Group III secreted phospholipase A₂ transgenic mice spontaneously develop inflammation

Hiroyasu SATO*†, Yoshitaka TAKETOMI*‡, Yuki ISOGAI*§, Seiko MASUDA*†, Tetsuyuki KOBAYASHI§, Kei YAMAMOTO* and Makoto MURAKAMI*†

*Biomembrane Signaling Project, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan; †Department of Health Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan; ‡Center for Biotechnology, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan; §Department of Biological Sciences, Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo 112-8610, Japan, and †PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho Kagawachi, Saitama 332-0012, Japan

INTRODUCTION

PLA₂ (phospholipase A₂) enzymes catalyse the hydrolysis of the sn-2 position of glycerophospholipids to give rise to fatty acids and lysophospholipids. They are subdivided into several groups: high-molecular-mass intracellular PLA₂s with a catalytic serine residue, including cPLA₂ (cytosolic PLA₂) and iPLA₂ (Ca²⁺-independent PLA₂) enzymes, and low-molecular-mass disulfide-rich Ca²⁺-dependent sPLA₂ (secretory PLA₂) family with a catalytic histidine residue. To date, ten catalytically active sPLA₂ enzymes have been identified in mammals (IB, IIA, IIC, IID, IIE, IIF, III, V, X and XIIA). On a structural basis, these enzymes exhibit unique tissue and cellular localizations and enzymatic properties, suggesting their distinct tissue-specific roles in various pathophysiologic events.

Because group IIA sPLA₂ (PLA2G2A; or synovial sPLA₂) is strongly induced during inflammatory conditions such as rheumatoid arthritis or sepsis and because sPLA₂s from snake secretory PLA₂ (secretory PLA₂) that is homologous with bee venom PLA₂ rather than with other mammalian sPLA₂s. In the present paper, we show that endogenous group III sPLA₂ (PLA2G3) is expressed in mouse skin and that Tg (transgenic) mice overexpressing human PLA2G3 spontaneously develop skin inflammation. Pla2g3-Tg mice over 9 months of age frequently developed dermatitis with hyperkeratosis, acanthosis, parakeratosis, erosion, ulcer and sebaceous gland hyperplasia. The dermatitis was accompanied by infiltration of neutrophils and macrophages and by elevated levels of pro-inflammatory cytokines, chemokines and prostaglandin E₂.

In addition, Pla2g3-Tg mice had increased lymph aggregates and mucus in the airway, lymphocytic sialadenitis, hepatic extramedullary haemopoiesis, splenomegaly with increased populations of granulocytes and monocytes/macrophages, and increased serum IgG₁. Collectively, these observations provide the first demonstration of spontaneous development of inflammation in mice with Tg overexpression of mammalian sPLA₂.

Key words: dermatitis, phospholipase A₂, phospholipid, prostaglandin, splenomegaly, transgenic mouse.

Abbreviations used: BRAK, breast- and kidney-expressed chemokine; CAG, chicken α-globin (β-globin) promoter; CAG, chicken β-globin; COX, cyclo-oxygenase; ESI, electrospray ionization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDC, histidine decarboxylase; HDL, high-density lipoprotein; IFN, interferon; IL, interleukin; LDL, low-density lipoprotein; LNL, Lox³-neomycin-resistance gene-Lox³; LPS, lipopolysaccharide; LTB₄, leukotriene B₄; MCP-1, monocyte chemoattractant protein-1; mCMP, mouse mast cell protease; mPGES, microsomal prostaglandin E synthase; MPO, myeloperoxidase; NK, natural killer; PAF, platelet-activating factor; PAS, periodic acid–Schiff; PC, phosphatidylcholine; PGE₂, prostaglandin E₂; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; sPLA₂, secretory PLA₂; PLA2G2A (etc.), sPLA₂ group IIA (etc.); RT, reverse transcription; Tg, transgenic; TNFα, tumour necrosis factor α; WT, wild-type.

1 To whom correspondence should be addressed (email murakami-mk@igakuken.or.jp).
in vivo [16]. In the present study, we have demonstrated another aspect of Pla2g3-Tg mice in that they spontaneously develop inflammation in multiple organs as they age. This mouse model will therefore be useful for analysing and understanding the molecular events in inflammation caused by increased expression of sPLA2.

MATERIALS AND METHODS

Animals

All mice were housed in climate-controlled (21 °C) specific pathogen-free facilities with a 12 h light/12 h dark cycle, with free access to standard laboratory food (Picolab mouse diet 20; Laboratory Diet) and water. All procedures involving animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Showa University, in accordance with the Standards Relating to the Care and Management of Experimental Animals in Japan. The generation of Pla2g3-Tg mice has been reported previously [16].

