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*Original article*

# Protective role of endogenous secretory RAGE against contact hypersensitivity in mouse ear skin

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## Abstract

***Aims*:** The receptor for advanced glycation end-products (RAGE) is involved in the pathogenesis of diabetic complications, aging-related diseases, and inflammation. A splice variant of RAGE, called endogenous secretory RAGE (esRAGE), which is distinguishable from the membrane-bound full-length RAGE (mRAGE), was identified and found to have defensive properties as a decoy receptor against inflammatory reactions. However, the contribution of mRAGE and esRAGE to contact hypersensitivity is not well understood. In this study, we examined the role of RAGE using 2,4-dinitro-1-fluorobenzene (DNFB) to induce contact hypersensitivity in a mouse model.

***Methods*:** Ear swelling was induced in wild-type (*Ager*$^{+/+}$) and RAGE-null (*Ager*$^{-/-}$) mice using DNFB. The degrees of ear swelling, vascular permeability, and inflammatory cell accumulation were assessed and TNF-α, IL-6, histamine, and VEGF levels were assayed.

***Results*:** Expression of esRAGE was observed in the epidermis of mouse ear skin. Compared with *Ager*$^{+/+}$ mice, DNFB-induced ear swelling was significantly more severe in *Ager*$^{-/-}$ mice at 24 h. Vascular permeability, infiltrated mast cell number, and IL-6 expression were significantly higher in *Ager*$^{-/-}$ than *Ager*$^{+/+}$ mice. Supplementation of recombinant esRAGE protein could ameliorate the ear swelling observed in *Ager*$^{-/-}$ mice.

***Conclusions*:** RAGE-null conditions in the skin accelerated contact hypersensitivity, suggesting that esRAGE-deficiency may be implicated in this acute reaction. We conclude that esRAGE may play a protective role during contact hypersensitivity in the skin and that esRAGE application may be a new strategy for the treatment of allergic contact dermatitis.

***KEY WORDS*:** RAGE (receptor for advanced glycation end-products), endogenous secretory RAGE (esRAGE), ear skin, contact hypersensitivity

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## Introduction

Allergic contact dermatitis, also known as contact hypersensitivity, is one of the most common inflammatory skin diseases with high socio-economic impact. Acute or chronic inflammatory reactions can be induced by allergens or irritants such as nickel, lacquer tree, and hair dye, manifesting as obvious skin inflammation that is characterized by itching, erythema, edema, and crusting. These skin lesions are accompanied by spongiotic changes in the epithelium, dilated vessels, plasma extravasation, and perivascular immune cell infiltration [1]. In addition, inflammatory cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α) and vascular permeability factors such as histamine and vascular endothelial growth factor (VEGF) play a central role in the initiation and progression of skin inflammatory reactions [2,3].

The receptor for advanced glycation end-products (RAGE) is known to play critical roles in regulating innate immune responses and inflammatory reactions [4,5]. RAGE was first described due to its ability to bind advanced glycation end-products (AGEs), which are generated through a non-enzymatic reaction between reducing sugars and free amino groups of proteins, lipids, or nucleic acids [4,5]. Presently, RAGE is known as a multi-ligand receptor and a member of the pattern-recognition receptor (PRR) like the toll-like receptors. The RAGE ligands are composed of high-mobility group box1 (HMGB1), calcium-binging S100 protein group, β2-integrin Mac/CD11b, amyloid β-peptide, β-sheet fibrils, complement C3a, lipopolysaccharides (LPS), and phosphatidylserine on the surface of apoptotic cells [4,5]. Ligand-RAGE interaction causes cellular activation mediated by the activation of Erk1/2 (p44/p42) MAP kinases, p38 and SAPK/JNK MAP kinases, and NF-κB [4,5,6]. Thus, RAGE actively participates in the regulation of innate and adaptive immunity and inflammation via partly mediating inflammatory cell recruitment.

RAGE, which belongs to the immunoglobulin (Ig) superfamily, is composed of an extracellular region containing one V-type and two C-type Ig domains and a hydrophobic transmembrane-spanning domain, followed by a highly charged 43-amino acid short cytoplasmic tail that is essential for post-RAGE signaling [7]. The abovementioned signal transducing type of RAGE was designated as membrane-bound full-length RAGE (mRAGE). Soluble RAGE isoforms have also been previously reported, including alternative mRNA splicing and proteolytic cleavage isoforms of mRAGE [4,5,8]. The major alternative spliced variant of RAGE is an endogenous secretory RAGE (esRAGE) that lacks the transmembrane and cytoplasmic tail of mRAGE [9,10]. The esRAGE is known to be widely expressed in the cell surface and cytoplasm of neuron, endothelial cells, pneumocytes, mesothelium, pancreatic β-cells, monocytes, macrophages, salivary glands, digested tracts, renal tubules, prostate, skin, thyroid, and bronchioles of human and mouse models [11]. The esRAGE can function as a decoy blocking the activation of RAGE signaling. However, the effects of mRAGE and esRAGE on the development of allergic contact dermatitis in the skin are not well understood. To address this, we investigated the skin lesions and swelling, cytokine levels, and histopathological changes in RAGE-null (lacking both mRAGE and esRAGE; *Ager*$^{-/-}$) and wild-type control (*Ager*$^{+/+}$) mice during 2,4-dinitrofluorobenzene (DNFB)-induced contact dermatitis.

