

Effect of Protease-Activated Receptor-2 Deficiency on Allergic Dermatitis in the Mouse Ear

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Received August 29, 2001 Accepted October 15, 2001

ABSTRACT—To investigate the involvement of protease-activated receptor-2 (PAR-2) in allergic dermatitis, we generated PAR-2-deficient (PAR-2^{-/-}) mice. Ear thickness, contact hypersensitivity (CH) induced by topical application of picryl chloride (PC) or oxazolone (Ox) after sensitization, and vascular permeability after ear passive cutaneous anaphylaxis (PCA) were compared between wild-type (WT) and PAR-2^{-/-} mice. Ear thickness was almost the same in untreated WT and PAR-2^{-/-} mice. Topical application of PC or Ox thickened the ears at 6, 24 and 48 h after challenge with a peak at 24 h in WT mice. In PAR-2^{-/-} mice, the ear swelling induced by both PC and Ox was suppressed at every time point, and significant inhibition was found at 24 h in PC-induced CH and at 24 and 48 h in Ox-induced CH. Histopathological observation of the ears at 24 h after challenge revealed that PC- or Ox-induced ear edema and infiltration of inflammatory cells in WT mice were greatly attenuated in PAR-2^{-/-} mice. The vascular permeability in the ears after PCA was not different between WT and PAR-2^{-/-} mice. These results strongly suggest that PAR-2 plays a crucial role in type IV allergic dermatitis but not in type I allergic dermatitis.

Keywords: Protease-activated receptor-2, Knockout mice, Allergic dermatitis, Contact hypersensitivity, Passive cutaneous anaphylaxis

Protease-activated receptors (PARs) are a family of G-protein-coupled seven transmembrane receptors, currently consisting of four members, PAR-1 to PAR-4, and the activation mechanism of PARs is very unique (1–3). A neo-terminus tethered ligand produced by proteolytic cleavage of the extracellular N-terminus or a short synthetic peptide based on the tethered ligand sequence, with the exception for PAR-3, activates the corresponding receptor (1–3). Among these, PAR-2 was identified in 1994 (4) and reported to be activated by the tissue factor/factor VIIa, Xa (5), the sperm protease, acrosin (6), and a trypsin-like serine protease isolated from rat brain (7) as well as trypsin, tryptase, and a synthetic peptide having its tethered ligand sequence (1–3).

Physiological and pathological roles of PAR-2 have been investigated in several kinds of systems and tissues

including skin (8–10) using different approaches such as immunohistochemistry and pharmacology with synthetic PAR-2-activating peptide and PAR-2-deficient (PAR-2^{-/-}) mice. So far, many reports suggested detrimental effects of PAR-2 activation (11–17), although PAR-2 activation also showed protective effects in myocardial ischemia-reperfusion injury (18), airways (19, 20) and cerebral circulation during chronic hypertension (21).

Among the detrimental effects of PAR-2 activation, a pro-inflammatory effect has mainly been suggested from the observations in which trypsin, tryptase and PAR-2-activating peptide induced extravasation of plasma proteins (14–16), leukocyte rolling and adhesion (22) and infiltration of neutrophils (15, 23), and stimulated secretion of proinflammatory cytokines (24, 25) and nuclear factor kappa B-DNA binding (26). Furthermore, a recent study using PAR-2^{-/-} mice showed that lack of PAR-2 led to the delayed onset of inflammation (27). However, direct evidence for the involvement of PAR-2 in allergic dermatitis

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has not yet been investigated.

In the present study, we created a null (knock-out) allele of the PAR-2 gene in embryonic stem (ES) cells and generated PAR-2^{-/-} mice to investigate the involvement of PAR-2 in allergic dermatitis in the mouse ear. Ear thickness, contact hypersensitivity (CH) induced by topical application of picryl chloride (2,4,6-trinitrochlorobenzene, PC) or oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, Ox) after sensitization and vascular permeability after ear passive cutaneous anaphylaxis (PCA) were compared between wild-type (WT) and PAR-2^{-/-} mice.