RT (reverse transcription)–PCR

Total RNA was extracted from mouse tissues with TRIzol® reagent (Invitrogen). For RT–PCR, first-strand cDNA synthesis was carried out using SuperScript III reverse transcriptase kit (Invitrogen). Total RNA (5 μg) was used in reactions primed with oligo(dT) (12–18-mer) primer (Invitrogen) to obtain cDNA. Then, 1 μl of the synthesized cDNA was used as the template for the mRNA-amplification reactions. The PCR amplification was performed on a GeneAmp9600 PCR system (PerkinElmer) with TaqMan® RT (reverse transcription)–PCR reagent (Invitrogen). For RT–PCR, first-strand cDNA synthesis with oligo(dT) (12–18-mer) primer (Invitrogen) to obtain cDNA. Total RNA (5 μg) was used in reactions primed with oligo(dT) (12–18-mer) primer (Invitrogen) to obtain cDNA. Then, 1 μl of the synthesized cDNA was used as the template for the mRNA-amplification reactions. The PCR amplification was performed on a GeneAmp9600 PCR system (PerkinElmer) with TaqMan® RT (reverse transcription)–PCR reagent (Invitrogen). For RT–PCR, first-strand cDNA synthesis was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Showa University, in accordance with the Standards Relating to the Care and Management of Experimental Animals in Japan. The generation of Pla2g3-Tg mice has been reported previously [16].

Quantitative RT–PCR

For quantitative RT–PCR, cDNA synthesis was performed using the high-capacity cDNA reverse transcription kit (Applied Biosystems). The reaction was carried out on an ABI Prism 7000 sequence detection system (Applied Biosystems) with TaqMan gene expression master mix (Applied Biosystems). Pre-designed TaqMan assays (Applied Biosystems) were used to determine gene expression: Mm00439636 for mouse CD68, Mm00478374 and Mm00478374 for mouse COX (cyclooxygenase)-1 and -2, respectively. Mm00447040 for mouse cPLA2-α, and Mm00452105 and Mm00460181 for mouse mPGES (microsomal prostaglandin E synthase)-1 and -2, respectively.

Histochemistry

Formalin-fixed tissues were embedded in paraffin, sectioned, mounted on glass slides, deparaffinized in xylene and rehydrated in ethanol with increasing concentrations of water. The cell type was identified from conventional haematoxylin and eosin staining. For PAS (periodic acid–Schiff) staining, tissue sections were deparaffinized, rehydrated and placed in 0.5 % periodic acid solution for 5 min. After washing in water, the sections were placed in Coleman’s Schiff Reagent for 15 min. Washed in water for 10 min and counterstained with haematoxylin solution for 15 min. After washing in water for 15 min, the sections were dehydrated and mounted with resinous medium.

Measurement of eicosanoids

The contents of PG_E2 (prostaglandin E2) and LTB4 (leukotriene B4) in mouse skins were quantified using PGE2 and LTB4 enzyme immunoassay kits (Cayman Chemicals).

Measurement of MPO (myeloperoxidase) activity

The tissue samples were homogenized in 50 mM potassium phosphate buffer (pH 6.0) containing 1 % (w/v) hexadecyltrimethylammonium bromide and centrifuged at 12,000 g for 15 min at 4 °C. An aliquot (30 μl) was added to 180 μl of the buffer containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005 % (v/v) hydrogen peroxidase (Sigma–Aldrich). After appropriate periods of incubation at 37 °C, the absorbance at 460 nm was measured. MPO activity was calculated using a standard curve generated with human MPO (Sigma), and values were normalized to protein concentration.

Determination of cell types in blood circulation

Blood was collected from mouse tail biopsy with 10 mM EDTA Blood was collected from mouse tail biopsy with 10 mM EDTA. Determination of cell types in blood circulation was performed using a blood cell analyser VetScan HM (Abaxis) to determine the numbers of lymphocytes, granulocytes, monocytes, erythrocytes and platelets, and the haemoglobin content and the haematocrit value.
Flow cytometry (FACS)
Suspensions of 10⁶ splenic cells were treated on ice with anti-
mouse CD16/CD32 (FcγRII/RII) monoclonal antibody (BD Biosciences) to block cell-surface Fcγ receptors. Then the cells were incubated with FITC-, phycoerythrin- or Alexa Fluor® 488-labelled monoclonal antibodies against mouse CD3, CD4, CD8, CD11b (Mac1), CD11c, CD21, CD23, CD45R, Gr-1, NK1.1, slgD and slgM (BD Biosciences). Flow cytometric analysis was carried out on a FACS Aria cell-sorting system (BD Biosciences).

