Allergology International. 2010;59:277-284
DOI: 10.2332/allergolint.09-OA-0149

ORIGINAL ARTICLE

Amphiregulin is Not Essential for Induction of Contact Hypersensitivity

Akiko Yagami[1], Naoki Kajiwara[1,2], Keisuke Oboki[1], Tatsukuni Ohno[1], Hideaki Morita[1,3], Susan W Sunnarborg[4], Ko Okumura[2], Hideoki Ogawa[2], Hirohisa Saito[1,2] and Susumu Nakae[1,2,5]

ABSTRACT

Background: Amphiregulin (AR) is expressed in Th2 cells, rather than Th1 cells, and plays an important role in Th2 cell/cytokine-mediated host defense against nematodes. We also found earlier that AR mRNA expression was strongly upregulated in inflamed tissue during Th2 cell/cytokine-mediated fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS), suggesting a contribution of AR to the induction of those responses.

Methods: To elucidate the role of AR in the induction of FITC- or dinitrofluorobenzene (DNFB)-induced CHS, AR-deficient mice were sensitized and/or challenged with FITC or DNFB epicutaneously. The levels of FITC-mediated skin dendritic cell (DC) migration and FITC-specific lymph node cell proliferation and cytokine production were assessed by flow cytometry, [3H]-thymidine incorporation and ELISA, respectively, after FITC sensitization. The degree of ear swelling, the activities of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) in inflammatory sites and the levels of FITC-specific immunoglobulin (Ig) in sera were determined by histological analysis, colorimetric assay and ELISA, respectively, after FITC challenge.

Results: DC migration and FITC-specific lymph node cell proliferation and cytokine production were normal in the AR-deficient mice. Ear swelling, tissue MPO and EPO activities and FITC-specific serum Ig levels were also similar in AR-deficient and -sufficient mice.

Conclusions: Amphiregulin is not essential for the induction of FITC- or DNFB-induced CHS responses in mice.

KEY WORDS

amphiregulin, contact dermatitis, EGF, mast cells, Th2 cell/cytokine

INTRODUCTION

Contact hypersensitivity (CHS) responses, which are induced by epicutaneous exposure to chemicals, are classically considered to be an IFN-γ-producing Th1 and Tc1 cell-mediated allergic disorder.[1] This is supported by the facts that increased levels of IFN-γ and accumulation of IFN-γ^{+} Th1 cells were observed at local inflammatory sites in CHS.[2,3] However, results from studies using IFN-γ- and/or IFN-γR1-deficient mice did not always support the concept, since they showed normal development of CHS in response to oxazolone[4] and dinitrofluorobenzene (DNFB).[5,6] CHS induced by trinitrochlorobenzene (TNCB) in acetone and olive oil developed normally in IFN-γ- and IFN-γR 1-deficient mice,[4,7] while induction by TNCB in ethanol and acetone was attenuated in IFN-γ-deficient mice.[8] Although fluorescein isothiocyanate (FITC)-induced CHS was attenuated in IFN-γR2-deficient mice on a 129 × B6 mixed background,[9] a normal response was observed in IFN-γ-deficient mice on a C57 BL/6 background.[8,10] These observations suggest that IFN-γ may not to be crucial for induction of CHS. In contrast, it was shown that DNFB- and/or TNCB-

[1]Department of Allergy and Immunology, National Research Institute for Child Health and Development, [2]Atopy Research Center, Juntendo University, [3]Department of Pediatrics, Keio University, School of Medicine, [5]Frontier Research Initiative, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan and [4]Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina, NC, USA.
Authors' contributions: AY and NK contributed equally to this paper.
Correspondence: Susumu Nakae, PhD, Frontier Research Initiative, Institute of Medical Science, University of Tokyo, 4–6–1 Shirokanedai, Minato, Tokyo 108–8639, Japan.
Email: snakae@ims.u–tokyo.ac.jp
Received 28 September 2009. Accepted for publication 28 February 2010.

induced CHS responses were significantly decreased in IL-4-deficient mice, while oxazolone-induced CHS developed normally in those mutant mice.[11-14] Although IL-13-deficient mice showed normal development of DNFB-induced CHS,[15] STAT-6-deficient mice, which lack both IL-4- and IL-13-mediated immune responses, exhibited decreased inflammation during DNFB-, TNCB-, oxazolone- and FITC-induced CHS responses.[16] Therefore, these observations indicate that Th2 cytokines/Th2 cells, rather than Th1 cytokines/Th1 and Tc1 cells, are important in the pathogenesis of CHS.