MATERIALS AND METHODS

Animals

Generation of PAR-2^{-/-} mice: A knock-out allele of the PAR-2 gene was generated by gene targeting in E14Tg2a ES cells (129/Ola strain) by standard procedures using a targeting vector design similar to that previously described (28). This resulted in the replacement of almost the entire exon 2 coding sequence with a cassette consisting of a β -galactosidase coding sequence preceded by an internal ribosome entry site sequence and followed by a neomycin resistance gene (29). This therefore brings expression of the β -galactosidase coding region under PAR-2 transcriptional control. Male chimeras generated with PAR-2-targeted ES cells were test crossed with C57BL/6 females. Germ-line transmission of the null allele was confirmed in the agouti coat colored test cross offspring by Southern blot analysis of restriction enzyme-digested DNA obtained by tail biopsy and hybridization with probes flanking each side of the vector integration position. Mice heterozygous for the null allele (PAR-2^{+/-}) from the test cross were then intercrossed to generate PAR-2^{-/-} mice with a mixed 129/Ola and C57BL/6 genetic background. PAR-2^{-/-} mice were identified from the intercross (by genotyping as above) at a normal Mendelian ratio relative to the PAR-2^{+/-} and PAR-2^{+/+} genotype classes, indicative of no effect of the knock-out allele on viability. The PAR-2^{-/-} mice were of the same size, vitality and fertility when compared to PAR-2^{+/-} and PAR-2^{+/+} littermates, and as previously reported, they exhibited no evidence of macroscopic abnormalities (27). Lines of PAR-2^{-/-} and PAR-2^{+/+} mice (for use as WT controls) were established by separate interbreeding of each genotype class and maintained in parallel by full-sibling mating in successive generations. In all the experimental analyses, male WT and PAR-2^{-/-} mice aged 6–9 weeks were compared from the same generation number.

All animals were housed under specific pathogen-free conditions and had free access to a standard laboratory diet and water in a air-conditioned room at 23 ± 3°C and relative humidity of 55 ± 15%. The present experiments were carried out in accordance with the Guidelines for the

Care and Use of Laboratory Animals of Kowa Co., Ltd.

Reagents

The following reagents were obtained from commercial sources: Ox and chicken egg albumin (OA) (Sigma, St. Louis, MO, USA); PC and Evans blue dye (Tokyo Kasei, Tokyo).

PC- and Ox-induced CH

Experiments were carried out by a method basically similar to that of Nakano (30). The mice were sensitized by topical application of 100 μ l of a 7% solution of PC or a 0.5% solution of Ox in ethanol on shaved abdominal skin. Six and five days after sensitization with PC and Ox, respectively, mice were challenged by applying 20 μ l of a 1% solution of PC in olive oil or a 0.5% solution of Ox in acetone on both sides of the right ear, and respective vehicle on both sides of the left ear, after measuring ear thickness with a dial thickness gauge (Peacock G-1A; Ozaki Mfg. Co., Ltd., Tokyo). Ear thickness was measured at 6, 24 and 48 h after challenge, and ear swelling was determined as the difference in ear thickness before and after challenge. For histopathological evaluation, the ears from mice sacrificed under ether anesthesia by cervical dislocation at 24 h after the challenge with PC or Ox were fixed and embedded in paraffin by standard procedures. Paraffin sections (3 μ m) were stained with hematoxylin and eosin (H&E), and assessed by light microscopy.

Vascular permeability increase induced by PCA in the mouse ear

The method was based on that in previous reports (31, 32). Briefly, mice under ether anesthesia were passively sensitized by intradermal injection of mouse anti-OA antiserum prepared in-house at an appropriate dilution into the ears in a volume of 5 μ l/site. Forty-eight hours after sensitization, PCA was elicited by intravenous administration of saline solution containing OA (10 mg/kg) and Evans blue dye (50 mg/kg) in a volume of 10 ml/kg. After 30 min, mice were anesthetized with ether and sacrificed by cervical dislocation and vascular permeability was assessed by measuring the amount of extravasated dye in the ears. The ears obtained from each mouse were dissolved in 0.25 ml of 1 N KOH solution in a stoppered tube at 37°C overnight, and 1.5 ml of a mixture of 0.6 N H₃PO₄ solution and acetone (5:13) was added. After vigorous shaking and centrifugation at 3,000 rpm for 10 min, the amount of dye in the supernatant was determined colorimetrically at 620 nm.