ESI (electrospray ionization)–MS
Lungs of mice were lavaged with 1 ml of SET buffer (250 mM sucrose, 0.5 mM EDTA and 20 mM Tris/HCl, pH 7.4), and the resulting bronchoalveolar lavage fluid was used after centrifugation at 160 g for 10 min at 4 °C to remove the cells and tissue debris. Lipids were extracted from the bronchoalveolar lavage fluid using the method of Bligh and Dyer [16a]. PC with C₆₁₋₁₇-C₆₁₃ (diacyl) was added to the samples (1 nmol of internal standard per 1 mg of protein equivalents) as an internal standard. MS spectra were obtained on a Quattro Micro tandem mass spectrometer equipped with ESI, as described previously [11]. Lipid extracts were reconstituted in 2:1 chloroform/methanol (100–300 μmol of phosphorus/l), and 2 μl of the sample was injected per run. The samples were introduced by means of a flow injector into the ESI chamber at a flow rate of 4 μl/min in a solvent system of acetonitrile/methanol/water (2:3:1, by vol.) containing 0.1% (v/v) ammonium formate (pH 6.4). The mass spectrometer was operated in the positive-and negative-scan modes. The flow rate of the nitrogen drying gas was 12 l/min at 80 °C. The capillary and cone voltages were set at 3.7 kV and 30 V respectively, argon at (3–4)×10⁵ Torr (1 Torr = 133.3 Pa) was used as the collision gas, and a collision energy of 30–40 V was used for obtaining fragment ions for precursor ions.

Determination of serum immunoglobulin levels
Serum titres of IgM, IgG₁, IgG₂ and IgE were determined using a mouse IgX ELISA quantification kit (Bethyl Laboratories).

RESULTS

Pla2g3-Tg mice develop dermatitis
Pla2g3-Tg mice appeared normal at birth and showed no apparent abnormality up to 9 months of age under our housing conditions (except that the Tg mice had altered plasma lipoproteins, as described in [16]). Subsequently, however, we noted that about one-third of PLA2G3 mice, both male and female, experienced gradual hair loss on their facial, neck and dorsal skin, which subsequently (typically within 2–3 months) developed into focally alopecic lesions that were often filled with pus (Figure 1A). Behaviour suggestive of increasing itching eventually worsened the lesions further. Neither age-matched non-Tg WT (wild-type) mice nor Tg mice expressing LNL (LoxP–neomycin-resistance gene–LoxP)–PLA2G3 (i.e. mice harbouring the PLAG3 transgene in a silent state [16]) exhibited these skin abnormalities (Figure 1A), implying that the phenotype resulted from Tg overexpression of PLA2G3. Subsequent studies mostly focused on aged (9–12 months) PLA2G3 mice that developed dermatitis, with age-matched WT mice as controls.

Histological examination revealed many abnormalities in the skin of Pla2g3-Tg mice (Figure 1B, panels b–i) as compared with the normal appearance of the skin of WT mice (Figure 1B, panel a). Pla2g3-Tg mice (1-year-old) showed dense cellularity in the dermis with markedly increased numbers of haematoxylin-stained nuclei, which were mostly ascribed to infiltrating polymorphonuclear neutrophils and macrophages (Figure 1B, panels b–i). Pla2g3-Tg mice had thickened epidermis (acanthosis) with dense cornified layers, sebaceous gland hyperplasia and epidermal rete peg formation (downward papillary projection of the epidermis) (Figure 1B, panels b and c). In contrast with hair shafts that emerged straight out of the hair canals in WT mice (Figure 1B, panel a), those in Pla2g3-Tg mice often bent over within the canals, probably because highly thickened interfollicular and follicular keratinocyte layers prevented the protrusion of the hair shafts (Figure 1B, panel b). Parakeratosis, which represents unusual retention of nuclei in the outermost cornified layers, was frequently observed in severe lesions in Pla2g3-Tg mice (Figure 1B, panels c–e), where the epidermis was focally lost resulting in cutaneous erosion and ulcers with massive neutrophil infiltration (Figure 1B, panels d, e, g and h).

