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Bronchial hyperreactivity, increased endotoxin lethality and melanocytic tumorigenesis in transgenic mice overexpressing platelet-activating factor receptor

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Although platelet-activating factor (PAF) has been shown to exert pleiotropic effects on isolated cells or tissues, controversy still exists as to whether it plays significant pathophysiological roles *in vivo*. To answer this question, we established transgenic mice overexpressing a guinea-pig PAF receptor (PAFR). The transgenic mice showed a bronchial hyperreactivity to methacholine and an increased mortality when exposed to bacterial endotoxin. An aberrant melanogenesis and proliferative abnormalities in the skin were also observed in the transgenic mice, some of which spontaneously bore melanocytic tumors in the dermis after aging. Thus, PAFR transgenic mice proved to be a useful model for studying the basic pathophysiology of bronchial asthma and endotoxin-induced death, and screening of therapeutics for these disorders. Furthermore, our findings provide new insights regarding the role of PAF in the morphogenesis of dermal tissues as well as the mitogenic activity of PAF and PAFR *in vivo*.

Keywords: β-actin promoter/hyperplasia/lipopolysaccharide/melanocytic tumor/methacholine

Introduction

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a phospholipid mediator with pleiotropic and potent biological effects on a variety of cells and tissues (Hanahan, 1986; Prescott *et al.*, 1990; Chao and Olson, 1993; Izumi and Shimizu, 1995). Activation of peripheral leukocytes by PAF is thought to be a key step in the pathogenesis of many allergic and inflammatory disorders. Several lines of evidence have also suggested that PAF has various pathophysiological effects on cardiovascular, respiratory and gastrointestinal systems (Stewart and Delbridge, 1993). Moreover, PAF is considered to play physiological roles in the reproductive (O'Neill, 1992; Pike *et al.*, 1992; Toyoshima *et al.*, 1995) and central nervous systems (Bazan, 1990; Kato *et al.*, 1994). The involvement of PAF in these processes has been postulated either by examining effects of exogenously added PAF *in vivo* or in isolated tissue preparations, by determining the endogenous PAF level, or by examining the modulatory effects of a wide range of PAF antagonists.

PAF mediates its biological effects through activation of a G-protein-coupled seven transmembrane receptor (Honda *et al.*, 1991; Chao and Olson, 1993; Izumi and Shimizu, 1995). We and others have cloned PAF receptor (PAFR) cDNAs of guinea-pig (Honda *et al.*, 1991), human (Nakamura *et al.*, 1991; Ye *et al.*, 1991; Kunz *et al.*, 1992; Sugimoto *et al.*, 1992) and rat (Bito *et al.*, 1994) and a PAFR gene of mouse (Ishii *et al.*, 1996). Using these PAFR probes, ubiquitous expression of PAFR mRNA in tissues was demonstrated (Honda *et al.*, 1991; Ye *et al.*, 1991; Bito *et al.*, 1994; Ishii *et al.*, 1996). Notably, leukocytes accumulate enormous amounts of PAFR mRNA.

We found two species of human PAFR transcripts, transcript 1 and 2, with identical open reading frames (Mutoh *et al.*, 1993, 1994a,b, 1996). These are driven by individual promoters and are expressed tissue specifically; transcript 1 is ubiquitous and most abundant in leukocytes, while transcript 2 is located in heart, lung, spleen and kidney but not in leukocytes or brain. Such dual promoter systems facilitate the high expression of PAFR mRNA in leukocytes, while maintaining the expression at a lower level in other cells and tissues, under physiological conditions. Augmentation of the message level in other tissues is closely related to the pathogenesis of various disorders; PAFR mRNA significantly increased in the lung tissue of asthmatic patients (Shirasaki *et al.*, 1994). This report is of particular interest since deficiency of plasma PAF acetylhydrolase is associated with severe respiratory symptoms in asthmatic children (Miwa *et al.*, 1988; Stafforini *et al.*, 1996).

To elucidate the pathophysiological roles of PAF and PAFR *in vivo*, we attempted to establish transgenic mice ubiquitously overexpressing PAFR by selecting the β-actin promoter and cytomegalovirus (CMV) enhancer (Niwa *et al.*, 1991). Northern blot analysis demonstrated the expression of the transgene in heart, skeletal muscle, eye, skin, trachea and aorta. PAFR transgenic mice exhibited bronchial hyperreactivity to PAF or methacholine, and an increased lethality to bacterial endotoxin (lipopolysaccharide, LPS). Various unexpected phenotypes included an abnormal breeding pattern, and epidermal and dermal hyperthickening with dermal melanosis. In some aged transgenic mice with a pronounced histopathology in the skin, melanocytic tumors arose spontaneously in the dermis. Thus, these transgenic mice are a pertinent model of bronchial asthma and endotoxin-induced lethality. Furthermore, our findings provide important insights into the novel roles of PAF in cell proliferation and subsequent tumor formation in the skin.

Results

Establishment of PAFR transgenic mice

The guinea-pig PAFR cDNA was placed under the regulation of the chicken β-actin promoter and the CMV immediate early enhancer (Figure 1). A 4.1 kb *Bam*HI–*Bam*HI fragment of the transgene construct (Figure 1) was microinjected into the pronuclei of fertilized eggs. Of 87 offspring, four founders (F_0 mice) were identified by PCR screening; at 7–8 weeks old, two of them spontaneously developed necrosis in the hind legs or in the eyes. Though all founders were fertile, only one male founder ($F_0$55) transmitted the transgene through the germline. Subsequent analysis of tail DNA of $F_0$55 and its offspring transgenic mice by Southern blotting revealed that this line segregated into two sublines based on the copy number of integrated transgenes. This is probably due to two transgene integration sites in the chromosomes of $F_0$55 mouse. These two sublines contained ~50 and 100 copies of the transgene (data not shown), and were designated 55-L and 55-H, respectively. Northern blot analysis of RNAs extracted from heart and skeletal muscle of F_2 or F_3 mice confirmed the establishment of the two sublines (Figure 2A). When the expression of the transgenic PAFR mRNA was compared in the two groups, 55-H showed 5- to 10-fold higher expression than 55-L.

Northern blot analysis, using a guinea-pig-specific PAFR probe, of tissue RNAs from 55-H transgenic mice showed a restricted pattern of transgene expression: high levels of expression were seen in heart and skeletal muscle, medium levels in eye, skin, trachea and aorta, and barely detectable levels in neutrophils, brain, lung, liver, spleen, kidney, small intestine, uterus and testis (Figure 2B). Transgenic mRNAs were detected above 18S rRNA. In the control mice, hybridization signals were not observed (data not shown). The membrane was re-hybridized with a murine PAFR probe. Although we have reported the ubiquitous expression of murine PAFR mRNA (Ishii *et al.*, 1996), the murine PAFR mRNA was detected below 28S rRNA only in neutrophils under these conditions because of the low amount of total RNA (3 μg) loaded on the membrane (Figure 2B). The transgene expression pattern of 55-L transgenic mice was similar to that of 55-H (data not shown). Unless stated otherwise, 55-H transgenic mice (F_2–F_4) were used in the following studies, with their wild-type littermates as control.