## Materials and Methods

### Animals

Female RAGE-null (*Ager*$^{-/-}$) mice [12,13] between 8 and 12 weeks of age that were backcrossed with C57BL/6J strain (>F7) and wild-type (*Ager*$^{+/+}$) were used. All animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University and performed in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

### Induction of contact hypersensitivity in mice

Experiments were carried out as described previously [14]. The shaved back skin of female mice (*Ager*$^{-/-}$ or *Ager*$^{+/+}$ mice) was sensitized with 100 μL 0.5% 2,4-dinitro-1-fluorobenzene (DNFB; Sigma-Aldrich, St. Louis, MO, USA) diluted with an olive oil: acetone (1:4 v/v) solution. After five days, 20 μL 0.5% DNFB and olive oil: acetone mixture was also applied to the front and dorsal surface of the right ear, whereas the left ear was treated with vehicle only [14]. The ear swelling was measured for 7 days. To determine the effect of esRAGE, 100 μg esRAGE was injected intravenously prior to challenge. The recombinant human esRAGE protein was purified from conditioned media of human esRAGE-encoding cDNA-transfected COS-7 cells as previously described [9].

### Miles vascular permeability assay

Miles assay was performed as described previously [15]. One hundred μL of 1% Evans blue solution in 0.9% NaCl was injected into the tail vein of *Ager*$^{-/-}$ and *Ager*$^{+/+}$ mice with or without challenging 0.5% DNFB mixture. After 30 min, the vasculature was perfusion-fixed for 1 min using 1% paraformaldehyde in 50 mM citrate buffer (pH 3.5). The animals were anesthetized and mouse ears were removed and dried for 6 h at 55°C. Evans blue was extracted by incubating the tissues in 1 mL formamide (Sigma-Aldrich, St. Louis, MO, USA) for 5 days. The Evans blue content was measured using a spectrophotometer at 610 nm.

### Immunohistochemistry and hematoxylin-eosin staining

Basic immunostaining was performed using a microwave technique [11]. To evaluate mouse esRAGE expression levels, frozen sections were fixed with 95% (v/v) alcohol for 1 min and treated with 3% (v/v) $H_2O_2$ for 5 min to exhaust endogenous peroxidases. After washing with Tris-buffered saline (pH 7.4) containing 0.1% (v/v) Tween-20 (TBS-T) and blocking with 5% (w/v) BSA (Sigma-Aldrich, St. Louis, MO, USA) for 1 min, the sections were incubated with a mouse esRAGE-specific polyclonal antibody (1:200 dilution) overnight in a wet chamber. The mouse esRAGE-specific antibody against the unique C-terminal amino-acid peptide (PAEGEGLD) was raised in rabbits and affinity-purified with an antigen peptide-conjugated column [10]. After washing again with TBS-T, the sections were incubated with an anti-rabbit IgG peroxidase-conjugated polymer (EnVision™, Dako, Carpinteria, CA, USA) for 10 min in a wet chamber

and subjected to intermittent microwave irradiation. After another round of washing with TBS-T, immune complexes were visualized using 3-3′-diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA). The sections were counterstained with Meyer's hematoxylin and cover-slipped for microscopic observation. Brown-colored areas were judged as positive based on the manufacturer's information (EnVision™, Dako, Carpinteria, CA, USA).