RT–PCR showed that pro-inflammatory cytokines, including TNFα, IL-1β and IL-6, and CCL2/MCP-1 were highly expressed in the inflamed skin lesions of Pla2g3-Tg mice, but not in control mice (Figure 2A). CXCL14 (BRAK), a skin chemokine, was faintly detected in WT skin and was also increased in Tg skin. The T₃₀ cytokine IL-4 was weakly, but significantly, detected in Tg, but not WT, skin, whereas the T₃₀ cytokines IFNγ and IL-12 were below the detection limit in both genotypes (Figure 2A). Moreover, MPO activity, which mirrors neutrophil infiltration, in skin sections was markedly elevated in Pla2g3-Tg mice compared with WT mice (Figure 2B). Quantitative RT–PCR demonstrated a marked increase in CD68, a macrophage marker, in Pla2g3-Tg skin relative to WT skin (Figure 2C), consistent with macrophage infiltration. In contrast, the expression levels of mast cell markers, including mMCP-4, -5 and -6 (mast cell proteases), were unchanged between control and PLA2G3 skin (Figure 2D), indicating that the mast cell population was unaffected by Tg expression of PLA2G3. Therefore increased expression of HDC (a histamine-biosynthetic enzyme) in the Tg skin (Figure 2D) might reflect the increase in macrophages, in which a trace level of HDC is expressed [17], or, alternatively, the expression of HDC, but not mMCPs, in mast cells might be specifically controlled by PLA2G3.

Furthermore, a marked increase in PGE₂ was observed in the skin of Pla2g3-Tg mice compared with that of WT mice (Figure 3A). Consistent with this, expression of COX-2 and mPGES-1, which are inducible enzymes that act sequentially in the PGE₂-biosynthetic pathway downstream of PLA₂ [18], was higher in Pla2g3-Tg skin than in WT skin (Figure 3B). In particular, the induction of COX-2 was markedly higher in Tg skin relative to WT skin, whereas cutaneous expression levels of cPLA₂-α, COX-1 and mPGES-2 were similar between the genotypes (Figure 3B). Therefore increased expression of pro-inflammatory cytokines by infiltrating macrophages or hyperplastic epidermal keratinocytes may contribute to the induction of COX-2 and mPGES-1, which in turn leads to increased PGE₂ synthesis in Pla2g3-Tg skin. Although we also measured LTβr, a leuokiniotene produced by neutrophils and macrophages, in skin homogenates as well as serum of Pla2g3-Tg mice, it was below the detection limit probably because of its instability.

The expression of various sPLA₂ enzymes in the skin of WT and Tg mice is shown in Figure 3(C). Interestingly, transcripts of endogenous mouse PLA2G3 were clearly detected in control skin (Figure 3C). To our knowledge, this is the first demonstration that PLA2G3 is intrinsically expressed in mouse skin. Expression of PLA2G2D and PLA2G5 was increased in Tg lesion skin.
compared with unaffected Tg skin and with WT skin, whereas expression of PLA2G10 was below the detection limit in the skin of both genotypes (Figure 3C). Another intriguing finding is constitutive expression of PLA2G2E and PLA2G2F at relatively high levels in the skin of both genotypes (Figure 3C).

Inflammation in other tissues

The finding that several inflammatory markers were elevated in the skin lesions of Pla2g3-Tg mice (Figures 2 and 3) prompted us to examine whether other tissues in these mice also display inflammatory symptoms. We showed previously that Pla2g5-Tg mice die at birth because of lung injury resulting from aberrant hydrolysis of lung surfactant phospholipids, and that the lungs of newborn Pla2g5-Tg mice are characterized by abnormally thickened alveolar walls with formation of hyaline membranes lining the alveolar surfaces [11]. In marked contrast, the alveolar architecture of Pla2g3-Tg mice was largely indistinguishable from that of age-matched WT mice (Figure 4A, panels a and b). When surfactant lipids were extracted from bronchoalveolar lavage fluid of WT and Pla2g3-Tg mice and were subjected to ESI–MS analysis, the composition of the surfactant PC (Figure 4B) and phosphatidylglycerol (results not shown) was similar in WT and Pla2g3-Tg mice. However, goblet cell hyperplasia, which was visualized by PAS staining, was evident in the bronchial epithelium of Pla2g3-Tg mice compared with
WT mice, suggesting increased mucus secretion in the former (Figure 4A, panels c and d). In addition, perivascular lymph aggregates were frequently found in the lungs of Pla2g3-Tg mice compared with WT mice (Figure 4A, panels e and f). These observations suggest that PLA2G3 elicits airway inflammation through a mechanism independent of increased lung surfactant hydrolysis.