To verify transgene overexpression in the heart, ligand binding assays were done on cardiac membranes by using a ^{3}H-labeled PAF antagonist, WEB 2086 (Casals-Stenzel, 1987). The membranes were prepared by homogenizing whole hearts from the transgenic and control mice. While membranes of control mice ($n = 5$) showed no binding activities under our assay conditions, those of the transgenic mice showed high PAFR densities (3.24 ± 0.73 pmol/mg protein, mean ± SEM, $n = 4$).

Anatomical and biochemical examinations

No gross morphological abnormalities were detected in three males each of the transgenic mice and their littermate control mice (23 or 35 weeks old) and were found to be normal except for the skin of the ear and tail of the transgenic mice (see below). Light microscopic evaluation of heart, skeletal muscle, thymus, spleen, bone marrow and mesenteric lymph nodes showed no abnormalities. Measurement of body weight of 14- to 17-week-old mice revealed that the transgenic mice were ~20% lighter than their control littermates of either gender (data not shown).

The following parameters were all normal: erythrocyte, platelet and leukocyte counts, proportion and morphology of neutrophils, eosinophils, monocytes and lymphocytes, and serum levels of aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, creatine phosphokinase, alkaline phosphatase, total cholesterol and creatinine (data not shown).

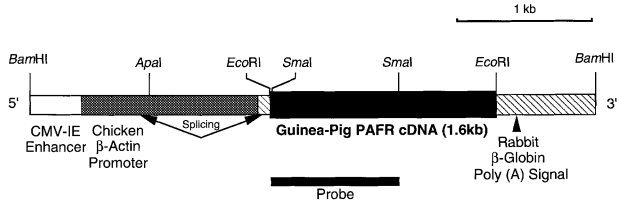

Fig. 1. Guinea-pig PAFR transgene expression construct. Open, dotted, hatched and closed boxes represent CMV immediate early (CMV-IE) enhancer, chicken β-actin gene, rabbit β-globin gene and guinea-pig PAFR cDNA, respectively. A polyadenylation [poly(A)] signal is indicated by an arrow head. Splice sites are indicated by arrows. The hybridization probe (a *Sma*I–*Sma*I fragment of the guinea-pig PAFR cDNA) used for Southern and Northern blot analysis is also shown.

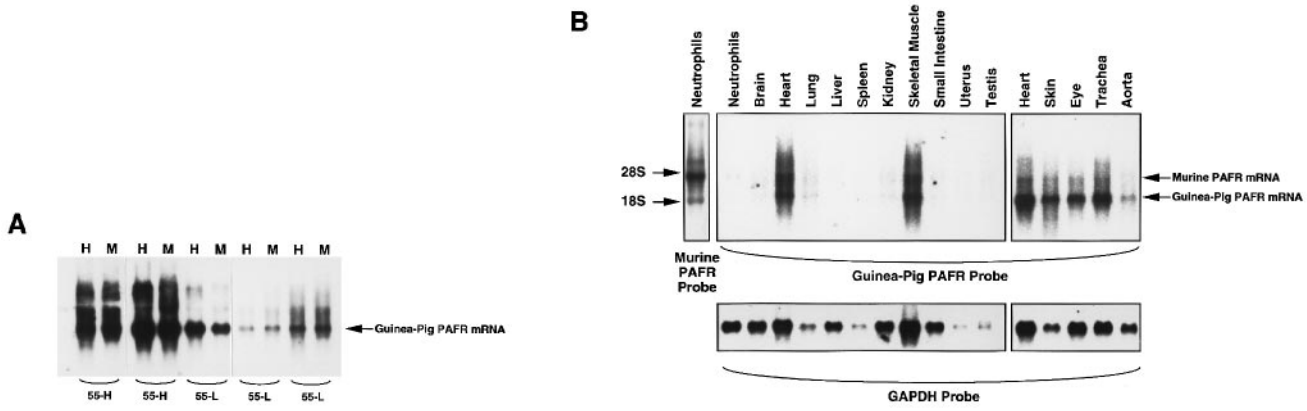

Fig. 2. Northern blot analyses. (**A**) Expression of the PAFR transgene in heart (H) and skeletal muscle (M) of two 55-H and three 55-L transgenic mice. Total RNA (5 μg/lane) was hybridized with the guinea-pig PAFR cDNA probe shown in Figure 1. (**B**) Expression of the transgene in various tissues of 55-H transgenic mice. Total RNA (3 μg/lane) was hybridized with either the guinea-pig PAFR cDNA probe, the murine PAFR genomic DNA probe or the human GAPDH cDNA probe.

Reduced fertility and gender bias of offspring

The transgenic heterozygous females produced rare transgenic progenies: out of 20 pups, only two were transgenic. Thereafter, progeny were generated by mating heterozygous F_1 to F_3 males to wild-type (BDF_1) females. However, the proportion of transgenic progeny to control progeny, 1:2.3 (Table I), was significantly lower than that expected from Mendelian distribution (1:1). In addition, the transgenic ratio among females (1:3.0) was significantly lower than the transgenic ratio among males (1:1.8) (Table I). Each generation of transgenic mice showed similar results. Post-natal deaths were rarely observed.

The fertility of 55-L transgenic male was normal with regard to the sex (data not shown) and the transgenic ratio of the progeny (Table II). The mean litter size was 8.2 ± 0.9 (mean ± SEM, $n = 10$). This value is significantly larger ($P < 0.005$; two-tailed, unpaired t test) than that observed with the 55-H males that produced progeny under the same conditions [5.5 ± 0.4 (mean ± SEM, $n = 24$)] (Table II). The reduction of the litter size of the 55-H line roughly corresponded to the decrease of the transgenic ratio.

Table I. Gender and genotype of progeny of 55-H transgenic mice

	Transgenic	Control
Female	66[a,b]	197
Male	103[c]	186
Total	169	383

The heterozygous transgenic males (F_1–F_3) of the 55-H line were mated to wild-type (BDF_1) females. Data shown are the numbers of progeny of each genotype and gender.
P values derived from the χ^2 test: [a]$P < 0.001$ versus the female control group, [b]$P < 0.001$ versus the male transgenic group, [c]$P < 0.005$ versus the male control group.

Table II. Mean litter sizes of PAFR transgenic mice

Subline	No. in litter	No. of progeny			Mean litter size ± SEM
		Transgenic	Control	Total	
55-H	24	32	99	131	5.5 ± 0.4[a]
55-L	10	40	42	82	8.2 ± 0.9

The heterozygous transgenic males (F_1 or F_2) of either 55-H or 55-L line were mated to wild-type (BDF_1) females.
[a]$P < 0.01$ versus 55-L group, as determined by two-tailed, unpaired t test.