Data analyses and statistics

Results were expressed as values of the mean ± S.E.M. Data were evaluated by Student's *t*-test with the software, SAS system for Windows Ver. 6.12 (SAS Institute Inc.,

Cary, NC, USA) supported by EXSAS ver 5.00 (Arm Co., Ltd., Tokyo), and $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of the PAR-2 knockout on ear thickness in untreated mice

The ear thickness in untreated WT mice ($22.7 \pm 0.4 \times 10^{-2}$ mm, $n = 10$) was not statistically different from that in untreated PAR-2^{-/-} mice ($22.0 \pm 0.4 \times 10^{-2}$ mm, $n = 10$).

Inhibitory effect of the PAR-2 knockout on PC-induced mouse ear swelling

In WT mice, topical application of PC increased ear thickness at 6, 24 and 48 h after challenge with a peak at 24 h (Table 1). In PAR-2^{-/-} mice, although ear thickness increased by the challenge with PC until 48 h with a maximal response at 48 h, the degree of ear swelling was reduced at every time point as compared with WT mice (Table 1). A statistical difference in ear swelling between WT and PAR-2^{-/-} mice was observed at 24 h after challenge (Table 1, $P < 0.01$), and the result at this time point was highlighted in Fig. 1A. The challenge with vehicle (olive oil) did not affect ear thickness in both WT and PAR-2^{-/-} mice. Topical application of PC greatly thickened the ears in WT mice, but PC-induced ear swelling was significantly suppressed in PAR-2^{-/-} mice. The degree of the ear swelling in PAR-2^{-/-} was only one-fourth of that in WT mice.

Table 1. Time course of PC- or Ox-induced ear swelling in WT and PAR-2^{-/-} mice

Genotype	n	Time after challenge (h)		
		6	24	48
PC-induced ear swelling				
WT	10	1.1 ± 0.3	9.2 ± 1.8	7.3 ± 1.3
PAR-2 ^{-/-}	10	0.4 ± 0.3	$2.3 \pm 1.2^{**}$	3.5 ± 1.3
Ox-induced ear swelling				
WT	5	1.0 ± 0.6	11.4 ± 2.0	9.3 ± 0.7
PAR-2 ^{-/-}	5	-0.1 ± 0.4	$-0.9 \pm 0.6^{**}$	$4.1 \pm 1.8^*$

The animals were sensitized with PC or Ox by painting on shaved abdominal skin. Six days for PC or five days for Ox after sensitization, vehicle and the hapten were topically applied on both sides of the left and right ears, respectively. The ear thickness was measured using a dial thickness gauge before and at 6, 24 and 48 h after challenge. The difference in ear thickness before and after challenge was taken as ear swelling. Each value represents the mean \pm S.E.M. of 10 or 5 mice ($\times 10^{-2}$ mm). * $P < 0.05$, ** $P < 0.01$, compared with WT at the same time point.

Inhibitory effect of the PAR-2 knockout on Ox-induced mouse ear swelling

The results were almost the same as those obtained in the experiment using PC as a sensitizer. In WT mice, topical application of Ox increased ear thickness at 6, 24 and 48 h after challenge with a peak at 24 h (Table 1). In PAR-2^{-/-} mice, Ox did not affect ear thickness at 6 and 24 h but moderately increased it at 48 h (Table 1). Statistical difference in ear swelling between WT and PAR-2^{-/-} mice was observed at 24 ($P < 0.01$) and 48 ($P < 0.05$) h after challenge (Table 1), and the result at 24 h was highlighted in Fig. 1B. Although the vehicle for Ox (acetone) used at challenge was different from that for PC (olive oil), it also had little effect on ear thickness in both WT and