We also found notable inflammatory changes in salivary glands (Figure 4C) and liver (Figure 4D) of Pla2g3-Tg mice. Compared with WT mice (Figure 4C, panel a), the salivary glands of Pla2g3-Tg mice showed marked glandular swelling accompanied by infiltration of lymphocytes in the interstitium (Figure 4C, panels b and c); this histological feature resembles lymphocytic salienadenitis in humans. In addition, foci of extramedullary haemopoiesis, an event that often occurs during systemic inflammation, were found in the liver of Pla2g3-Tg mice (Figure 4D, panel b), whereas they were absent from WT mice (Figure 4D, panel a).

Furthermore, Pla2g3-Tg mice, even at 5 months of age at which time no skin inflammation was apparent, frequently showed splenomegaly (Figure 5A), such that spleen weight in the Tg mice was markedly increased compared with that in WT mice (Figure 5B). We therefore examined splenic cell populations by FACS analysis using antibodies against cell lineage-specific markers. Figure 5(C) summarizes the results. Essentially, there were no appreciable differences in populations of effector T-cells, including CD4+ and CD8+ T-cells, various differential stages of B-cells, including CD21+, IgM+, IgD+ and CD23+ cells, and NK (natural killer) (NK1.1+) cells (Figure 5C). In contrast, splenic Gr-1+ neutrophils, CD11b+ monocytes/macrophages and CD11c+ dendritic cells were increased >2-fold in Pla2g3-Tg mice relative to WT mice (Figure 5C). Therefore PLA2G3 overexpression preferentially affects the haemopoietic, rather than the lymphocytic, lineages of splenic cells at this stage.

In agreement with the above observations, numbers of circulating granulocytes and monocytes were increased in 9–10-month-old Pla2g3-Tg mice compared with WT mice, whereas they were similar in young adults (2–3-months-old) of both genotypes (Figure 6A, panels a and b). The number of circulating lymphocytes tended to increase in young, rather than aged, PLA2G3 mice relative to WT mice, although this difference was not statistically significant (Figure 6A, panel c). Platelet and erythrocyte counts were unaffected in Pla2g3-Tg mice throughout the experimental periods (Figure 6A, panels d and e). Likewise, haemoglobin levels and haematocrit values in the blood were indistinguishable between the genotypes (Figure 6B). In addition, serum IgG, was increased >7-fold in aged Pla2g3-Tg mice relative to WT mice, whereas serum IgM, IgG2a and IgE levels were unchanged (Figure 6C). To examine further the potential contribution of PLA2G3 to the inflammatory response, we examined PGE2 generation ex vivo by peritoneal macrophages derived from Pla2g3-Tg and WT mice. Spontaneous production of PGE2 over 12 h of culture was higher in macrophages from Tg mice than in those from control mice (Figure 6D, panel a). After stimulation of macrophages with LPS (lipopolysaccharide) for 24 h, WT-derived cells showed vigorous production of PGE2, which was further augmented 3-fold in replicate Tg-derived cells (Figure 6D, panel b). These ex vivo experiments agree with our previous observations that PLA2G3 has the capacity to augment arachidonic acid metabolism in various cultured cells when overexpressed [14,15]. Moreover, LPS-stimulated Pla2g3-Tg macrophages produced more IL-6 than did replicate WT cells (Figure 6E). Even in the absence of LPS stimulation, IL-6 release was significantly increased in macrophages of Pla2g3-Tg mice compared with those of control mice (Figure 6E, inset). Thus overexpression of PLA2G3 enhances the inflammatory response in macrophages.

**DISCUSSION**

Because of the ability of PLA2 enzymes to release arachidonic acid (a precursor of various lipid mediators such as prostaglandins and leukotrienes) and lysophospholipids such as lysophosphatidic...
Figure 3  Elevations of PGE₂ and its biosynthetic enzymes in skin of Pla2g3-Tg mice

(A) Skin homogenates from WT mice and Pla2g3-Tg mice (III-Tg) were subjected to PGE₂ enzyme immune assay. (B) Expression of PGE₂-biosynthetic enzymes in skins of WT and Pla2g3-Tg (III-Tg) mice was assessed by quantitative RT–PCR (means ± S.D., n = 3). The PGE₂-biosynthetic pathway is indicated. PGH₂, prostaglandin H₂. (C) Expression of sPLA₂s in WT and Pla2g3-Tg (Tg) skin was assessed by RT–PCR.