Enhanced sensitivity to PAF

First, we assessed the bronchopulmonary effects of PAF by measuring lung resistance in anesthetized, tracheostomized mice. Figure 3A shows typical tracings of tracheal pressure before and after the i.v. administration of 10 μg/kg PAF to the transgenic mouse and to the littermate control mouse. While the control mouse did not respond to PAF, the transgenic mouse showed a marked bronchoconstriction. The response occurred immediately after the administration, peaked in 7 min and continued for >10 min (data not shown). The peak total lung resistance of the transgenic mice was remarkably higher than that of the control mice (2.33 ± 0.40 versus 0.49 ± 0.02 cmH_2O/ml/s, mean ± SEM, $n = 8$ each, $P < 0.001$; Mann–Whitney U test). Baseline values of the total lung resistance did not differ significantly between transgenic and control mice. A prominent bronchoconstriction and infiltration of blood cells in parenchymal tissues were observed in the transgenic mice (Figure 3B), whereas these changes were not

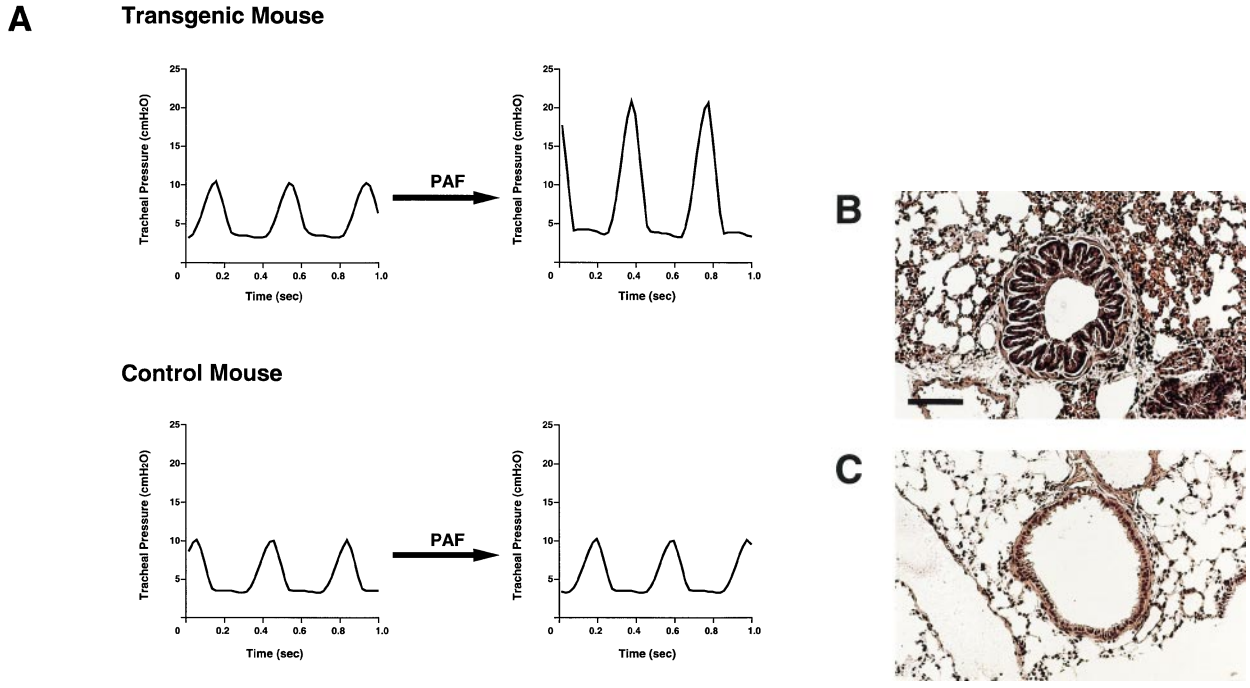

Fig. 3. Bronchopulmonary effects of PAF. (**A**) Tracheal pressure tracings before and after PAF administration. Mechanically ventilated mice were intravenously administered with PAF (10 μg/kg) and the tracheal pressure changes were monitored continuously. Results shown are arbitrary tracings for 1 s before and after the PAF administration and are representative of the results of eight mice in each group. Typical results of light microscopic analyses of the lungs of (**B**) PAFR transgenic mouse and (**C**) control mouse. Note the contracted airway smooth muscle, wrinkled epithelium and blood cell infiltration in alveoli of the transgenic mouse. Hematoxylin and eosin staining. Scale bar (shown in B), 100 μm.

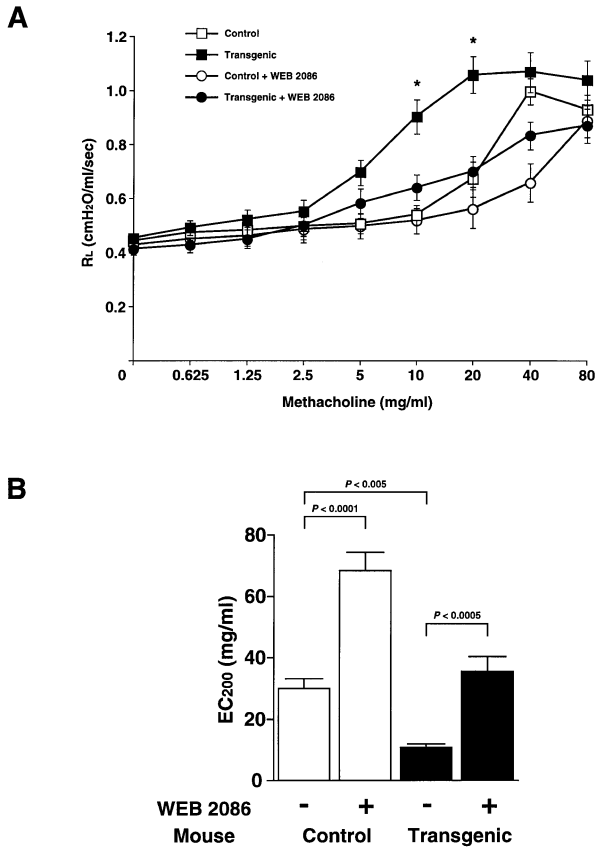

Fig. 4. Effects of the PAFR transgene and PAF antagonist on reactivity to methacholine. (**A**) Dose–response curves to methacholine. PAFR transgenic mice or their littermate control mice were administered cumulatively with methacholine aerosols via trachea. The total lung resistance at each dose is represented as R_L. WEB 2086 (1.0 mg/kg) was injected i.v. 2 min prior to methacholine. Each group consisted of six mice. Data points represent the mean ± SEM. $*P < 0.002$ versus each other group, as determined by analysis of variance (ANOVA) with Fisher's PLSD test for pair-wise comparisons. (**B**) Evaluated EC_{200} values by the dose–response curves. Statistical significance was determined by ANOVA with Fisher's PLSD test for pair-wise comparisons.

observed in the control mice (Figure 3C). The vehicle of PAF had little or no effect in either group.