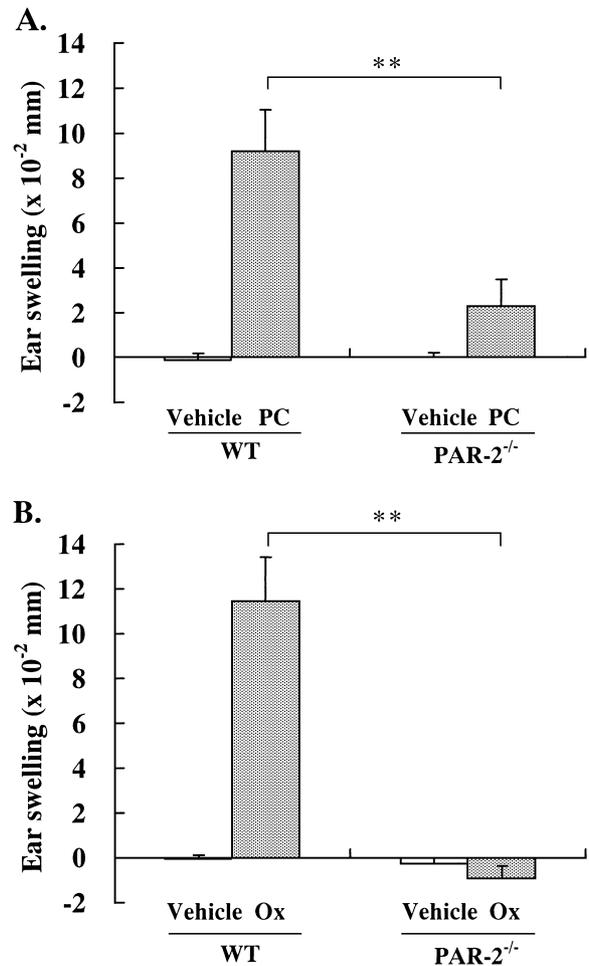


Fig. 1. PC- and Ox-induced ear swelling at 24 h after challenge in WT and PAR-2^{-/-} mice. The animals were sensitized with PC (A) or Ox (B) by painting on shaved abdominal skin. Six days for PC or five days for Ox after sensitization, the vehicle and sensitizer were topically applied on both sides of the left and right ears, respectively. The difference in ear thickness before and after challenge was taken as ear swelling. Each column and bar represents the mean \pm S.E.M. of 10 or 5 mice. ** $P < 0.01$.

PAR-2^{-/-} mice. The challenge with Ox greatly thickened the ears in WT mice, but did not produce ear swelling in PAR-2^{-/-} mice.

Effect of the PAR-2 knockout on histopathological changes after challenge with PC or Ox

Histopathological observation of the ears at 24 h after the challenge with PC and Ox supported the assessment of

ear swelling using the dial thickness gauge.

Typical histopathological changes of the ears in WT and PAR-2^{-/-} mice at 24 h after the challenge with PC and Ox are presented in Figs. 2 and 3. Topical application of vehicles, olive oil and acetone (data not shown), did not show any histopathological changes in the ears of WT (Fig. 2A) and PAR-2^{-/-} (Fig. 2B) mice. In WT mice, the challenge with PC (Fig. 2C) and Ox (Fig. 2E) induced