Recently, it was shown that amphiregulin (AR), which is a member of the EGF family of growth factors/cytokines and involved in mammary gland development and the progression of breast cancer,[17,18] is expressed in Th2 cells but not Th1 cells[19] and plays an important role in Th2 cell-mediated host defense against nematodes.[19] In addition, in our pilot study using DNA microarrays, we found that expression of AR mRNA was increased in local inflammatory sites during Th2 cell/cytokine-mediated FITC-induced CHS. These observations suggest that AR produced by Th2 cells and/or other immune cells may contribute to the induction of CHS responses. However, the precise role of AR in the pathogenesis of CHS is unknown. In the present study, we used AR-deficient mice and demonstrated that AR is not necessary for FITC- or DNFB-induced CHS.

METHODS

MICE

Amphiregulin-deficient ($AR^{-/-}$) mice on a 129 × B6 mixed background were originally generated as described elsewhere.[20] Littermates ($AR^{+/+}$, $AR^{+/-}$ and $AR^{-/-}$ mice) were used in all experiments. Mice were housed under specific-pathogen-free conditions in the National Research Institute for Child Health and Development, and the animal protocols were approved by the Institutional Review Board of the National Research Institute for Child Health and Development.

SKIN DC MIGRATION

Skin DC migration was determined as described elsewhere.[21] In brief, mice were epicutaneously treated with 40 µl of 0.5% (w/v) FITC isomer I solution in a mixture of acetone and dibutylphthalate (1 : 1) (20 µl to the surface of each left ear) and the vehicle alone (20 µl to the surface of each right ear). Twenty-four hours later, submaxillary lymph nodes (LNs) were separately collected from both the FITC-treated left and vehicle-treated right ears. LN single-cell suspensions were prepared and incubated with anti-CD16/CD32 mAb (2.4 G2; BD Biosciences, San Jose, CA, USA) on ice for 15 minutes for FcR blocking. Then the cells were incubated with PE-anti-mouse CD11c mAb (N418; eBioscience) and APC-anti-mouse I-A/I-E mAb (M5/114.15.2; eBioscience, San Diego, CA, USA). The proportion of $FITC^+$ cells among 7-aminoactinomycin D-negative, MHC class II^{hi}, $CD11c^+$ cells was determined using a FACS Calibur (BD Biosciences).

FITC-SPECIFIC LN CELL RESPONSES

FITC-specific LN cell proliferative responses were measured as described elsewhere.[21] Briefly, mice were sensitized with 2.0% FITC on both the left and right ears (20 µl on one surface of each ear). Six days later, submaxillary LNs were collected, and single-cell suspensions were prepared. The LN cells were cultured in the presence or absence of 40 µg/ml FITC at 37℃ for 72 hours. FITC-specific LN cell proliferative responses were determined by pulsing with 0.25 µCi [^{3}H]-labeled thymidine for 6 hours.

MEASUREMENT OF CYTOKINES

Cytokine levels in the culture supernatants of FITC-specific LN cells were determined with mouse IFN-γ, IL-4, IL-10 and IL-17 ELISA kits obtained from BD Biosciences or eBioscience.

INDUCTION OF CHS

FITC- or DNFB-induced CHS was induced as described elsewhere.[21-23] Briefly, 2 days after shaving the back with clippers, mice were treated with 200 µl of a 0.5 or 2.0% (w/v) FITC isomer I (SIGMA) (0.5% FITC solution and 2.0% FITC suspension, respectively) in a mixture of acetone and dibutylphthalate (1 : 1) or 25 µl of a 0.5% (v/v) DNFB (Wako, Osaka, Japan) in a mixture of acetone and olive oil (4 : 1). Five days later, the animals were challenged with 40 µl of a 0.5% (w/v) FITC isomer I solution in a mixture of acetone and dibutylphthalate (1 : 1) (20 µl to the surface of each left ear) and 40 µl of the vehicle alone (20 µl to the surface of each right ear) or with 20 µl of a 0.2% (v/v) DNFB solution in a mixture of acetone and olive oil (4 : 1) (the left ear) and 20 µl of the vehicle alone (the right ear). Ear thickness was measured before and after FITC challenge using an engineer's caliper (Ozaki MFG. Co. Ltd., Tokyo, Japan).