Increased bronchopulmonary response to methacholine

After nebulized methacholine was administered to mice through the trachea, the total lung resistance was calculated. While high doses of methacholine elicited a substantial bronchoconstriction in the control mice, resultant methacholine dose–response curves (Figure 4A) revealed a significant bronchial hyperreactivity in PAFR transgenic mice. On the basis of the dose–response curves, we evaluated the EC_{200}, the dose required to obtain a 100% increase in the total lung resistance of the baseline. The EC_{200} of the transgenic mice was significantly smaller than that of the littermate control mice (Figure 4B), suggesting that the airways of transgenic animals were more sensitive to methacholine than those of the controls. The hyperreactivity to methacholine was practically reversed by the prior i.v. administration of a PAF antagonist, WEB 2086 (Figure 4). Even in the control mice, the airway response to methacholine was reduced by the PAF antagonist (Figure 4).

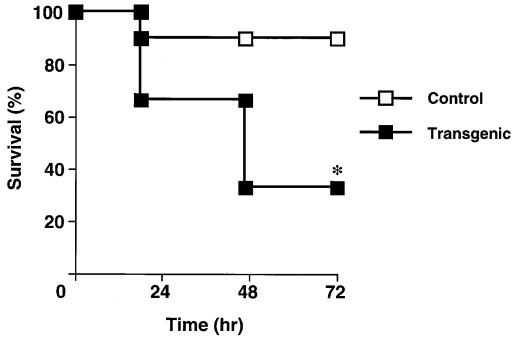

Fig. 5. Survival curves of mice given bacterial endotoxin. PAFR transgenic females ($n = 9$) or their littermate control females ($n = 10$) were injected i.v. with endotoxin (12.5 mg/kg) and survival was monitored for 3 days. $*P < 0.02$ versus control mice, as determined by Fisher's exact test.

Table III. Protection by WEB 2086 from bacterial endotoxin-induced death of the transgenic mice

	Endotoxin	Endotoxin + WEB 2086[a]
Dead	4	0
Alive	1	4

Survival was monitored for 3 days after the i.v. injection of endotoxin. Numbers of mice are shown.
[a]$P < 0.05$ versus the endotoxin group, as determined by Fisher's exact test.

Hypersensitivity to endotoxin

When we gave *Escherichia coli* endotoxin (10.0 mg/kg or 12.5 mg/kg i.v.) to the transgenic mice and the littermate control mice, all mice showed clear symptoms of murine endotoxic shock: decreased motor activities, ruffled fur and diarrhea. With a dose of 12.5 mg/kg, the mortality of the transgenic mice was significantly higher ($P < 0.02$; Fisher's exact test), as compared with that of the control mice (Figure 5): 10% (1/10) of the control mice died within 72 h, whereas 66.7% (6/9) of the transgenic mice died. With a dose of 10.0 mg/kg, there was a trend toward increased sensitivity to the bacterial endotoxin: a mortality of 50% (5/10) for the transgenic mice and 20% (2/10) mortality for the control littermates. Simultaneous injection of WEB 2086 (10 mg/kg) with endotoxin (15 mg/kg) afforded good protection of the transgenic mice from endotoxin-induced death (Table III).

Hyperplasias of various cutaneous cells and melanocytic tumor formation

The transgenic mice had remarkable black patches on the skin of the ear and the tail, irrespective of coat color (Figure 6A); this phenotype was more noticeable in females and was also present in the 55-L transgenic line. Skin pigmentation was appreciable at ~3–4 weeks old, and increased with age (data not shown). The areas with severe pigmentation were thicker than normal, though the degree of hyperpigmentation differed between individuals. When ear sections of the control mice were stained with hematoxylin and eosin, pigment-containing cells were observed occasionally in the dermis. The epidermis of the

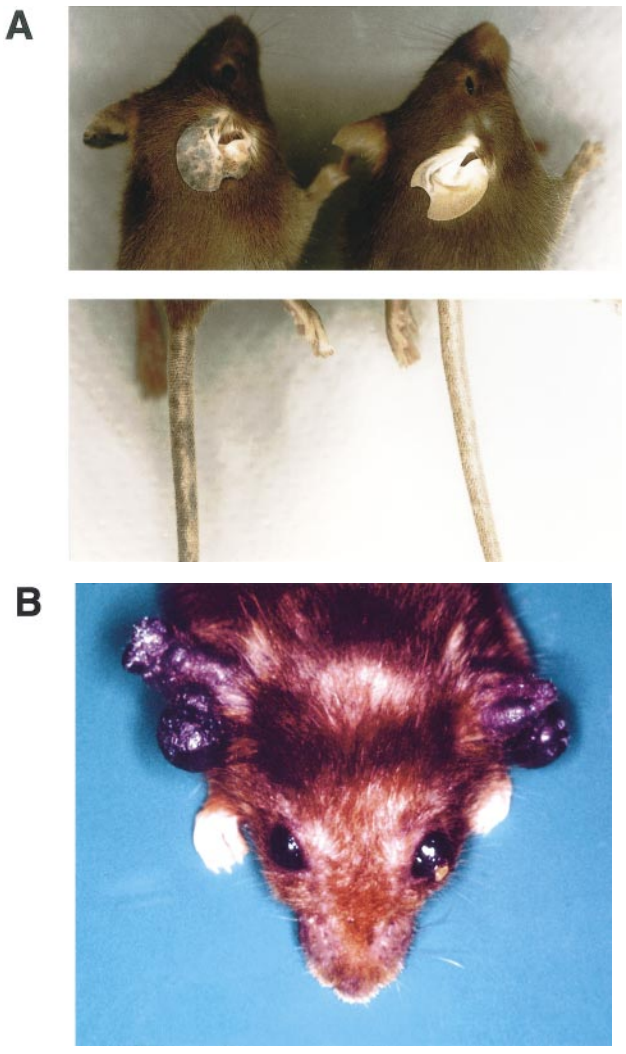

Fig. 6. Gross features of PAFR transgenic mice. (**A**) Ears (upper) and tails (lower) of a 46-week-old transgenic female (left) and a 44-week-old control female (right) with a similar coat color. Note the black patches on the ear and tail of the transgenic mouse. (**B**) A severely pigmented transgenic mouse (28 months old) with a melanocytic tumor. Several nodules of the tumor are clearly visible.