For the detection of CD3 lymphocytes and mast cells, the mice were anesthetized and transcardially perfused with 4% paraformaldehyde in PBS (pH 7.4). The mouse ears were removed and immersion-fixed for 24 hr. Rabbit polyclonal antibodies against murine CD3 (Abcam, Cambridge, UK) and mast cell tryptase (Abcam, Cambridge, UK) were used as the primary antibodies. The sections were deparaffinized, dehydrated, and treated for antigen retrieval using TRS-buffer solution (TRS) (Dako, Carpinteria, CA, USA) in a wet chamber inside a microwave oven (maximum 500 W; Type RE-11, Sharp, Tokyo, Japan) for 15 min. The sections were allowed to cool at room temperature for 30 min, rinsed under running water for 1 min, and sequentially treated with 3% (v/v) $H_2O_2$ for 5 min. After washing with TBS-T and blocking with 5% normal bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) for 1 min, the sections were incubated with a primary antibody in a wet chamber, with intermittent microwave irradiation (MI-77, Azumaya, Japan) for 10 min. After washing with TBS-T, the peroxidase-labeled polymer for rabbit polyclonal antibody (EnVision™, Dako, Carpinteria, CA, USA) and goat polyclonal antibody (Histofine, Simple Stain, Nichirei Bioscience, Tokyo, Japan) were applied for 10 min in a wet chamber with intermittent microwave irradiation. After washing with TBS-T, the color was developed with 3-3′-diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA). The sections were counterstained with Meyer's hematoxylin and cover-slipped for microscopic observation.

### *Enzyme-linked immunosorbent assays (ELISA)*

Mouse IL-6, TNF-α, and VEGF immunoreactivity levels in ear tissues were quantified using ELISA kits (R&D Systems, McKinley Place, MN, USA) following the manufacturer's instructions. Mouse histamine levels were also measured using an ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA). Whole ear tissues from *Ager*$^{+/+}$ or *Ager*$^{-/-}$ mice were homogenized in 500 µL lysis buffer ($Ca^{2+}$- and $Mg^{2+}$-free PBS containing 1% (v/v) Nonidet P-40, 0.5% (w/v) deoxycholate, 10 mM EDTA, 0.1% (w/v) SDS, and 1 mM PMSF) using a Polytron tissue homogenizer as previously described [10]. The homogenates were centrifuged at 20,000 × g for 30 min and 100 µL supernatants were used for assays.

### *Statistical analysis*

Results are presented as means ± SEM (standard error mean). Comparisons between groups were assessed using Student's *t*-test or ANOVA combined with Scheffe's multiple comparison test ($p < 0.05$).

## *Results*

We previously reported that esRAGE was observed with a supranuclear dot-like granular staining pattern in basal cells of the epidermal squamous epithelia and in the ductal epithelia of the accessory gland in human skin specimens [11,16]. Using *Ager*$^{+/+}$ mouse ear skin, esRAGE expression was clearly observed in basal cells of the epidermis (***Fig. 1***); this is similarly observed in human skin. In addition, some esRAGE-positive cells were seen in the dermis of the skin. By contrast, the positive signals were not observed in *Ager*$^{-/-}$ mouse skin.

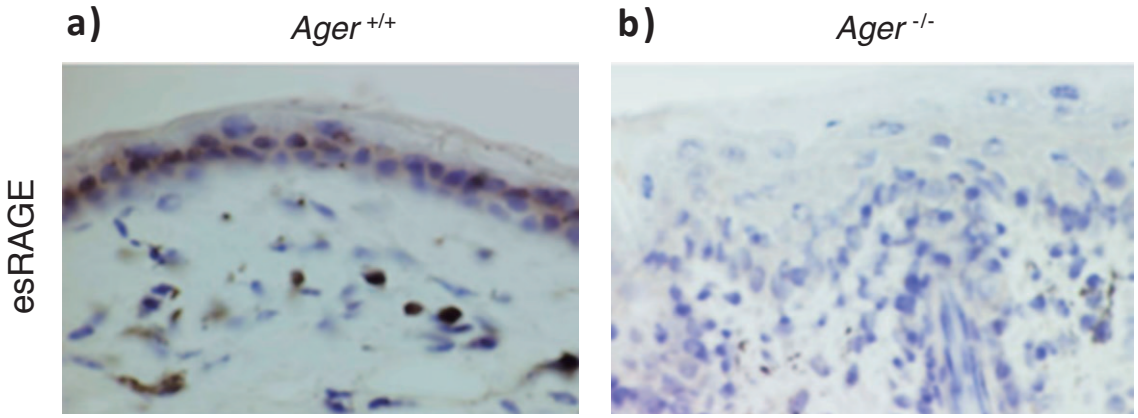


***Fig. 1.*** **Immunostaining for esRAGE protein.**

In *Ager*$^{+/+}$ **(a)** and *Ager*$^{-/-}$ **(b)** mouse ear skins, esRAGE expression was observed in a dot-like supranuclear pattern in basal cells of the epidermal squamous epithelia. esRAGE, endogenous secretory RAGE; RAGE, receptor for advanced glycation end-products.