HISTOLOGY

Twenty-four hours after FITC challenge, ear tissues were harvested, fixed in Carnoy's fluid and embedded in paraffin. Then sections were prepared and stained with hematoxylin-eosin.

MEASUREMENT OF MYELOPEROXIDASE (MPO) AND EOSINOPHIL PEROXIDASE (EPO) ACTIVITIES

Twenty-four hours after FITC challenge, ear tissues were harvested and homogenized in a 0.5% cetyltrimethylammonium chloride solution (Sigma-Aldrich, St Louis, MO, USA). After passing the homogenates through a nylon mesh and centrifuging at 3,000 rpm for 20 minutes at 4℃, the supernatants

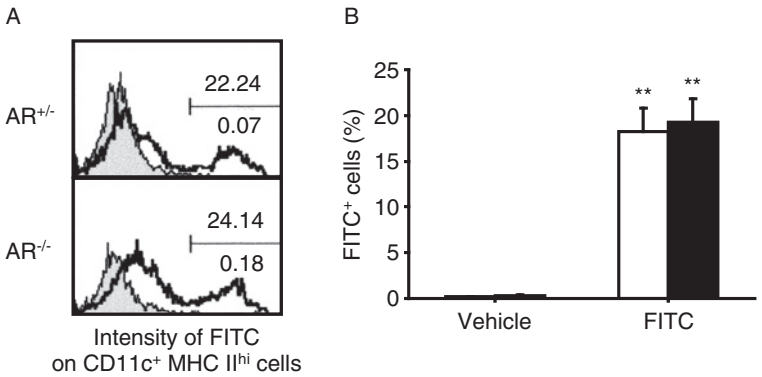

Fig. 1 AR is not required for migration of FITC-bearing LCs/DCs from skin to draining LNs. At 24 hours after epicutaneous treatment with FITC (left ear skin) and vehicle (right ear skin), the submaxillary LNs on the left and right sides were separately collected. The proportion of FITC-positive cells among 7-aminoactinomycin D$^-$ MHC class II^{hi} CD11c$^+$ cells was determined by flow cytometry. (**A**) Representative flow cytometry data. Shaded area = LNs draining vehicle-treated ears (lower number [%]); solid lines = LNs draining FITC-treated ears (upper number [%]). (**B**) Data show the mean + SEM of values for individual mice. Open columns = AR$^{+/-}$ mice (n = 7), and solid columns = AR$^{-/-}$ mice (n = 8). $^{**}p < 0.01$ *vs.* LNs draining vehicle-treated ears.

were collected. For measurement of MPO activity, the supernatants were diluted with 10 mM citrate buffer (pH 5.0), followed by addition of an equal volume of substrate solution for MPO assay (3 mM 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich), 120 mM resorcinol (Wako), 2.2 mM H_2O_2 (Wako) in distilled water). For measurement of EPO activity, the supernatants were diluted with 50 mM PIPES-NaOH (pH 6.5) containing 6 mM KBr, followed by addition of an equal volume of substrate solution for EPO assay (3 mM o-phenylenediamine (Sigma-Aldrich), 8.8 mM H_2O_2 (Wako) in 50 mM PIPES-NaOH (pH 6.5) containing 6 mM KBr). After 0.5 to 2 minutes, 2N H_2SO_4 was added to stop the reaction, and the absorbance was measured using a plate reader (MPO, 450 nm; EPO, 490 nm). The amount of total protein in ear tissue homogenate was measured using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). MPO and EPO activities were shown as the absorbance per gram of total protein in ear tissue homogenates.