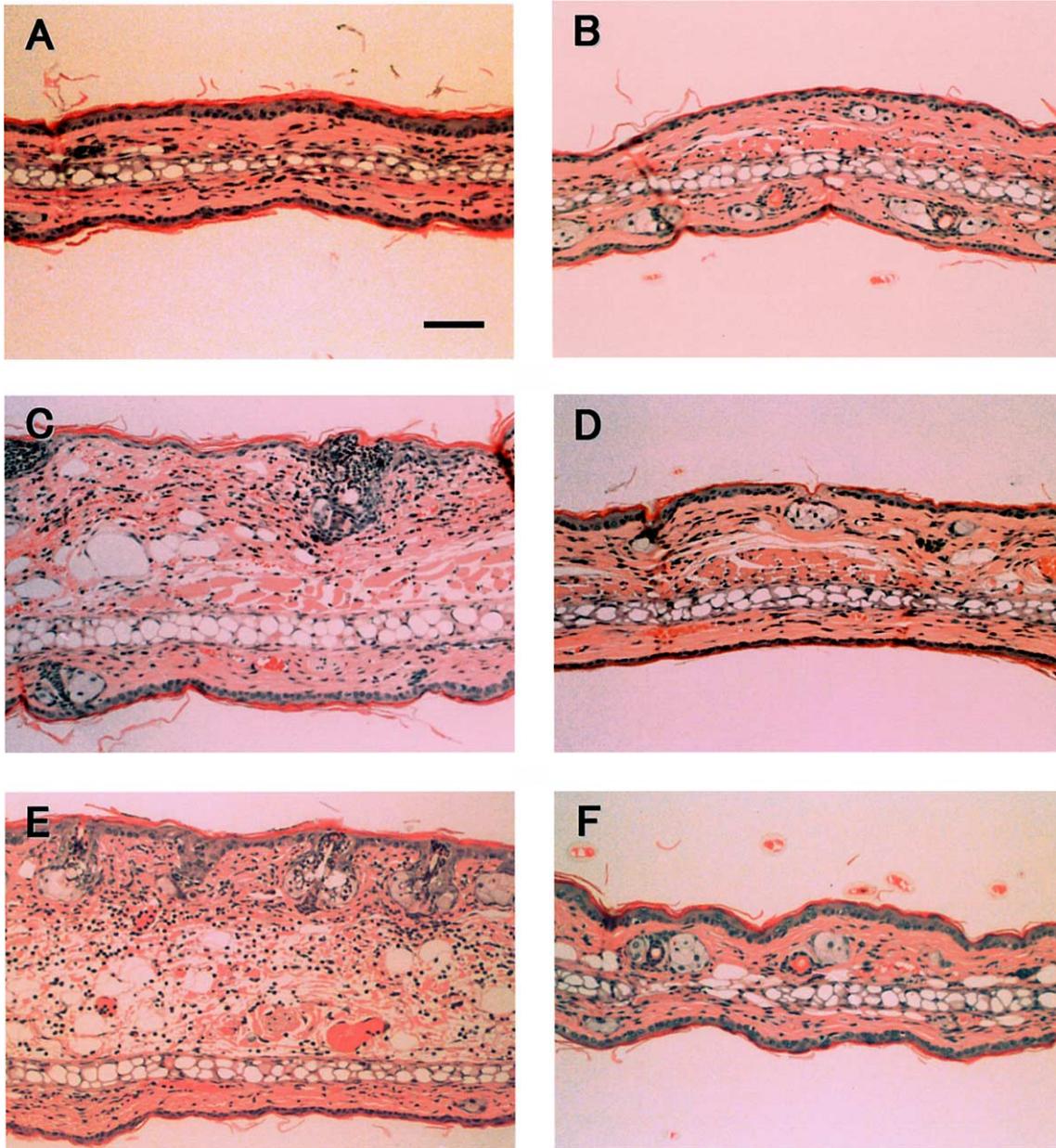


Fig. 2. Histopathological findings in the ears at 24 h after the challenge with PC or Ox in WT and PAR-2^{-/-} mice. After sensitization, vehicle for PC (A and B) or Ox (data not shown), PC (C and D) or Ox (E and F) was topically applied on the ears in WT (A, C and E) or PAR-2^{-/-} (B, D and F) mice. Microscopic observation of the sections stained with H&E showed that vehicle had no effect on the ears in both WT (A) and PAR-2^{-/-} (B) mice and that ear swelling, edema and infiltration of inflammatory cells induced by the challenge with both PC (C) and Ox (E) in WT mice were markedly inhibited or abolished in PAR-2^{-/-} mice (D and F). Bar = 100 μ m.



Fig. 3. High magnification photograph of histopathological findings in the ears at 24 h after the challenge with PC in WT mice. Microscopic observation of the sections stained with H&E at high magnification conclusively demonstrates an extremely large degree of edema and infiltration of neutrophils (N), macrophages (M), lymphocytes (L) and eosinophils (E) after the challenge with PC in WT mice. Bar = 20 μ m.

similar pathological changes in the ears. The severe edema occurred corresponding to ear swelling determined using the dial thickness gauge. The infiltration of inflammatory cells including mononuclear and polymorphonuclear cells was observed throughout the subcutis and dermis, and several pustules were found in the epidermis (Fig. 2: C and

E). The observation of the ear sections at higher magnification revealed that neutrophils, macrophages and lymphocytes predominated and a smaller number of eosinophils also presented in the dermis at 24 h after challenge (Fig. 3). In PAR-2^{-/-} mice, however, these histopathological changes after the challenge with both PC and Ox were significantly inhibited (Fig. 2: D and F).