We also examined the roles of mRAGE and esRAGE during contact hypersensitivity using 2,4-dinitro-1-fluorobenzene (DNFB) in *Ager*$^{+/+}$ and *Ager*$^{-/-}$ mice. Results show that ear swelling occurred with ~1.5-fold ear weight gain (right ear, DNFB-treated vs. left ear, negative control) in *Ager*$^{+/+}$ mice 24 h after DNFB challenge (***Fig. 2***). Moreover, the ear swelling in the acute phase after 24 h of contact dermatitis was unexpectedly more severe in *Ager*$^{-/-}$ than *Ager*$^{+/+}$ mice (***Fig. 2***). Allergic contact dermatitis with DNFB during the chronic phase (48 h to 7 days) was not significantly different between *Ager*$^{-/-}$ and *Ager*$^{+/+}$ mice. To prove the acute reaction in the skin of *Ager*$^{-/-}$ mice, we checked the vascular permeability and assessed the plasma extravasation using Evans blue in Miles assay. Macroscopic findings showed blue-colored ear skin in the DNFB-challenged right side at 24 h as compared to the control left side (***Fig. 3***). Additionally, the clear blue staining with the plasma extravasation was more distinct in *Ager*$^{-/-}$ than *Ager*$^{+/+}$ mice. Quantitative evaluation of the blue dye revealed significant vascular leakage in *Ager*$^{-/-}$ mice (***Fig. 3***). We further evaluated the histological changes in the mouse ear 24 h after challenging with DNFB. The exposed ear lesion on the right side was more edematous in *Ager*$^{-/-}$ mice as observed in H&E staining (***Fig. 4***). Immunohistological examinations after 24 h revealed that mast cell infiltration was more evident in the DNFB-exposed ear lesions of *Ager*$^{-/-}$ mice, but displayed no difference in the lymphocyte (CD3-positive cells) number between *Ager*$^{-/-}$ and *Ager*$^{+/+}$ mice. Quantitative analysis showed that the mast cell number was significantly ~2-fold higher even in the non-challenged control side of *Ager*$^{-/-}$ mice when compared with *Ager*$^{+/+}$ mice. DNFB-challenge induced greater mast

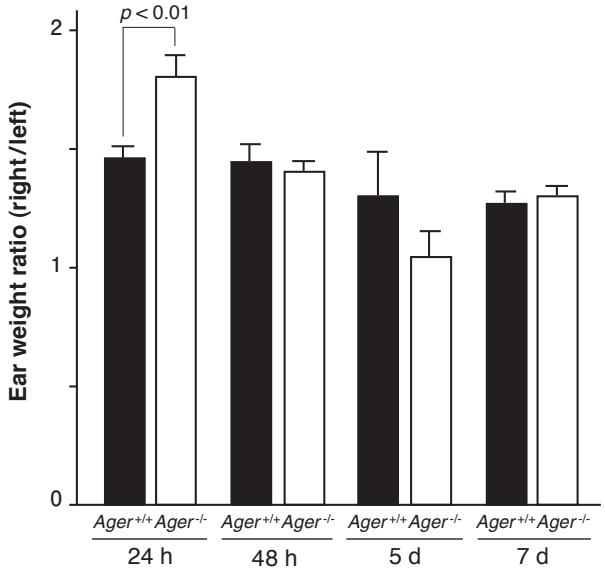


***Fig. 2.*** **Contact hypersensitivity and ear swelling.**

Female *Ager*$^{+/+}$ and *Ager*$^{-/-}$ mice (8 weeks old) with shaved back skin were sensitized with 100 µL of 0.5% DFNB (Sanger's reagent) diluted with a 4:1 acetone and olive oil solution. Five days after the induction, 20 µL of the same DNFB mixture was applied to the right ear of the sensitized mice. As a control, 20 µL of DNFB-free acetone and olive oil solution was applied to the left ear. After the challenge, the ear swelling was measured and evaluated 24 h, 48 h, 5 days, and 7 days after the DNFB-exposure. n = 4-13. Data represent mean ± SEM. DFNB, 2,4-dinitrofluorobenzene; SEM, standard error mean.