MEASUREMENT OF FITC-SPECIFIC Ig LEVELS

Serum was collected from naïve mice and mice 8 days after FITC challenge during FITC-induced CHS responses. FITC-conjugated OVA was prepared as described elsewhere.[21,24] A 96-well ELISA plate (Nunc, 442404) was coated with 2 μg/ml FITC-OVA at 4℃ overnight. After the wells were blocked with PBS containing 10% FCS, optimally diluted serum samples (IgG1, 1/1000; IgG2a, 1/10; IgG2b, 1/10; IgG3, 1/10; and IgE, undiluted) were applied, and the plates were incubated at room temperature for 1 hour. After washing, biotinylated anti-mouse IgG1 (A 85-1; BD Biosciences), IgG2a (R19-15; BD Biosciences), IgG2b (R12-3; BD Biosciences), IgG3 (R 40-82; BD Biosciences) or IgE (R35-118; BD Biosciences) mAb was added, followed by incubation at room temperature for 1 hour. Then, after washing, HRP-conjugated streptavidin (BD Biosciences) was added, followed by incubation at room temperature for 1 hour. For enzymatic reaction, TMB substrate (KPL) was used as a substrate. The reaction was stopped by addition of 2N H_2SO_4, and then the absorbance was measured using a plate reader at 450 nm. Data show the absorbance value at 450 nm.

STATISTICAL ANALYSES

Data show the mean +/± SEM. Differences were evaluated by the two-tailed Student's t test and considered significant at a P value of less than 0.05.

RESULTS

AMPHIREGULIN IS NOT ESSENTIAL FOR MIGRATION OF SKIN DENDRITIC CELLS FROM SKIN TO DRAINING LNs AFTER EPICUTANEOUS FITC TREATMENT

Langerhans cells (LCs) and dermal dendritic cells (DCs) are important in the sensitization and elicitation phases of CHS. Activated keratinocyte-derived cytokines such as IL-1 and TNF are crucial for migration of LCs/dermal DCs from the skin to draining LNs.[2,3,7] AR can enhance activation of keratinocytes,[25] implying the ability of AR to promote LC/dermal DC

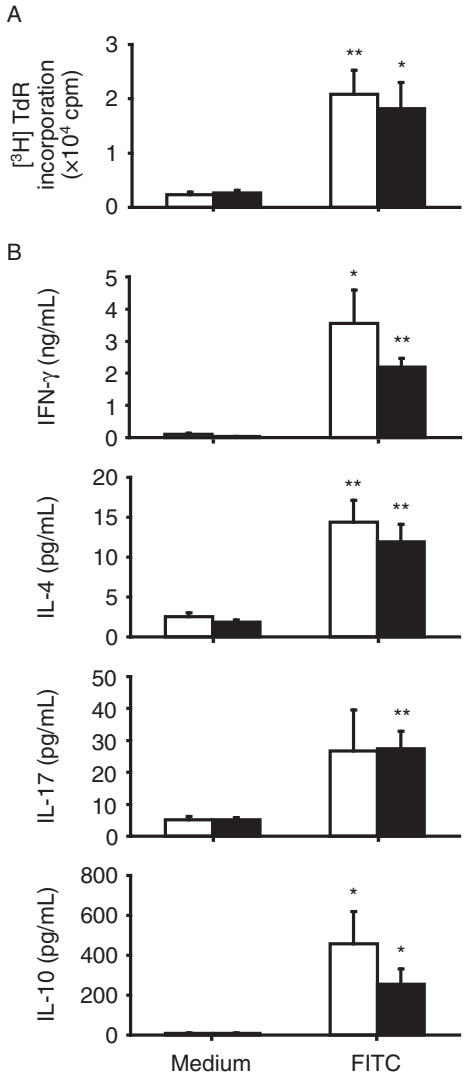

Fig. 2 AR deficiency did not influence FITC-specific LN cell responses. Mice were epicutaneously treated with 2.0% FITC on the ear skin. Six days later, submaxillary LN cells were collected and cultured in the presence and absence of 40 μg/ml FITC for 72 hours. (**A**) Incorporation of [3H]-thymidine and (**B**) the levels of IFN-γ, IL-4, IL-10 and IL-17 in the culture supernatants shown in (**A**) were determined. Data show the mean + SEM of values for individual mice. Open columns = $AR^{+/-}$ mice (n = 6), and solid columns = $AR^{-/-}$ mice (n = 6). *p < 0.05 and **p < 0.01 *vs.* LNs draining vehicle-treated ears.