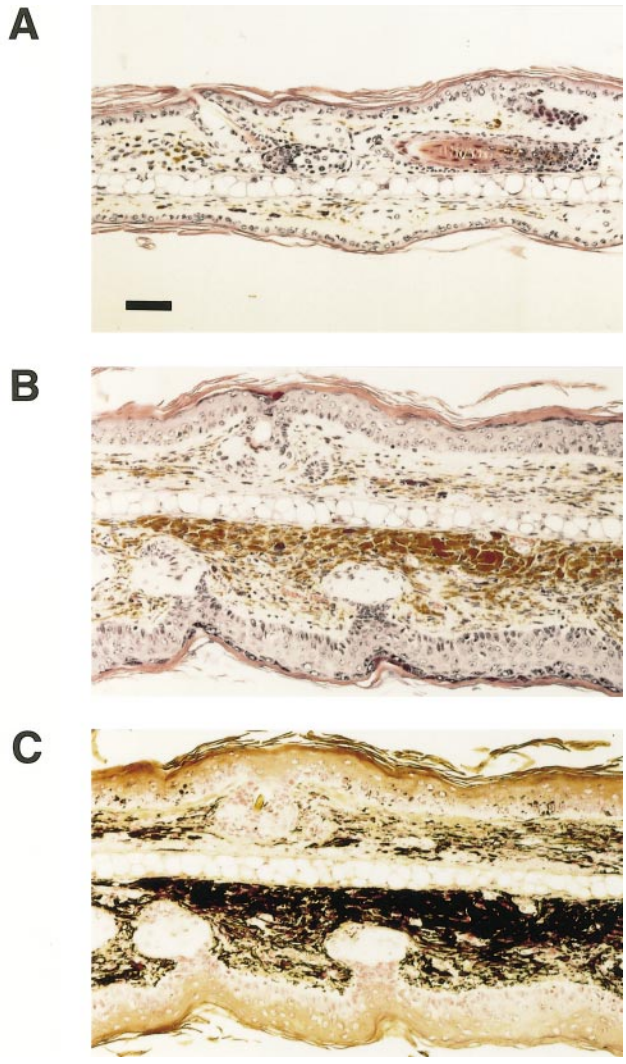

Fig. 7. Histopathology of PAFR transgenic mice. (**A**) The ear section of the control mouse shown in Figure 6A. Hematoxylin and eosin staining. (**B**) The pigmented skin of the PAFR transgenic ear shown in Figure 6A. Considerable numbers of pigment-containing cells and fibroblasts are present in the dermis. Epidermal hyperthickening (acanthosis) is also observed. Hematoxylin and eosin staining. (**C**) Fontana–Masson staining of the same section as (B). The pigment-containing cells are mostly positive. Scale bar (shown in A), 50 μm.

control mice consisted of one or two cell layers (Figure 7A). In contrast, the transgenic mice had a large number of pigment-containing cells in the dermis, where hyperplasia of fibroblasts was also observed. In the epidermis, acanthosis (hyperplasia of the epidermis) was evident (Figure 7B). Due to these hyperplasias, the pinna was markedly thickened (compare Figure 7A and B). There were trends for nests of the pigment-containing cells to be present beneath the acanthotic epidermis, and for severe pigmentation to occur at the lateral and the dorsal region of the pinna. To identify the nature of the pigment, these sections were subjected to Fontana–Masson staining, a diagnostic staining for melanin. Since most colored cells were strongly stained (Figure 7C), the pigmentation proved to be due to the increased number of melanin-containing cells. Both hyperthickening of the skin and melanosis in the dermis were also present in the tail (data not shown). In the dermis of the transgenic ear, the number of mast cells increased (data not shown), consistent with a persistent scratching behavior of the transgenic mice.

Electron microscopic examination of the melanin-containing cells in the dermis revealed that they were melanocytes, but not melanophages (Figures 8A and C). The dermal melanocytes of both the control and transgenic mice contained many melanosomes. The majority of them were in mature stage IV (Figures 8B and D). However, in the transgenic mice, melanosomes in developmental stage II and III were frequently observed, indicating the active melanogenesis in PAFR transgenic mice. The melanocytes of transgenic mice were characterized further with enlarged perikarya with well-developed endoplasmic reticula, numerous mitochondria and hypertrophic Golgi complexes (Figure 8D).

In three out of 14 aged female transgenic mice (>15 months old), melanocytic tumors arose spontaneously in the ear (Figure 6B). One of them also developed a tumor in the tail (data not shown). Histopathological examination revealed that the tumors were located in the dermis (data not shown), suggesting that the tumors derived from

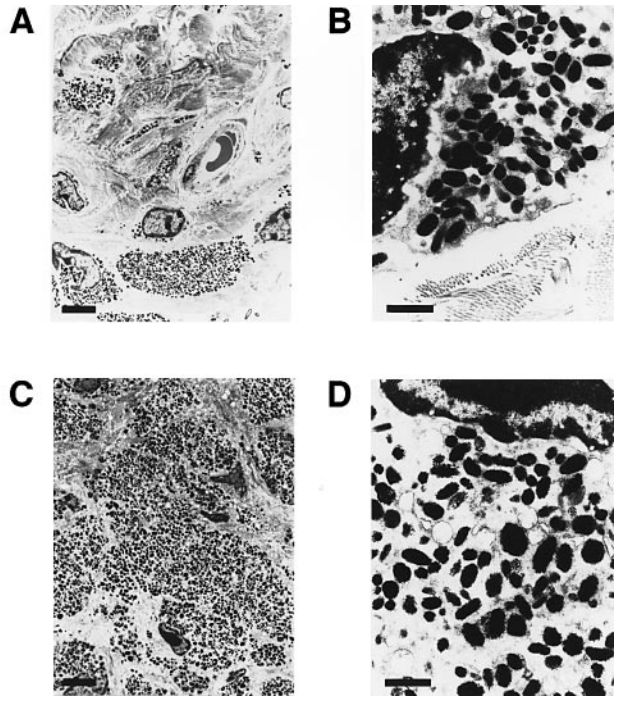

Fig. 8. Electron micrographs of melanocytes in the ear. (**A**) The dermis of an 11-month-old control female. Scale bar, 5 μm. (**B**) High-power view of (A). Scale bar, 1 μm. (**C**) The severely pigmented dermis of a 13-month-old PAFR transgenic female. Scale bar, 5 μm. (**D**) High-power view of (C). A larger number of premature melanosomes are present than in (B). Melanin contents are low and melanized lamellae are visible. Scale bar, 1 μm.

the hyperplastic melanocytes. The tumor cells did not metastasize after several months. The control mice showed no such tendency to tumor formation.

In summary, multiple abnormalities were observed in the transgenic mice overexpressing PAFR in various tissues, including breeding abnormality, hyperresponsiveness to methacholine or bacterial endotoxin, and aberrant pigmentation and subsequent tumor formation.