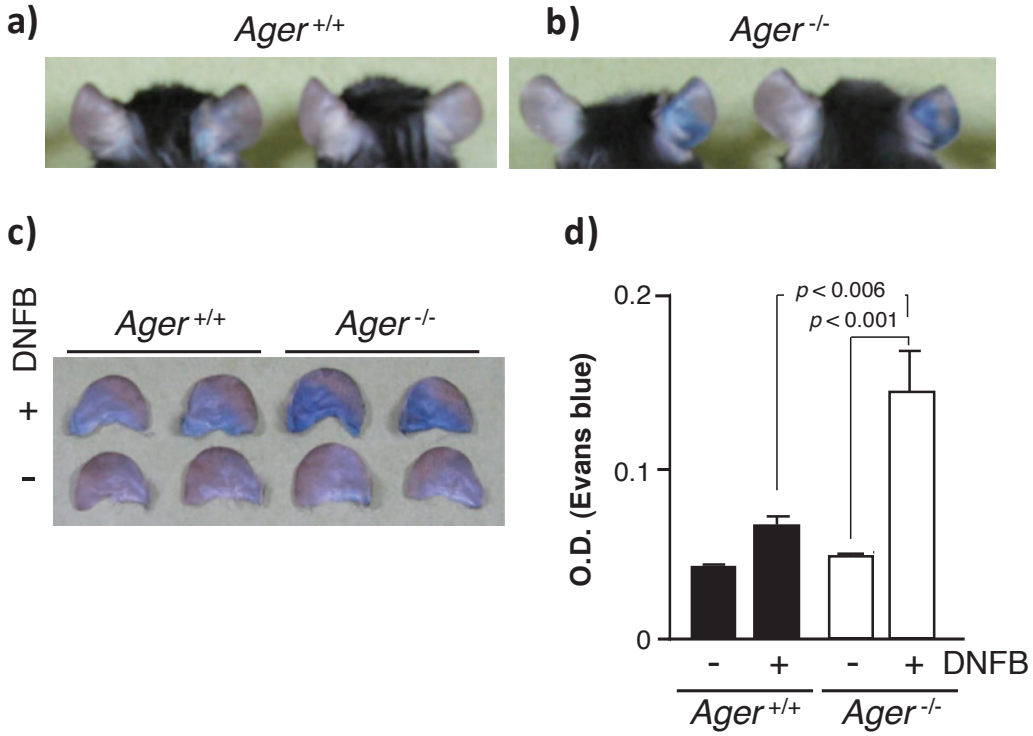


***Fig. 3.*** **Miles vascular permeability assay.**

**a, b)** Evans blue dye (100 µL of a 1% solution in 0.9% NaCl) was injected into the tail vein of *Ager*$^{+/+}$ **(a)** and *Ager*$^{-/-}$ **(b)** mice. **c)** After 30 min, the vasculature was perfusion-fixed (1% paraformaldehyde in 50 mM citrate buffer, pH 3.5) for 1 min. Vascular permeability was then evaluated by blue dye leakage at 4 h after DNFB-challenge. **d)** Evans blue was extracted from the tissues and measured using a spectrophotometer at 610 nm. n = 3-6. Data represent mean ± SEM. DFNB, 2,4-dinitrofluorobenzene; SEM, standard error mean.