acid and 1-O-alkyl lysophosphatidylcholine [a precursor of PAF (platelet-activating factor)] from membrane phospholipids, it has long been thought that these enzymes participate in a wide variety of diseases, such as inflammation, atherosclerosis and cancer, in which lipid mediators play pivotal roles. Gene ablation of group IV A cPLA₂ α, a stimulus-coupled intracellular PLA₂ that is regulated by phosphorylation and Ca²⁺-dependent membrane translocation, has established the importance of this arachidonate-selective enzyme in the production of eicosanoids and PAF in most, if not all, cells and tissues [19]. Although several sPLA₂s are capable of releasing arachidonic acid from isolated or cultured cells in vitro [1–3], the pathophysiological relevance of this event in vivo has remained elusive. Although PL2G2A is a prototypic inflammatory PLA₂, whose expression is markedly elevated during various inflammatory states in humans and experimental animals (except in mice, in which the Pla2g2a gene is naturally disrupted (e.g. C57BL/6)) or expressed only in the intestine (e.g. BALB/c [20]), its contribution to lipid mediator production in vivo has not yet been proven conclusively. Tg mice overexpressing human or mouse PLA2G2A do not show any sign of inflammation, although they display a notable alopecic phenotype with hyperkeratosis [4,21]. Furthermore, animals overexpressing the mouse enzyme are sensitive to two-stage chemical carcinogenesis, with development of a higher number of skin papillomas and then of carcinomas than in replicate control mice [21]. Rather, the main function of PL2G2A is now recognized to be protection of the host from microbial invasion by degrading bacterial membranes, in which phosphatidylethanolamine and phosphatidylglycerol, two preferred substrates for PL2G2A, are major phospholipid components [9]. PL2G5 and PL2G10 have much higher capacity to interact with PC-rich outer plasma membranes to release arachidonic acid than does PL2G2A [22,23]. The involvement of both PL2G5 and PL2G10 in inflammation and associated lipid-mediator production, such as zymosan-induced peritonitis [5], methacholine-induced airway hypersensitivity [6], Th₂-biased asthmatic response [7] and ischaemia/reperfusion-induced myocardial damage [8], has been demonstrated in studies using Pla2g5-null or Pla2g10-null mice, even though it remains uncertain whether arachidonic acid is supplied directly through the membrane-hydrolytic action of these sPLA₂s or indirectly through the activation of cPLA₂α or other enzymes in those situations.
In the present study, we found that Tg mice carrying the transgene for human PLA2G3, another sPLA2 that is capable of efficient hydrolysis of PC-rich membranes [14,15], frequently developed inflammation as they aged. This is, to our knowledge, the first demonstration that Tg overexpression of a particular form of sPLA2 induces inflammation in the skin. By 1 year of age, Pla2g3-Tg mice displayed dermatitis associated with acanthosis, hyperkeratosis, parakeratosis, sebaceous gland hyperplasia, erosion and ulcer, with massive dermal infiltration of inflammatory cells, such as neutrophils and macrophages,
and elevated expression of pro-inflammatory cytokines and chemokines, such as IL-1β, IL-6, TNFα and CCL2 (Figures 1 and 2). A moderate but significant expression of IL-4, but not IFNγ and IL-12, suggests a tendency to a Th2 response rather than a Th1 response. The affected skin in Pla2g3-Tg mice is histologically similar to that of patients with atopic dermatitis, but is distinct from that of Pla2g2a-Tg mice [4], which develop alopecia and epidermal hyperplasia in the absence of inflammation. Therefore the mechanisms underlying the skin phenotypes induced by PLA2G3 (with inflammation) and PLA2G2A (without inflammation) may not be identical.

PGE2, as well as its biosynthetic enzymes (COX-2 and mPGES-1), was markedly increased in skin lesions of Pla2g3-Tg mice (Figure 3), indicating that PLA2G3 facilitates arachidonic acid metabolism. At present, it is unclear whether the elevation of this arachidonate metabolite reflects increased PLA2G3-directed membrane hydrolysis or a secondary effect involving the activation of other PLA2s (such as cPLA2α) in infiltrating leukocytes, keratinocytes or other cell types in response to elevated levels of pro-inflammatory cytokines/chemokines. Nevertheless, peritoneal macrophages from Pla2g3-Tg mice produced more PGE2 and IL-6 than did those from control mice, particularly after stimulation with LPS (Figures 6D and 6E), an observation that accords with studies showing that PLA2G3 can promote arachidonic acid release in multiple types of cultured cells [14,15] and that the skin lesions in Pla2g3-Tg mice showed elevated levels of pro-inflammatory cytokines and PGE2 with a concomitant infiltration of macrophages (Figures 2 and 3). The inflammatory signs are not restricted to the skin, but are also seen in other tissues including salivary gland, lung, liver, spleen and even blood (Figures 4–6). In this context, a likely explanation for the inflammatory phenotype is that sustained, albeit moderate, increases in levels of lipid mediators within the affected tissues might lead to a gradual onset of inflammation in Pla2g3-Tg mice. For instance, PGE2, through its receptor EP2, promotes epidermal hyperplasia and dermal macrophage infiltration [23a], which are reminiscent of Pla2g3-Tg skin (Figure 1). In accordance, episodes of infection with environmental micro-organisms through cutaneous lesions, particularly wounded areas as a result of increasing scratching behaviour, might facilitate the expansion, migration and activation of neutrophils and macrophages, thereby leading to exacerbation of local and systemic inflammatory responses.