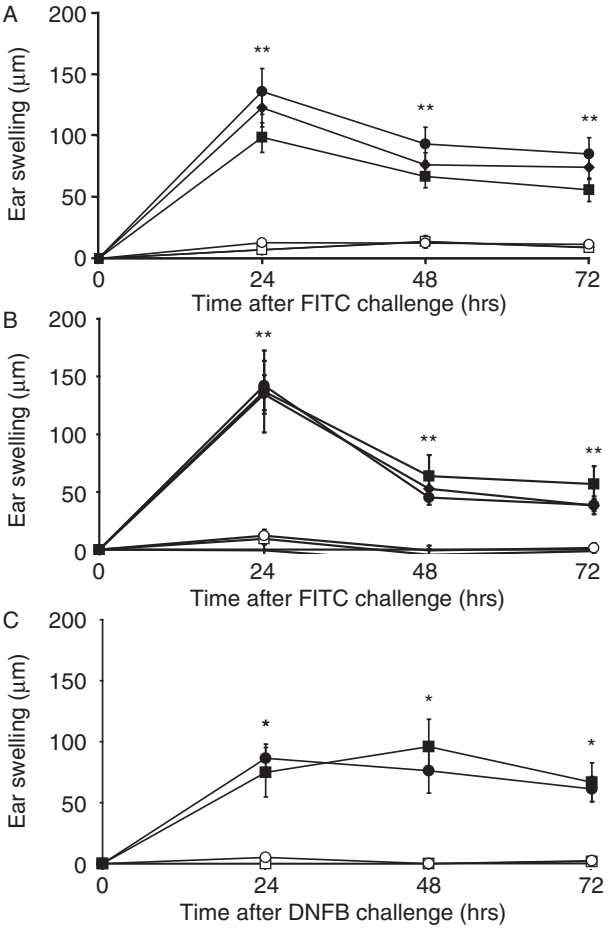

Fig. 3 AR is not required for development of FITC- and DNFB-induced CHS. Five days after epicutaneous sensitization with 0.5% (**A**), 2.0% FITC (**B**), and 0.5% DNFB (**C**), mice were challenged with 0.5% FITC (**A, B**) or 0.2% DNFB (**C**) (left ears) and vehicle alone (right ears). Before (0) and after challenge, ear thickness was measured using an engineer's caliper. Data show the mean ± SEM of values for individual mice. Open symbols = vehicle-treated ears, and closed symbols = FITC-treated ears. Diamonds = $AR^{+/+}$ mice (**A**; n = 7, **B**; n = 9), squares = $AR^{+/-}$ mice (**A**; n = 21, **B**; n = 8, and **C**; n = 6) and circles = $AR^{-/-}$ mice (**A**; n = 22, **B**; n = 7, and **C**; n = 4). *p < 0.05 and **p < 0.01 *vs.* vehicle-treated ears.

migration. To assess the role of AR in skin LC/DC migration, we treated AR-deficient and -sufficient mice with FITC epicutaneously. Twenty-four hours after FITC treatment, the proportion of FITC-positive cells among MHC class II^{hi} $CD11c^{+}$ cells in draining LNs was determined by flow cytometry. The proportion of FITC-positive cells in draining LNs from AR-deficient mice was equivalent to that from the littermate control mice (Fig. 1). These results indicate that AR is not essential for skin DC migration after epicutaneous FITC treatment.