Discussion

Highly deviated expression of the transgene and abnormal breeding

Ubiquitous expression of endogenous PAFR mRNA has been reported in all species investigated (Honda *et al.*, 1991; Ye *et al.*, 1991; Bito *et al.*, 1994; Ishii *et al.*, 1996). In an attempt to overexpress PAFR mRNA ubiquitously in transgenic mice, we used the chicken β-actin promoter and CMV enhancer (Niwa *et al.*, 1991), which is known to direct a widespread gene expression (Shimada *et al.*, 1993; Honda *et al.*, 1995). However, the transgene expression was not ubiquitous (Figure 2B). The restricted expression pattern suggests that the ubiquitous overexpression of PAFR may be embryonically lethal to mice. In fact, PAF has been shown to be involved in ovulation, implantation, fetal lung maturation and the initiation and maintenance of parturition (O'Neill, 1992; Pike *et al.*, 1992; Toyoshima *et al.*, 1995). Moreover, failure in the regulation of PAF metabolism has been suggested to elicit premature delivery and infertility (Narahara *et al.*, 1995; Toyoshima *et al.*, 1995). Expression of the PAFR transgene in the early embryonic stage can be deduced from studies of transgenic mice, using the same expression unit (Ikematsu *et al.*, 1993; Araki *et al.*, 1995). A fraction of 55-H transgenic mice, despite the restricted pattern of transgene expression, may not survive gestation (Table II). Thus, even a limited overexpression may still be lethal to some transgenic embryos. The reason for this partial penetrance remains elusive, although the presence of modifying genes that affect the level of the PAFR transgene expression is plausible, as suggested in studies on exencephaly of p53-deficient embryos (Sah *et al.*, 1995). The female transgenic embryos seem to be more difficult to develop than male transgenic embryos (Table I), possibly because the transgene might be expressed more effectively in the female embryos. Alternatively, there might be a sex-dependent effect of PAFR signaling on the embryo. Fertility of the transgenic female is affected by the transgene to a greater extent than that of transgenic males. A higher expression of PAFR in the female reproductive system might easily cause the disorders in fertilization.

Enhanced sensitivity to PAF

We found abundant expression of PAFR protein in the heart of the transgenic mice. These mice had remarkable bradycardia and arrhythmia immediately after the administration of PAF in doses that elicited a moderate tachycardia in the control mice (unpublished data). The airways of transgenic mice were capable of responding to PAF (Figure 3). Taken together, the overexpressed guinea-pig PAFR protein is capable of enhancing the susceptibility to PAF *in vivo*, with successful coupling to murine intracellular effector systems.

Due to the airway constriction shown in Figure 3B, the total lung resistance seemed to be elevated (Figure 3A). High level transgenic mRNA was seen in the trachea, but the messenger was hardly detectable in the lung (Figure 2B). This may reflect the fact that the proportion of airway smooth muscle cells is likely to be lower in the lung tissue than that in the trachea. Alternatively, intravenously administered PAF may stimulate production of other mediators which in turn cause bronchoconstriction.

Bronchial hyperreactivity

Reversible bronchial hyperreactivity underlies the pathophysiology of asthma, yet the precise mediators of the response are unclear. PAF administration to both experimental animals (Mazzoni *et al.*, 1985; Chung *et al.*, 1986) and humans (Cuss *et al.*, 1986) induces a bronchial hyperreactivity that resembles the pathology seen in asthmatics. Though a number of reports have referred to the mechanisms of PAF-induced bronchial hyperreactivity, much remains to be elucidated (Chung and Barnes, 1991; Page, 1992). Since the transgenic mice showed a heightened airway responsiveness to PAF (Figure 3), response of the airway to methacholine, a spasmogen widely used to diagnose bronchial hyperreactivity, was studied. Airway contractile tissues in mice respond to intravenously administered methacholine (Martin *et al.*, 1988).

Airways of the transgenic mice showed hyperreactivity to methacholine aerosols (Figure 4). Because a specific PAF antagonist, WEB 2086, blocked it, this phenomenon relates directly to the interaction of PAF and PAFR. Additionally, even in the control mice, this PAF antagonist

decreased the reactivity to methacholine (Figure 4). In both transgenic and control mice, endogenous PAF may be the physiological regulator of airway contraction by muscarinic stimulation. However, endogenous PAF does not seem to have any effects on resting tone of the airways, because no significant difference was observed in baseline values of the total lung resistance between the transgenic and control mice (Figure 4A).

Eosinophil products have been postulated to contribute to bronchial hyperreactivity through damage to the airway epithelium, leaving underlying smooth muscle more susceptible to non-specific contractile mediators (Gleich *et al.*, 1988). PAF-induced bronchial hyperreactivity has been reported to be associated with eosinophil infiltration into the airway (Coyle *et al.*, 1988). PAFR transgenic mice, under resting conditions, showed no evidence of airway eosinophil infiltration (unpublished data). Also, the onset and resolution of airway contractile responses to methacholine were rapid. Thus, it seems unlikely that eosinophils could mediate the hyperreactivity seen in the transgenic mice. As Page (1992) suggested, PAF is probably capable of causing acute bronchial hyperreactivity, without inflammation. The uncoupling of airway eosinophilia and bronchial hyperreactivity has been demonstrated by other *in vivo* systems; pre-treatment of guinea-pig with a PAF antagonist abrogated the antigen-induced airway hyperreactivity to acetylcholine with no effect on the eosinophilia (Ishida *et al.*, 1990).

It remains to be clarified whether methacholine induces synthesis and release of PAF, to which the transgenic airways respond remarkably, or whether the overexpression of PAFR increases the muscarinic receptor density and/or function. In any event, the PAFR overexpression *per se* seems to be sufficient to mimic the symptoms elicited by administration of PAF. Indeed, enhancement of myocardial function was evoked by overexpressing β_2-adrenergic receptor in transgenic mice (Milano *et al.*, 1994). Our data may, at least in part, account for the airway hyperreactivity of asthmatics who show an increase in PAFR mRNA expression level in the lung (Shirasaki *et al.*, 1994).

Endotoxin-induced death

Severe Gram-negative bacterial infection can lead to development of endotoxic shock, a state characterized by hypotension, multiorgan failure and, potentially, death (Bone, 1991). This syndrome is secondary to the effects of endotoxin, the LPS-containing portion of the bacterial cell wall. Because pharmacological studies have suggested that PAF is one of the potent mediators of endotoxic shock (Casals-Stenzel, 1987; Imanishi *et al.*, 1991; Terashita *et al.*, 1992), we determined whether the transgenic mice would be more sensitive to bacterial endotoxin. This possibility was analyzed by monitoring the mortality of mice after the i.v. injection of endotoxin. Our results indicate that overexpression of PAFR causes an increased sensitivity to endotoxin (Figure 5 and Table III). Although molecular mechanisms underlying the hypersensitivity to endotoxin are unclear, endotoxin is known to increase the serum level of PAF (Doebber *et al.*, 1985; Chang *et al.*, 1987), which may cause dysfunction of the target organs such as the heart. Alternatively, the released PAF may produce lethal amounts of other vasoactive mediators such as eicosanoids (Chao and Olson, 1993). The direct activation of PAFR by endotoxin is another possibility, as we previously demonstrated (Nakamura *et al.*, 1992; Waga *et al.*, 1993).