It is intriguing to note that the skin inflammation in Pla2g3-Tg mice was accompanied by increased expression of PLA2G5 and PLA2G2D (Figure 3C). This suggests that these two sPLA2s are associated with inflammation, either through transcriptional up-regulation in resident cells or through infiltration of inflammatory cells in which these sPLA2s are expressed. Both situations might be applied to PLA2G5, since this enzyme is known to be expressed...
in macrophages [24], a cell population which is increased in Pla2g3-Tg skin, and since its expression has been reported to be induced in cytokine-stimulated macrophage-like cells [25] and keratinocytes [26]. Increased expression of PLA2G2D has been reported in a mouse model of atopic dermatitis [27]. On the other hand, PLA2G2E and PLA2G2F, as well as endogenous PLA2G3, were consistently detected in WT and Pla2g3-Tg skin (Figure 3C), suggesting that these sPLA2 enzymes are constitutively expressed in certain resident cells, rather than in infiltrating leucocytes, within the skin niche. Importantly, judging from the intensity of RT–PCR bands, the expression of endogenous mouse PLA2G3 in WT skin is similar to that of human PLA2G3 overexpressed in Tg skin (Figure 3C), suggesting a potential contribution of this enzyme to skin inflammation under certain pathological conditions. In contrast with previous reports describing the expression of PLA2G10 in newborn mouse skin [28], we failed to detect its transcript in the skin of either WT or Pla2g3-Tg mice (Figure 3C); this might be due to the difference in ages or strains

Figure 6 Changes in circulating cell populations and serum immunoglobulin levels in Pla2g3-Tg mice

The numbers of circulating granulocytes, monocytes, lymphocytes, platelets and erythrocytes (A), as well as haemoglobin and haematocrit values (B), in young (11–13-week-old) and aged (38–41-week-old) WT and Pla2g3-Tg (Tg) mice were evaluated using VetScan HM (means ± S.D., n = 4–12, *P < 0.05). (C) Serum concentrations of IgG1, IgM, IgG2a and IgM were determined by sandwich ELISA (means ± S.D., n = 3, *P < 0.05). (D) Peritoneal macrophages from Pla2g3-Tg (III-Tg) and WT mice were cultured for the indicated periods in culture medium (left-hand panel) or for 24 h in medium with (+) or without (−) 1 μg/ml LPS (right-hand panel). The supernatants were taken for PGE2 enzyme immune assay. A representative result of two reproducible results (panel a) and means ± S.E.M. for four experiments (panel b) are shown. *P < 0.05 compared with replicate WT cells. (E) Peritoneal macrophages from Pla2g3-Tg (III-Tg) and WT mice were cultured for 24 h in culture medium with (+) or without (−) LPS (right-hand panel). The supernatants were taken for ELISA for IL-6 (means ± S.E.M., n = 4, *P < 0.05 compared with replicate WT cells). Inset, IL-6 production by the cells cultured in the absence of LPS is magnified.
examined (neonatal NMRI mice compared with aged C57BL/6 mice).