AMPHIREGULIN IS NOT ESSENTIAL FOR DEVELOPMENT OF HAPTEN-SPECIFIC MEMORY T CELLS

After hapten-carrying skin DCs migrate from skin to draining LNs, these cells present the antigens to naïve T cells and induce allergen-specific effector and memory T cells in the sensitization phase of CHS.[2,3] To elucidate the role of AR in hapten-specific memory

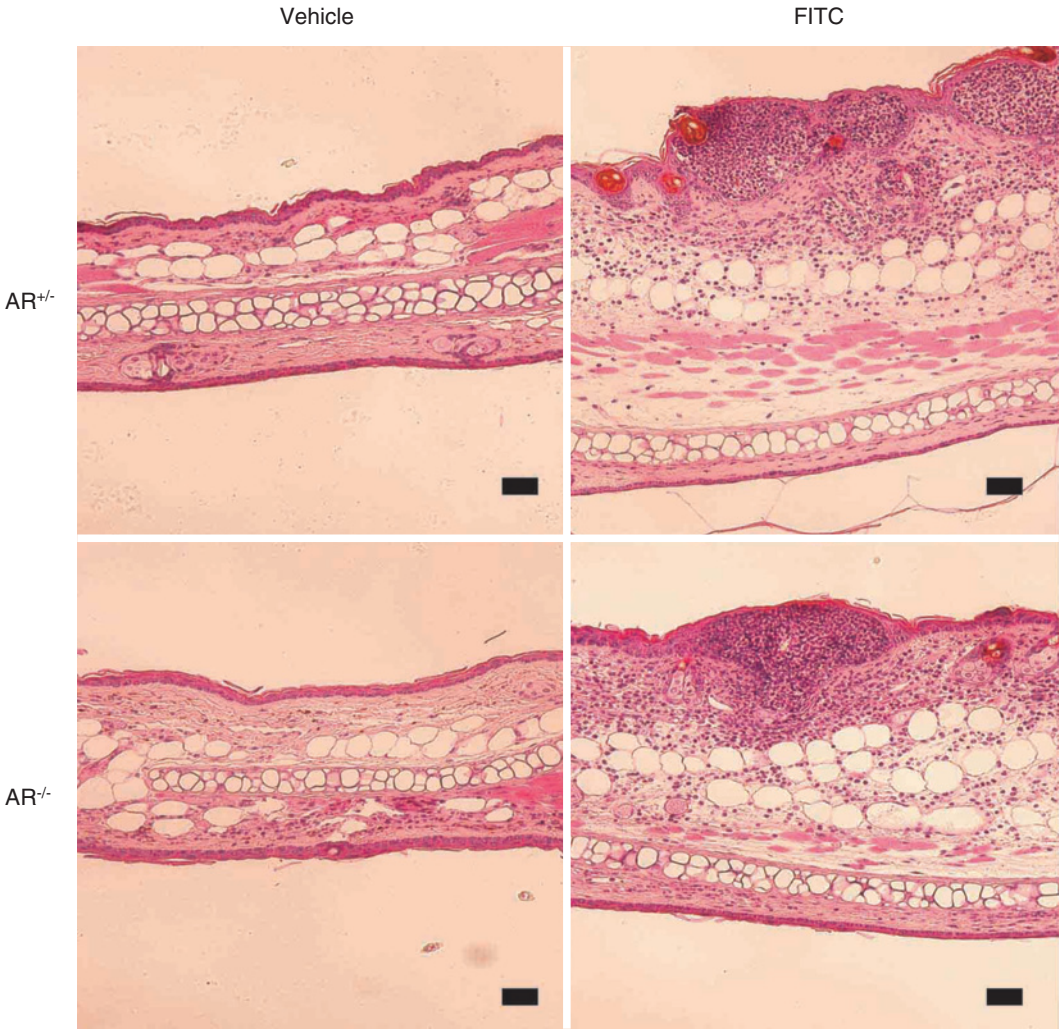

Fig. 4 Inflammation is normal in AR-deficient mice during FITC-induced CHS. At 24 hours after FITC and vehicle treatment, ear tissues were collected and embedded in paraffin. Sections of ear tissues were prepared and stained with hematoxylin and eosin. Representative data are shown. Scale bars = 100 μm.

T-cell induction, draining LN cells from FITC-treated mice were cultured in the presence of FITC *in vitro*. FITC-specific LN cell proliferative responses evaluated using [^{3}H]-labeled thymidine were comparable between AR-deficient and -sufficient mice (Fig. 2A). The AR-deficient and -sufficient mice also showed similar levels of IFN-γ, IL-4, IL-10 and IL-17 in their LN cell culture supernatants (Fig. 2B). These observations indicate that AR is not important for the generation of hapten-specific T cells in the sensitization phase of FITC-induced CHS.