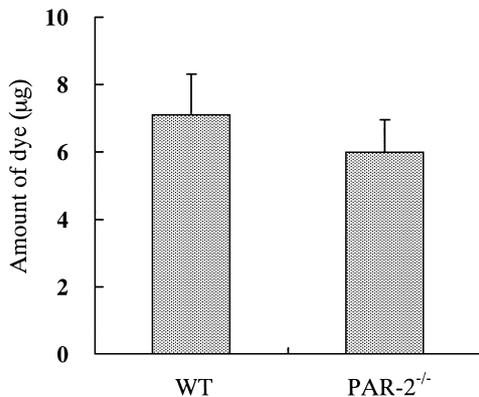


Fig. 4. PCA in the ears of WT and PAR-2^{-/-} mice. The animals were sensitized with mouse anti-OA antiserum by injecting into pinna of both left and right ears. Two days after passive sensitization, saline solution containing OA (10 mg/kg) and Evans blue dye (50 mg/kg) was administered intravenously. The mice were sacrificed under anesthesia 30 min after dye administration and permeated dye was extracted from the ears. The amount of dye was then determined colorimetrically. Each column and bar represents the mean \pm S.E.M. of 4 (for WT) or 5 (for PAR-2^{-/-}) mice.

Effect of the PAR-2 knockout on increased vascular permeability in mouse ear PCA

The effect of the PAR-2 knockout on mouse ear PCA is shown in Fig. 4. PCA extravasated 7.1 ± 1.2 (n=4) and 6.0 ± 1.0 (n=5) μ g of dye in the ears of WT and PAR-2^{-/-} mice, respectively. There was no statistical difference in the dye content of the ears between WT and PAR-2^{-/-} mice.

DISCUSSION

In the present study, both PC and Ox successfully increased ear thickness at 6, 24 and 48 h after challenge with a peak at 24 h in WT mice. Furthermore, histopathological observation of the ear sections revealed that edema and infiltration of inflammatory cells simultaneously occurred in WT mice. These results were well consistent with previous reports (30, 33–35). PCA also successfully extravasated the dye in the ears of WT mice at 30 min after antigen challenge as previously reported (31). Therefore, CH induced by the two different kinds of sensitizers and PCA

used here were thought to be reliable.

CH is elicited by repeated application of chemically reactive haptens such as PC, Ox, 2,4-dinitrofluorobenzene (DNFB) and 2,4-dinitrochlorobenzene (DNCB). The involvement of several types of cells and factors in CH and the effect of chemicals on CH have been investigated using CH induced by these different haptens (33, 34, 36–40). In STAT 6-deficient mice, ear swelling induced by three kinds of haptens, PC, Ox and DNFB, were similarly suppressed (39). However, in the experiments using IL-4- and IL-1 β -deficient mice, different results were obtained among CH induced by different haptens. The deficiency in IL-4 or IL-1 β inhibited CH to PC and DNCB, but not that to Ox (33, 41–43). Furthermore, different sensitivity to drugs has been reported between PC- and Ox-induced CH in the mouse ear (34). Because these results suggest that the kind and degree of the involved factors in CH varies among sensitizers used, experimental results using different hapten-induced CH may be needed to evaluate the effects of factors and drugs on CH. We, therefore, examined the effect of the PAR-2 knockout on CH induced by two different kinds of sensitizers, PC and Ox, in the present study. We found that PAR-2 deficiency greatly attenuated ear swelling and histopathological changes, edema and infiltration of inflammatory cells, in both PC- and Ox-induced CH. These results indicate that the inhibitory effect of PAR-2 deficiency on CH may not depend on the kind of hapten used to elicit CH. Furthermore, our results suggest that PAR-2 plays a crucial role in type IV allergic dermatitis because PC and Ox-induced CH is thought to be an animal model for this dermatitis.