As we have reported recently, alterations in plasma lipoproteins [a decrease in HDL (high-density lipoprotein) and an increase in atherogenic LDL (low-density lipoprotein)] occur in Pla2g3-Tg mice [16]. Interestingly, a potential linkage between altered lipoprotein levels and chronic inflammatory diseases has been proposed [29–32]. Mice deficient in LDL receptor and apolipoprotein A-I, in which plasma cholesterol levels were markedly altered, suffered from increasingly severe ulcerated cutaneous inflammation, and, in these mice, cholesterol accumulation in the skin was associated with macrophage infiltration and was accompanied by increases in TNFα and COX-2 [29,30]. Tg overexpression of apolipoprotein C1 decreased lipids in the sebaceous gland, epidermis and subcutaneous adipose tissue, leading to dry and scaly skin with loss of hair, epidermal hyperplasia, hyperkeratosis and atrophic sebaceous glands lacking sebum [31]. An increase in circulating HDL led to a significant inhibition of basal and IL-1α-induced E-selectin expression, suggesting the potential anti-inflammatory action of HDL [32]. These observations raise the possibility that systemic alterations in circulating LDL and HDL in Pla2g3-Tg mice [16] might be responsible, at least in part, for the acceleration of inflammation. Interestingly, Pla2g3a-Tg mice, which develop skin abnormalities [4], also show reduced plasma HDL levels [10], and disturbance of systemic lipid homeostasis has often been linked with skin abnormalities in various mouse models and even in human diseases [33–35]. Therefore potential causal relationships between altered systemic lipoprotein levels and skin abnormalities in mouse models with Tg overexpression of particular forms of sPLA2 should be taken into account. Moreover, given the recent recognition that atherosclerosis is a form of chronic inflammation in the vascular wall [36], the atherosclerotic [16] and inflammatory (the present study) phenotypes of Pla2g3-Tg mice may be interrelated.

Endogenous PLA2G3 mRNA in humans is detected in several tissues such as kidney, heart, liver and skeletal muscle [13], and immunoreactive PLA2G3 protein is localized in microvascular endothelial cells, macrophages, peripheral neuronal fibres, atherosclerotic plaques and tumour cells in tissues from human subjects with diseases (e.g. inflammation, ischaemia, atherosclerosis and cancer) [15,16,37,38]. In mice, endogenous Pla2g3 mRNA is detected at trace levels in various tissues (H. Sato, Y. Taketomi and M. Murakami, unpublished work), among which the nervous system (e.g. brain, spinal cord and dorsal root ganglion) shows the highest expression [37]. Essentially, unlike PLA2G2A, whose tissue expression profiles differ considerably between human and mouse [20], it appears that human and mouse PLA2G3 orthologues (∼82% homology in their core S domain) display a similar, if not entirely identical, tissue distribution pattern. In cell culture systems, forcible expression of PLA2G3-directed small interfering RNA partially suppresses some of these events, probably through altering the cellular levels of lyso phospholipids or eicosanoids [14,15,37,39,40]. Detection of endogenous PLA2G3 in mouse skin (Figure 3C) raises the intriguing possibility that this unique type of sPLA2 might function in skin biology. Besides the effect of PLA2G3 on lipid mediator generation, PLA2G3 might be coupled with a particular signalling pathway through interacting with the putative N-type sPLA2 receptor, to which bee venom PLA2 (a PLA2G3 homologue) is known to bind as a ligand [41]. It will be necessary to reconcile the precise in vivo actions, expression and dynamics of PLA2G3 by conducting studies with Pla2g3 gene-disrupted mice or with PLA2G3-specific inhibitors. Nevertheless, despite the limitations of Tg overexpression strategies, the mouse model described in the present paper might be useful to uncover the molecular actions of sPLA2 family enzymes, including tissue-specific target substrates and their metabolites, during the process of inflammation or other related diseases. Furthermore, this mouse line has potential for the screening of novel anti-inflammatory agents aiming at inhibiting sPLA2-mediated phospholipid hydrolysis.

**AUTHOR CONTRIBUTION**

This paper was organized and written by Makoto Murakami. Most of the experiments, including maintenance of animals and preparation of samples, were performed by Hiroyasu Sato. Specifically, PCR analysis of sPLA2s was assisted by Yoshitaka Taketomi, histochemistry by Seiko Masuda, experiments using peritoneal macrophages by Yuki Isogai and Tetsuya Kobayashi, and FACS analysis by Kei Yamamoto.

**ACKNOWLEDGMENTS**

We thank Dr Y. Ishikawa and Dr T. Ishii (Toho University, Tokyo, Japan) and Dr Y. Takeanazawa (University of Tokyo, Tokyo, Japan) for their assistance for histological and ESI–MS analyses respectively.

**FUNDING**

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Culture, Sports and Technology of Japan (grant numbers 21300027, 2179105 and 21790093), Precursory Research for Embryonic Science and Technology (PRESTO) from the Japan Science and Technology Agency, the Tokyo Biochemical Research Foundation, the Mitsubishi Pharma Research Foundation, the Novartis Foundation for the Promotion of Science and the Takeda Science Foundation.

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Published as BJ Immediate Publication 17 April 2009, doi:10.1042/BJ20082429
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Received 23 December 2008/9 March 2009; accepted 17 April 2009

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