80-positive macrophages.

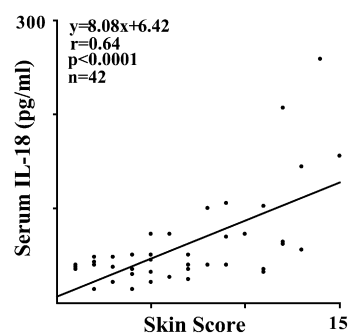


FIG E7. Comparison between serum IL-18 levels and skin scores in DS-Nh mice treated with PBS, hemin, or ZnPPiX (n = 14 for each group).