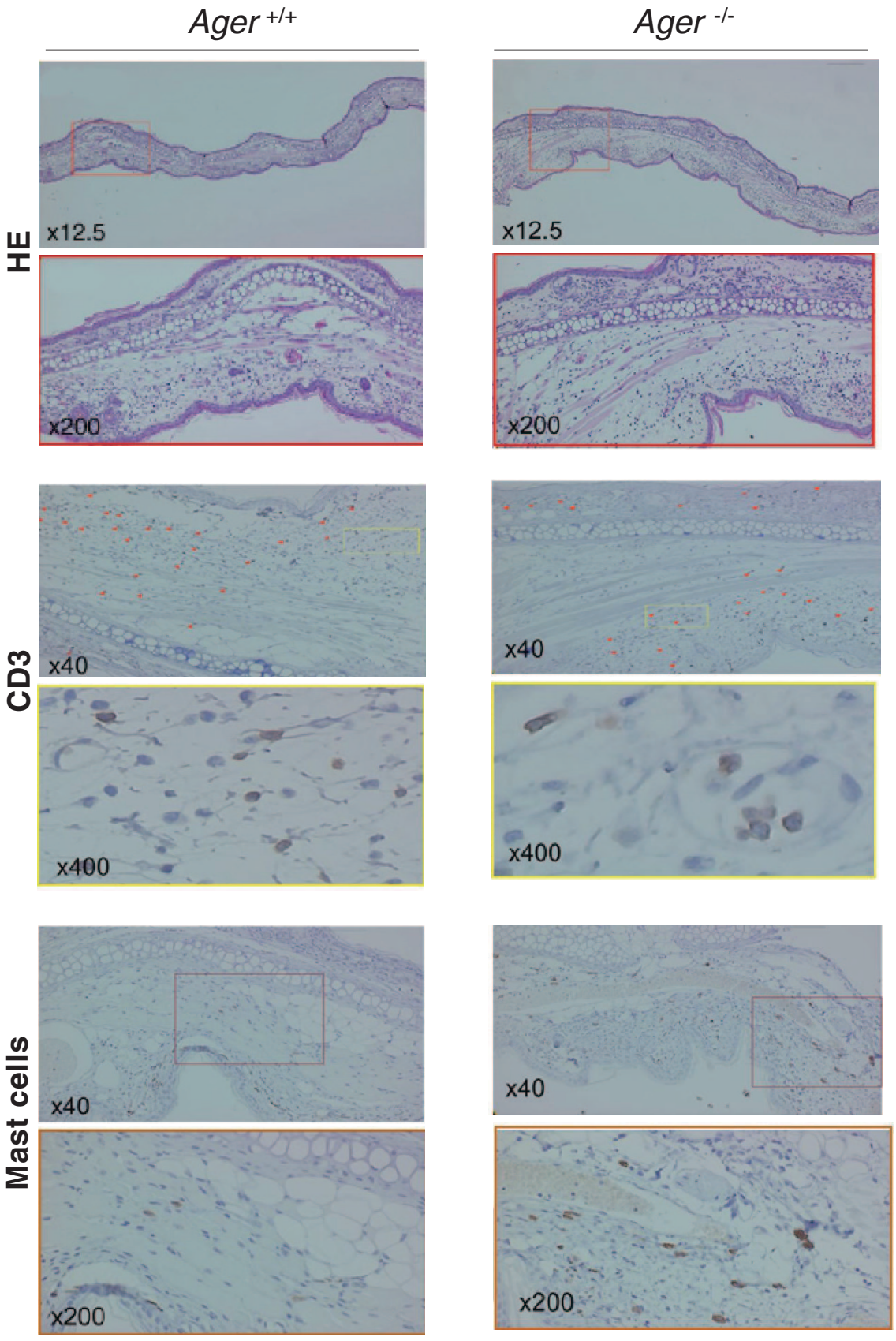


***Fig. 4.*** **Histology and immunohistochemistry.**

H&E staining of the right ear from *Ager*$^{+/+}$ and *Ager*$^{-/-}$ mice 24 h after DNFB-challenge. Immunohistochemical examination was performed for the detection of CD3-positive lymphocytes and mast cells. Each magnification is noted on figures. DFNB, 2,4-dinitrofluorobenzene.

cell infiltration in *Ager*$^{-/-}$ mouse ears (***Fig. 5***), indicating the involvement of this cell type in acute contact hypersensitivity development in RAGE-null conditions. In addition, we checked the levels of major cytokines and tissue permeability factors in the ear 24 h after the challenge using ELISA. Histamine levels were not altered among the four groups of *Ager*$^{-/-}$ and *Ager*$^{+/+}$ mice with or without DNFB-exposure (***Fig. 6***). TNF-α and VEGF levels were significantly higher in DNFB-challenge ears than the control, but no significant increase was observed in *Ager*$^{-/-}$ mouse ears. IL-6 significantly increased in DNFB-challenge ears of *Ager*$^{-/-}$ mice compared with *Ager*$^{+/+}$ mice.

Based on these results, we hypothesized that esRAGE in ear skin could defend against acute contact dermatitis induced by DNFB. To elucidate the function of esRAGE in the mouse model, 100 μg recombinant esRAGE protein was pre-treated prior to DNFB-exposure. After 24 hr, as expected, ear swelling in *Ager*$^{-/-}$ mice was significantly and completely ameliorated by the esRAGE injection (***Fig. 7***). However, esRAGE-supplementation did not affect the ear swelling in *Ager*$^{+/+}$ mice, suggesting that RAGE- or esRAGE-independent mechanisms are also involved in contact hypersensitivity.

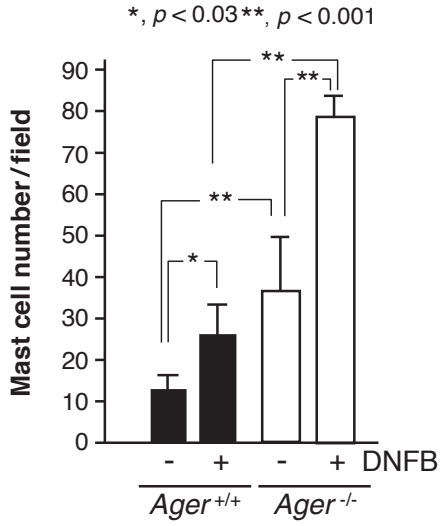


***Fig. 5.*** **Mast cell infiltration.**

The mast cell numbers between *Ager*$^{+/+}$ and *Ager*$^{-/-}$ mice calculated from the immunohistochemical examination in Fig.4. Data represent mean ± SEM. DFNB, 2,4-dinitrofluorobenzene; SEM, standard error mean.