CH involves various immunocomponent cells including Th1 and Th2 cells and mediators (37–40). Furthermore, participation of mast cells in CH has been suggested (44), and a defective CH was demonstrated in mast cell-deficient mice (45). Because activation of mast cells is important in PCA and the degree of PCA can be estimated by measuring the amount of extravasated dye in the reaction site (31, 32, 46, 47), we examined the effect of PAR-2 deficiency on the increased dye content after ear PCA. As shown in Fig. 4, PCA increased the dye content in the ears of WT mice and PAR-2 deficiency did not affect the increased dye content. This result indicates that PAR-2 deficiency has little effect on the function of mast cells and the increase of vascular permeability in PCA, suggesting that the mast cell activation similar to that occurring in PCA may not be related to the reduced CH in PAR-2^{-/-} mice. Furthermore, the absence of detectable PAR-2 mRNA in rat peritoneal mast cells (48) may also indicate that there is no direct activation of mast cells upon PAR-2 activation. In addition to the present results, we have also observed no effect of PAR-2 deficiency on IgE production after OA administration in mice in other experiments (data not shown),

suggesting that PAR-2 may not be important in type I allergic dermatitis.

Several recent reports suggest the pro-inflammatory effect of PAR-2 activation in allergic inflammation. Trypsin activates the infiltration of neutrophil and eosinophil (23) and stimulates the production and release of cytokines. PAR-2-activating peptide also stimulates the release of IL-8 from keratinocytes and nuclear factor kappa B-DNA binding (26), and produces rolling, adhesion and infiltration of neutrophils through the release of platelet activating factor without direct activation of neutrophils (22). Furthermore, the delayed onset of inflammation by an inhibition of P-selectin-mediated rolling of neutrophils in PAR-2^{-/-} mice (27) and PAR-2 activation-induced inflammation through a neurogenic mechanism (17) have been reported. However, direct evidence for the involvement of PAR-2 in allergic dermatitis has not yet been investigated. Although we proved the involvement of PAR-2 in type IV allergic dermatitis in the present study and inhibition of all of these PAR-2 activation-related effects may contribute to the marked suppression of CH in PAR-2^{-/-} mice, further experiments including the effect of PAR-2 deficiency on both induction and effector phases of CH will be needed to clarify the precise mechanism.

PAR-2 deficiency inhibited ear swelling in both PC- and Ox-induced CH at every time point, and significant suppression was found at 24 h in PC-induced CH and at 24 and 48 h in Ox-induced CH. However, a moderate increase in ear thickness was observed in PAR-2^{-/-} mice at 48 h in both PC- and Ox-induced CH. These results demonstrate that a pro-inflammatory mechanism other than PAR-2 activation may arise at 48 h and/or the inhibitory effect of PAR-2 deficiency on CH may not last until 48 h. In fact, the inhibitory effect of PAR-2 deficiency on neutrophil rolling was not sustained for a long period of time (27). Another experiment comparing the response at 24, 48, 72 and 96 h revealed a peak response at 48 h in both PC- and Ox-induced CH in PAR-2^{-/-} mice (data not shown), and the magnitude of the peak response at 48 h in PAR-2^{-/-} mice was calculated to be 38% (for PC) or 36% (for Ox) of that at 24 h in WT mice from the data in Table 1. Therefore, PAR-2 deficiency led to the reduced peak response to both PC and Ox in addition to the delayed onset of the response.

Although steroids are clinically used for the treatment of type IV allergic dermatitis, they are also reported to cause skin atrophy (49). From our results, it is anticipated that PAR-2 inhibitors will exert a beneficial effect against type IV allergic dermatitis because the PAR-2 knockout not only showed a strong prevention of hapten-induced CH but also exhibited no skin atrophy.

In summary, we suggest that PAR-2 plays a crucial role in type IV allergic dermatitis but not in type I allergic dermatitis.

Acknowledgments

This study was performed in a collaborative research with Scottish Biomedical (SB). The authors thank Drs. S. Hammond and M. McFarlane (SB) and Ms. C. Mitchell (Kowa Research Europe) for their sustained support. We also thank Drs. T. Doi and K. Abe and Mr. H. Kobayashi for their useful discussions and thank Misses T. Kanno, T. Okamura, N. Inoue and A. Nagato for their excellent technical assistance.

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