Cutaneous proliferative disorders

We detected a small number of melanocytes in the dermis of ear and tail of the normal mice (Figure 7A), indicating that the hyperplastic melanocytes were not ectopic. Since we could not observe melanophages in the hyperpigmented area, the melanocytes seem to proliferate abnormally without inflammation. The coat color of the transgenic mice was not altered substantially (for example, see Figure 6A). This is because the increase in melanocytes was restricted only to the dermis. Due to the lack of nerve cell-like dendrites, it is unlikely that the hyperplastic melanocytes discharge melanosomes into surrounding cells (Figure 8B). Melanocytes with such a morphology were also observed in the dermis of the pigmented area of nevus Ota, and were different from melanocytes in the hair follicles and the epidermis.

There are two kinds of animal models with melanosis in the dermis and subsequent tumor formation. In transgenic mice with the *ret* oncogene fused to the metallothionein-I promoter as a transgene (Iwamoto *et al.*, 1991), severe systemic melanosis was present at birth. At the age of several months, the transgenics developed melanocytic tumors that grew slowly and did not metastasize. On the other hand, malignant melanoma spontaneously arose in another transgenic mouse expressing the SV40 early region oncogenic sequences under the control of the tyrosinase promoter (Bradl *et al.*, 1991; Klein-Szanto *et al.*, 1991). In PAFR transgenic mice, aberrant melanogenesis was significant several weeks after birth and was accompanied by acanthosis. Furthermore, the melanocytic tumor did not metastasize. Therefore, our animal model is distinguishable from the above transgenic lines, suggesting a different mechanism of tumor formation.

PAFRs were identified on human keratinocytes (Travers *et al.*, 1995), and production of PAF by human fibroblasts (Michel *et al.*, 1988) and keratinocytes (Michel *et al.*, 1989) was demonstrated. Additionally, it has been suggested that PAF contributes to the development and maintenance of cutaneous inflammation and the pathogenesis of inflammatory dermatoses (Michel and Dubertret, 1992). However, there are, as far as we know, no reports regarding PAF-related hyperplasias of melanocytes, keratinocytes and fibroblasts, or tumor formation. Therefore, the proliferative effects of PAFR on the cutaneous cells are of particular interest. PAF has been reported to up-regulate the proliferation of lymphocytes (Behrens and Goodwin, 1990; Leprince *et al.*, 1991). We observed that PAFR stimulated mitogen-activated protein kinase (Honda *et al.*, 1994) which plays a key role in transducing extracellular mitogenic signals. Additionally, it is reported that repeated administration of arachidonic acid to the skin of mouse ears results in epidermal hyperplasia (Bouclier *et al.*, 1989). This report is consistent with our findings because arachidonic acid is known to be released in response to PAF stimulation (Chao and Olson, 1993).

Some types of human cutaneous cells release various growth factors regulating development and proliferation of the other types of cutaneous cells. Keratinocytes produce

growth factors for melanocytes, such as basic fibroblast growth factor (Halaban *et al.*, 1988) and endothelin-1 (Imokawa *et al.*, 1995). Fibroblasts and keratinocytes are reported to release stem cell factor, the ligand for the product of the c-*kit* proto-oncogene (Grabbe *et al.*, 1994). This factor can stimulate mast cell development, proliferation and mediator release, as well as melanocyte proliferation and melanin production (Grabbe *et al.*, 1994). In fact, there was an increased number of mast cells in the dermis of the transgenic mice, which may explain the skin itching observed in these mice. Although we have not examined which cells in the skin of our transgenic mice overexpress PAFR, PAFR overexpression may cause abnormal production of some growth factors. If so, then hyperplasias of several types of cutaneous cells may be the result of abnormal mitotic responses not only to PAF but also to an enhanced level of growth factors.

Conclusions

PAFR overexpression in the transgenic mice led to a hypersensitivity, not only to PAF but also to other ligands such as methacholine or bacterial endotoxin. Thus, these transgenic mice are a useful model for studying the basic pathophysiology of bronchial asthma and endotoxin-induced death, and screening of therapeutics for these disorders. The overexpression of PAFR led to pathologies in the skin and in the reproductive system. These findings provide new and important insights regarding morphogenesis of dermal–epidermal tissues by cell–cell interaction, and the underlying mechanisms of gender bias and infertility.

Materials and methods

Reagents

Materials and chemicals were obtained from the following sources. [α-^{32}P]dCTP (111 TBq/mmol) and transfer membrane (Hybond-N+) were from Amersham Corp., Tokyo, Japan; [^{3}H]WEB 2086 (521.7 GBq/mmol), from Du Pont/NEN, Tokyo; restriction enzymes and DNA-modifying enzymes, from Takara, Kyoto, Japan; agaroses (Sea Kem, Nu Sieve and Sea Plaque), from FMC, Rockland, ME; PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), from Cayman Chemical, Ann Arbor, MI; bovine serum albumin (BSA), methacholine (acetyl-β-methylcholine chloride) and endotoxin (LPS, *E.coli* serotype O127:B8, Code No. L-3129), from Sigma Chemical Co., St Louis, MO; and pyrogen-free saline, from Otsuka Pharmaceuticals, Tokyo, Japan. Unlabeled WEB 2086 was a generous gift from Boehringer Ingelheim, Ingelheim, Germany. Other materials and reagents were of analytical grade. PAF was dissolved in saline containing 0.25% BSA just before use. Methacholine was dissolved in saline. WEB 2086 was dissolved in saline by sonication at a concentration of 0.5 mg/ml. Endotoxin was suspended in saline.

Plasmid construction

The transgene was constructed as follows. A 1653 bp *Nco*I–*Nco*I fragment containing the coding region of guinea-pig PAFR was excised from Zp74 (Honda *et al.*, 1991). Cohesive ends were blunted with Klenow fragment and ligated to *Eco*RI linkers. The resulting fragment was introduced into the *Eco*RI site of pCXN2, a derivative of pCAGGS (Niwa *et al.*, 1991). The final construct consists of the CMV immediate early enhancer, 5′-flanking sequence, exon 1 containing 5′-untranslated sequence and the first intron of the chicken β-actin gene, the rabbit β-globin splice acceptor site followed by the guinea-pig PAFR sequence, and the rabbit β-globin polyadenylation site. To remove vector sequences prior to microinjection, cesium chloride-purified plasmid was digested with *Bam*HI, and the 4.1 kb *Bam*HI–*Bam*HI fragment was separated by agarose gel electrophoresis, extracted from the gel using GELase (Epicentre Technologies, Madison, WI) and purified with QIAGEN-tip (Qiagen Inc., Chatsworth, CA). The purified *Bam*HI–*Bam*HI fragment was dissolved in sterile 1/2× phosphate-buffered saline at a concentration of 5 μg/ml.