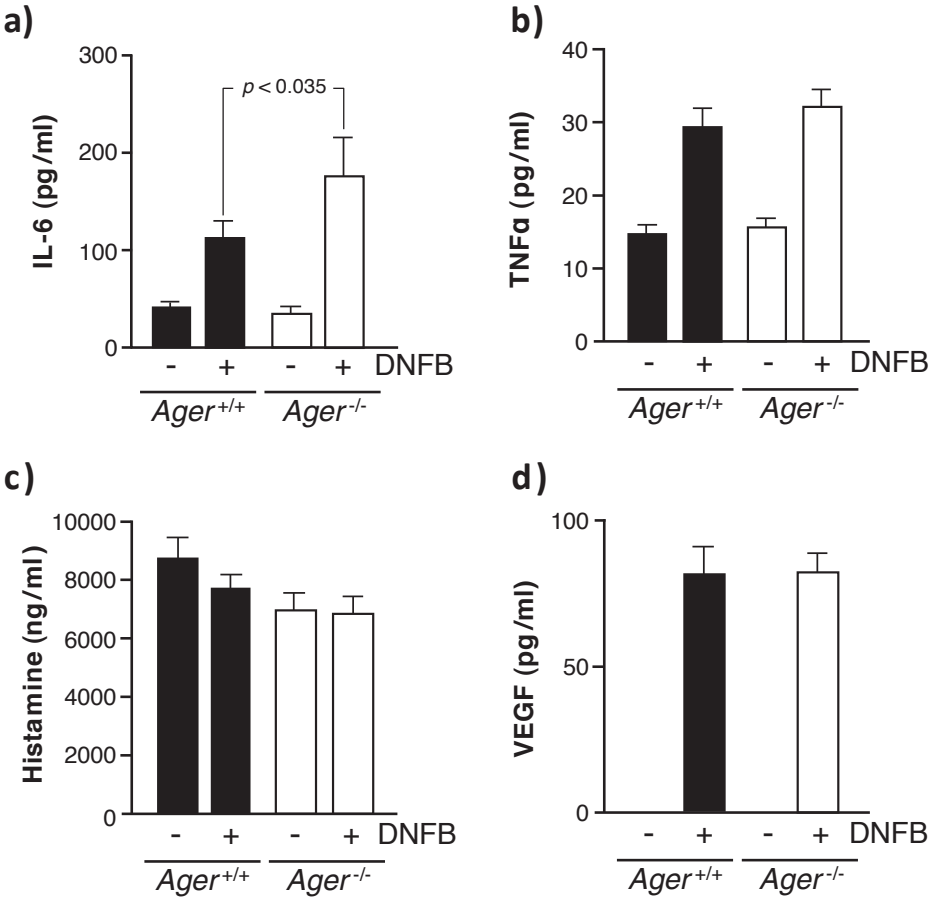


***Fig. 6.*** **Cytokines and vascular permeability factor.**

IL-6 **(a)**, TNF-α **(b)**, histamine **(c)**, and VEGF **(d)** levels were quantified by commercially available ELISA systems. n = 4-6. Data represent mean ± SEM. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; DFNB, 2,4-dinitrofluorobenzene; SEM, standard error mean; ELISA, enzyme-linked immunosorbent assay.

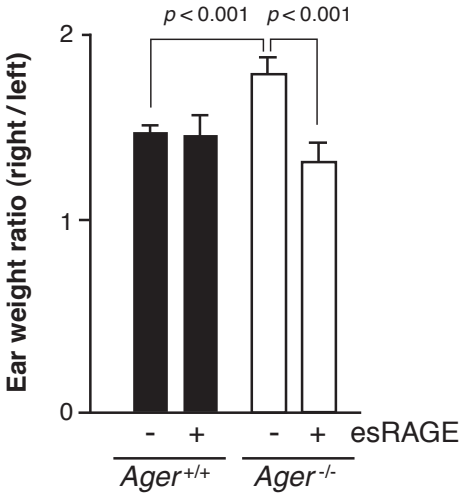


***Fig. 7.*** **Effect of recombinant esRAGE treatment.**

esRAGE treatment (100 μg per mouse) significantly prevented ear swelling in *Ager*$^{-/-}$ mice 24 h after DNFB-challenge. n = 3-10. Data represent mean ± SEM. esRAGE, endogenous secretory RAGE; DFNB, 2,4-dinitrofluorobenzene; SEM, standard error mean.