AMPHIREGULIN IS NOT ESSENTIAL FOR DEVELOPMENT OF FITC-INDUCED CHS

It is known that Th2 cytokines such as IL-4 and IL-5 and mast cells are important for the development of FITC-induced CHS.[16,21] Moreover, in the microarray analysis, we found that mRNA expression of AR was increased 10.47-fold in FITC-challenged ears in comparison with vehicle-treated ears at 24 hours after the treatment (n = 3, data not shown). These observations suggest that AR may be involved in the development of FITC-induced CHS. However, AR-deficient mice showed similar levels of ear swelling as compared with AR-sufficient mice during both FITC- and DNFB-induced CHS (Fig. 3). Also, histological analysis found the same degree of inflammation in AR-deficient and -sufficient mice at 24 hours after FITC challenge (Fig. 4).

The inflammation in FITC-induced CHS was associated with infiltration of neutrophils and eosinophils.[16] We examined the activities of MPO and EPO in the ear tissue homogenates at 24 hours after FITC challenge. Consistently, MPO and EPO activities in the ear tissue homogenates did not differ significantly between the AR-deficient and -sufficient mice during FITC-induced CHS (Fig. 5).

We next used ELISA to examine the levels of FITC-

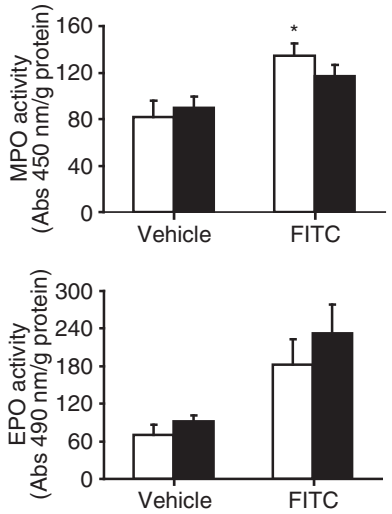

Fig. 5 MPO and EPO activities are normal in ear tissue homogenates from AR-deficient mice during FITC-induced CHS. At 24 hours after FITC and vehicle treatment, ear tissues were collected and homogenates were prepared. The activities of MPO and EPO in ear tissue homogenates were determined by colorimetric assay. Data show the mean + SEM of values for individual mice. Open columns = $AR^{+/-}$ mice (n = 3), and solid columns = $AR^{-/-}$ mice (n = 3). *p < 0.05 *vs.* vehicle-treated ears.

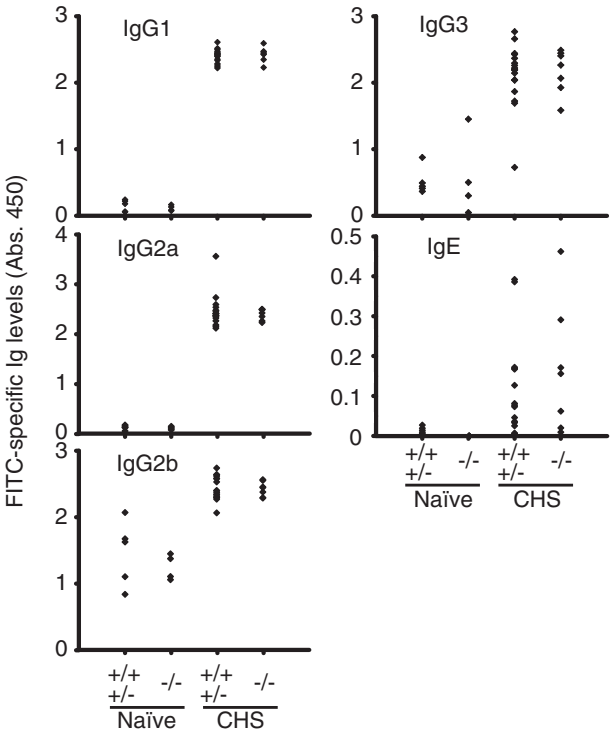

Fig. 6 Levels of FITC-specific Igs are normal in sera from AR-deficient mice during FITC-induced CHS. Blood was collected from naïve mice and mice 8 days after FITC challenge. The levels of FITC-specific IgG1, IgG2a, IgG2b, IgG3 and IgE in sera were determined by ELISA. Data show the mean + SEM of values for individual mice. Open columns = $AR^{+/-}$ mice (Naïve, n = 5; CHS, n = 8), and solid columns = $AR^{-/-}$ mice (Naïve, n = 4; CHS, n = 7). *p < 0.05 and **p < 0.01 *vs.* naïve mice.