Creation and identification of transgenic mice

Methods utilized in the creation of transgenic mice have been described previously (Hogan *et al.*, 1986). The purified DNA fragment was injected into the pronuclei of fertilized eggs (BDF_2) from superovulated BDF_1 females (Charles River Japan Inc., Yokohama, Japan) mated with BDF_1 males. Offspring were weaned at 4 weeks old. For genotyping, genomic DNAs were isolated from tail biopsies, and were subjected to 30 cycles of PCR amplification (1 min at 94°C; 2 min at 55°C; 2 min at 72°C). The primers specific to the guinea-pig PAFR cDNA were forward 5′-ACCACACTCCTGTCAATC-3′ and reverse 5′-TCAGGATCAGGT-CATGAT-3′. The PCR product consisted of 290 bp. Mice were housed in an air-conditioned room, and fed standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) *ad libitum*. Male transgenic mice were mated to BDF_1 females to maintain the transgenic lines.

Southern blot analysis

Transgene copy numbers were estimated by Southern blot analysis. Genomic DNAs (1.2 μg) of transgenic mice were digested with *Apa*I to release the transgene, electrophoresed in a 0.7% agarose gel, and transferred to a Hybond-N+ membrane using 0.4 M NaOH according to Chomczynski (1992). As a probe, a 933 bp *Sma*I–*Sma*I fragment of the guinea-pig PAFR cDNA (Honda *et al.*, 1991) was labeled with [α-^{32}P]dCTP by a Megaprime DNA labeling system (Amersham Corp., Tokyo). Hybridization and wash were performed under the same conditions described previously (Ishii *et al.*, 1996). Hybridization intensities were compared with standards composed of control mouse genomic DNA containing variable amounts of the transgene.

Northern blot analysis

Total RNAs were isolated from transgenic and control mouse tissues as described previously (Ishii *et al.*, 1996). Quantitative and qualitative assessments of the total RNAs were determined spectrophotometrically and electrophoretically on 1% agarose gels, respectively. To determine tissue expression, total RNAs were separated by electrophoresis and transferred to a Hybond-N+ membrane as described previously (Ishii *et al.*, 1996). The membrane was hybridized with the guinea-pig cDNA probe and washed under the conditions described previously (Ishii *et al.*, 1996). The same membrane was dehybridized sequentially and hybridized with the *Eco*47III–*Spe*I fragment of a murine PAFR gene DNA probe (Ishii *et al.*, 1996) or a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Human G3PDH cDNA Probe, Clontech, Palo Alto, CA).

Radioligand binding assay

Crude myocardial membranes were prepared by homogenizing a whole heart in ice-cold buffer [25 mM Tris–HCl (pH 7.4), 5 mM EDTA, 0.25 M sucrose, 100 μM leupeptin, and 40 kIU/ml aprotinin]. Nuclei were sedimented at 800 *g* for 20 min. Membranes were sedimented at 100 000 *g* for 60 min and washed once in the binding buffer [25 mM HEPES-NaOH (pH 7.4), 10 mM $MgCl_2$, and 0.25 M sucrose] before resuspension in 500 μl of the buffer. Ligand binding assays were performed in duplicate using 100 μl of the membrane suspensions (1.56–2.83 mg protein/ml). The concentration of the PAFR ligand, [^{3}H]WEB 2086, was 50 nM, and the non-specific binding was determined in the presence of 20 μM unlabeled WEB 2086. Assays were done at 25°C for 60 min, then the mixtures were passed through GF/C glass filters (Whatman International Ltd., Maidstone, UK), which were then washed and counted in a liquid scintillator (Scintisol; Dojindo Laboratories, Kumamoto, Japan). Specific binding was normalized to membrane protein. Protein concentration was determined by the method of Bradford (1976) with BSA as a standard.

Total lung resistance

The transgenic mice and their littermate controls (male, 13–24 weeks old) were anesthetized with a mixture of ketamine and pentobarbital (25 mg/kg each) by intraperitoneal injection. A metal cannula (inner diameter = 1.0 mm) was inserted in the trachea of a tracheostomized mouse. The animal was ventilated mechanically (Model 683; Harvard Apparatus, South Natick, MA) at a frequency of 150 breaths/min with a tidal volume of 8 ml/kg and a positive end-expiratory pressure of 3 cmH_2O. The thorax was opened wide by means of a midline sternotomy and wide lateral incisions along the costal diaphragmatic margins. A paralytic agent (pancuronium bromide, 0.1 mg/kg) was administered intraperitoneally to eliminate spontaneous respirations. During the experi-

ments, oxygen gas was supplied continuously to the ventilatory system. The tracheal pressure and the tracheal flow were measured using a piezoresistive microtransducer (Endevco 8510B-2, San Juan Capistrano, CA) and a Fleisch pneumotachograph (No. 00000, Metabo SA, Lauzanne, Switzerland), respectively. From these measurements, the total lung resistance was calculated as described previously (Bates *et al.*, 1989). The resistance of the cannula was estimated to be 0.225 cmH_2O/ml/s, which was subtracted from all total lung resistance values.

After recording of a baseline tracheal pressure, the vehicle of PAF (0.25% BSA in saline) and subsequent PAF (10 μg/kg) were injected in a volume of 3.0 ml/kg via the jugular vein.

Aerosols were from an ultrasonic nebulizer (Ultra-Neb 100, DeVilbiss, Somerset, PA). After saline aerosol exposure for 1 min, a series of methacholine aerosols (0.625–80 mg/ml) were delivered cumulatively into the trachea for 1 min each. WEB 2086 (1.0 mg/kg) in a volume of 3.0 ml/kg was injected i.v. 2 min prior to methacholine administration.

Endotoxin-induced lethality

The transgenic mice and their littermate controls (unanesthetized female, 12–15 weeks old or 18–25 weeks old) were injected via the tail vein with bacterial endotoxin (10.0 or 12.5 mg/kg) in a volume of 10 ml/kg. Another group of the transgenic females (27–32 weeks old) was injected with either endotoxin alone (15 mg/kg) or endotoxin (15 mg/kg) + WEB 2086 (10 mg/kg) in the same manner. Survival was monitored for 3 days. There were no deaths after 3 days.

Histology

The lungs were removed intact 10 min after i.v. PAF administration (10 μg/kg), and frozen with liquid nitrogen under a constant pulmonary pressure of 4 cmH_2O. The frozen lungs were fixed in Carnoy solution [60% (v/v) ethyl alcohol, 30% chloroform and 10% acetic acid] at –70°C for 18 h, and midsagittal slices of the lungs were embedded in paraffin as described by Nagase *et al.* (1996). Sections of 4 μm thickness were stained with hematoxylin and eosin. The skin tissues were fixed in 20% (v/v) formalin and embedded in paraffin. Sections were subjected to either hematoxylin and eosin staining or Fontana–Masson staining. Sections for electron microscopy were prepared following fixation in 2% (v/v) glutaraldehyde, post-fixing in 1% (w/v) osmium tetroxide, embedding in epoxy resin and slicing to 60–90 nm in thickness. They were stained with lead citrate and uranium acetate, and viewed in a transmission electron microscope (JEM-1200EX, JEOL).

Statistics

Values were expressed as mean ± standard error of the mean (SEM). P values <0.05 were considered to have statistical significance.

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