## *Discussion*

In the present study, we found that genetic RAGE-deletion, lacking both mRAGE and esRAGE, enhanced the DNFB-induced ear swelling, vascular leakage, mast cell infiltration, and IL-6 levels in a mouse model of contact hypersensitivity (***Fig. 2-7***). Chronic changes due to contact dermatitis after 48 h were not significantly different between *Ager*$^{-/-}$ and *Ager*$^{+/+}$ mice (***Fig. 2***). However, the acute phase changes observed 24 h after DNFB challenge were distinct and significant (***Fig. 2-6***). Additionally, the mast cell number was higher in the ear skin of *Ager*$^{-/-}$ than *Ager*$^{+/+}$ mice under basal conditions without DNFB exposure, indicating that RAGE-deletion could enhance the accumulation of residential mast cells. We speculate that the mast cells present could immediately respond to the chemical allergens in *Ager*$^{-/-}$ mice. DNFB stimulation coupled with RAGE-deletion enhanced mast cell infiltration and significantly increased the number of skin lesions (***Fig. 4, 5***). Skin esRAGE might also regulate the number of residential mast cells. Supplementation of esRAGE protein significantly attenuated ear swelling in *Ager*$^{-/-}$ mice 24 h after DNFB stimulation (***Fig. 7***), suggesting the functional role of esRAGE during acute contact hypersensitivity. Ear swelling was evident even in DNFB-exposed right side of *Ager*$^{+/+}$ mice compared with the non-exposed control side (left ear) (***Fig. 2, 7***). However, the systemic treatment of 100 μg esRAGE did not show any beneficial effects on the ear swelling in *Ager*$^{+/+}$ mice (***Fig. 7***). We believed that the endogenous expression level of esRAGE in *Ager*$^{+/+}$ mouse skin would be sufficient to control acute contact hypersensitivity. Otherwise, the skin residential mast cells in the same number were ready to be exposed to the allergen before systemic esRAGE application in *Ager*$^{+/+}$ mice. Therefore, the acute contact hypersensitivity reaction was not alleviated in the esRAGE treatment group. For the purpose of future human clinical treatment with esRAGE supplementation, we need further studies to elucidate the underlying pathological mechanism. Future studies focusing on the topical administration of esRAGE protein as a possible treatment for contact hypersensitivity are being considered.

Our group identified and reported that RAGE has isoforms – signal transducing mRAGE, alternatively spliced and soluble variant of esRAGE, and proteolytically cleaved soluble form from mRAGE in human and mouse [8-10]. It is known that the excess of esRAGE protein has physiological significance, since esRAGE could act as a decoy receptor and protect cells and tissues from ligand-dependent injury. Diabetic patients with higher serum esRAGE levels were reported to be resistant to the development of early retinal complications [17]. Plasma esRAGE may be a potential protective factor for metabolic syndrome and atherosclerosis [18]. Furthermore, lower circulating esRAGE is reported to be associated with reduced bone formation in patients with hip bone fracture and a higher incidence of schizophrenia [19, 20].

In human skin, we demonstrated the expression of esRAGE in basal cells of the epidermal squamous epithelia and the ductal epithelia of the accessory gland [11]. In this study, we observed a similar expression pattern of esRAGE in mouse ear skins (***Fig. 1***). In addition, mRAGE expression was detected in non-pathological and lesion-free normal human epidermis [16]. Using gene-manipulated animal models, it is revealed that the inflammatory hypertrophy of the epidermis was diminished in mouse ears with keratinocyte-specific deletion of RAGE, consequently lacking mRAGE and esRAGE [21]. Moreover, conventional RAGE-null mice were also protected from infiltration of leukocytes, such as monocytes/macrophages and neutrophils, and skin inflammation [22]. In the two mouse experiments above, skin inflammation was induced by the administration of 12-O-tetradecanoylphorbol 13-acetate (TPA), which was a stronger stimulus than the DNFB used in this study, as proven by the limited accumulation of monocytes/macrophages and neutrophils observed in the DNFB-exposed ear skin. Moreover, there was no difference in acute epidermal thickness 24 h after TPA stimulation between *Ager*$^{-/-}$ and *Ager*$^{+/+}$ mice, but the significant difference was obtained after 48 h [21]. Another ear inflammation model using UV irradiation (1,000 J/m$^2$), which was also a severe leukocyte infiltration model, demonstrated that ear swelling was significantly reduced after 3 days in conventional *Ager*$^{-/-}$ mice when compared with *Ager*$^{+/+}$ mice [22]. In addition, a topical administration of 120 ng soluble RAGE (sRAGE) successfully ameliorated UV-irradiated ear swelling and leukocyte accumulation in both *Ager*$^{-/-}$ and *Ager*$^{+/+}$ mice [22]. These findings are completely different from our results using the DNFB-induced ear swelling model with mild inflammatory changes.

In conclusion, our study is the first to report the observations regarding acute ear swelling due to DNFB-induced contact hypersensitivity under whole RAGE-null conditions. Skin esRAGE-deficiency could mediate this reaction. Endogenous esRAGE plays a protective role during contact hypersensitivity in the skin and excessive esRAGE expression may be a new therapeutic strategy against acute contact dermatitis.

## Conflict of interest statement

The authors declare no conflict of interest in this study.

## Acknowledgments

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