specific Igs in sera from FITC-treated mice during FITC-induced CHS. The levels of FITC-specific IgG1, IgG2a, IgG2b, IgG3 and IgE in sera from AR-deficient mice were equal to those from AR-sufficient mice after FITC challenge (Fig. 6). Taken all together, these results indicate that AR is not essential for the development of FITC-induced CHS.

DISCUSSION

AR, a member of the EGF family of growth factors/cytokines, plays an important role in the enhancement and/or inhibition of cell growth and is involved in physiological responses such as mammary gland development, blastocyst implantation, bone formation and nerve regeneration as a growth factor.[17,18] As another functional aspect of AR, it was shown that Th2 cell-derived AR is involved in the pathogenesis of Th2 cell/cytokine-mediated host defense against nematodes.[19] In addition, we and others have demonstrated that mast cells can produce AR by IgE-FcεRI crosslinking, and AR enhanced not only mucin gene expression in human epithelial cells but also proliferation of human lung fibroblasts,[26,27] implying a contribution of AR to the pathogenesis of Th2 cell/cytokine- and mast cell-mediated immune responses, such as allergic diseases.

CHS is classically considered to be an IFN-γ-producing Th1- and Tc1-mediated allergic disease of the skin.[1] However, Th2 cells/cytokines rather than Th1/Tc1 cells/cytokines seemed to be potentially responsible for the development of CHS.[10,28] In particular, Th2 cytokines and mast cells are responsible for the development of CHS induced by FITC.[16,21] These observations suggest that AR may be involved in the development of Th2 cell/cytokine- and mast cell-associated CHS.

In the sensitization phase of CHS, activation of skin LCs/DCs is considered to be important for induction of hapten-specific effector/memory T cells.[2,3] Keratinocyte-derived proinflammatory cytokines such as TNF and IL-1 are crucial for LC/DC migration and maturation.[2,3,7] Since AR can enhance activation of keratinocytes,[25] it might be expected to play a role in that step during CHS. However, we found that the levels of skin DC/LC migration and the proliferation and cytokine production of hapten-stimulated LN cells were normal in AR-deficient mice. These observations indicate that AR is not crucial for migration of LCs/DCs from the skin into draining LNs or for induction of hapten-specific effector/memory T cells in the sensitization phase of CHS.

In the elicitation phase of CHS induced by FITC, we found (by DNA microarray analysis) that AR mRNA expression was approximately 10-fold increased in FITC-challenged ear skin at 24 hours after treatment in comparison with vehicle-treated ear skin. However, local inflammation, assessed by ear swelling, histological analysis and the levels of MPO and EPO activities in tissue homogenates at 24 hours after the FITC challenge, and the levels of hapten-specific serum Igs were also comparable between AR-deficient and -sufficient mice. Therefore, AR is not necessary for induction of inflammation in the elicitation phase of CHS.

Taken all together, we conclude that AR is not essential for the development of FITC-induced CHS in mice.

ACKNOWLEDGEMENTS

We thank Shuhei Fukuda, Hiromi Wakita, Michiko Yamada, Akina Ishii and Noriko Hashimoto for their excellent technical assistance, Akane Nakamura for animal care, and Dr. David Lee for providing amphiregulin-deficient mice. This work was supported by a grant to H.S. from the National Institute of Biomedical Innovation and by Grants-in-Aid for Young Scientists (B) to N.K., K.O. and S.N., and by the Program for Improvement of Research Environment for Young Researchers, The Special Coordination Funds for Promoting Science and Technology to S.N. from the MEXT, Japan and supported by grant CA43793 from the U.S. National Institutes of Health (S.W.S.).

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