Expression of secretory phospholipase A₂ enzymes in lungs of humans with pneumonia and their potential prostaglandin-synthetic function in human lung-derived cells

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Although a number of sPLA₂ (secretory phospholipase A₂) enzymes have been identified in mammals, the localization and functions of individual enzymes in human pathologic tissues still remain obscure. In the present study, we have examined the expression and function of sPLA₂s in human lung-derived cells and in human lungs with pneumonia. Group IID, V and X sPLA₂s were expressed in cultured human bronchial epithelial cells (BEAS-2B) and normal human pulmonary fibroblasts with distinct requirement for cytokines (interleukin-1 β , tumour necrosis factor α and interferon- γ). Lentivirus- or adenovirus-mediated transfection of various sPLA₂s into BEAS-2B or normal human pulmonary fibroblast cells revealed that group V and X sPLA₂s increased arachidonate release and prostaglandin production in

both cell types, whereas group IIA and IID sPLA₂s failed to do so. Immunohistochemistry of human lungs with pneumonia demonstrated that group V and X sPLA₂s were widely expressed in the airway epithelium, interstitium and alveolar macrophages, in which group IID sPLA₂ was also positive, whereas group IIA sPLA₂ was restricted to the pulmonary arterial smooth muscle layers and bronchial chondrocytes, and group IIE and IIF sPLA₂s were minimally detected. These results suggest that group V and X sPLA₂s affect lung pathogenesis by facilitating arachidonate metabolism or possibly through other functions.

Key words: arachidonic acid, immunohistochemistry, lung, phospholipase A₂, prostaglandin E₂.

INTRODUCTION

PLA₂ (phospholipase A₂) enzymes hydrolyse cellular glycerophospholipids to release lysophospholipids and non-esterified fatty acids. Release of AA (arachidonic acid) by PLA₂s is considered to be of particular importance, since this polyunsaturated fatty acid is metabolized through the COX (cyclo-oxygenase) and lipoxygenase pathways to various eicosanoids such as PGs (prostaglandins) and leukotrienes, which exert diverse biological activities through their cognate G-protein-coupled (or possibly nuclear) receptors in target cells. A number of mammalian PLA₂s have been identified to date, including ten sPLA₂s (secretory PLA₂s; group IB, IIA, IIC, IID, IIE, IIF, III, V, X and XIIA), four cPLA₂s (cytosolic PLA₂s; α , β , γ and δ) and two intracellular Ca²⁺-independent PLA₂s (iPLA₂s; β and γ) [1,2].

The sPLA₂ family represents a group of structurally related, disulphide-rich, low-molecular-mass enzymes with strict Ca²⁺-dependence [1,2]. Group IB sPLA₂ (sPLA₂-IB) is abundantly present in pancreatic juice, and its primary role is supposed to be the digestion of dietary phospholipids [3]. sPLA₂-IIA is markedly induced by proinflammatory stimuli in several cells and tissues [4,5], and its plasma concentrations correlate with the severity of various inflammatory diseases [6]. sPLA₂-IIA often augments AA release by cytokine-primed cells in a manner dependent on its association with cellular HSPGs (heparan sulphate proteoglycans) [7,8]. In addition, sPLA₂-IIA (and possibly other sPLA₂s; see below) can release cellular AA before secretion [9]. Beyond this

function, sPLA₂-IIA has also been implicated in anti-bacterial defence [10,11], atherosclerosis [12,13] and lung surfactant hydrolysis [14].

Of novel sPLA₂ enzymes, sPLA₂-IIC, -IID, -IIE, -IIF and -V are often referred to as the group II subfamily, since the genes for these enzymes are clustered on human chromosome 1p34-36 [1,2]. sPLA₂-IIC, which is present in rodent testes, is not expressed as a functional protein in humans [15]; sPLA₂-IID [16] and sPLA₂-IIE [17], which are structurally closely related to sPLA₂-IIA, as well as sPLA₂-IIF, which has a unique and long C-terminal extension [18], are capable of augmenting cellular AA release when overexpressed in HEK-293 (human embryonic kidney 293 cells) cells [8,19]. sPLA₂-V exhibits more potent cellular AA-releasing activity than do other group II subfamily sPLA₂s [8,20–22]. The cellular action of sPLA₂-V depends on its ability to bind PC (phosphatidylcholine), a major phospholipid in the outer leaflet of the plasma membrane, and cell surface HSPGs [8,20–22]. sPLA₂-X also shows high affinity for PC, and releases cellular AA during secretion [9] and after secretion by acting on the PC-rich outer plasma membrane of cells with no dependence on HSPGs [8,23,24]. sPLA₂-III and -XII are more distantly related to the group I/II/V/X sPLA₂s [25,26]. sPLA₂-III is composed of the core group III sPLA₂ domain and unique N- and C-terminal domains, of which the core domain is sufficient for the enzymic function [27]. sPLA₂-XIIA has weak catalytic activity [26], whereas sPLA₂-XIIB has no catalytic activity because of replacement of an amino acid in the catalytic centre [28].

Abbreviations used: AA, arachidonic acid; COX, cyclo-oxygenase; PGE, prostaglandin E; cPGES, cytosolic PGE synthase; ER, endoplasmic reticulum; FCS, fetal calf serum; HEK-293 cells, human embryonic kidney 293 cells; HSPG, heparan sulphate proteoglycan; IFN- γ , interferon- γ ; IL-1 β , interleukin-1 β ; mPGES, microsomal PGE synthase; NHPF, normal human pulmonary fibroblast; PC, phosphatidylcholine; PG, prostaglandin; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; sPLA₂, secretory PLA₂; RT, reverse transcriptase; TNF α , tumour necrosis factor α ; VSMC, vascular smooth muscle cells.

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There is emerging evidence that the expression profiles of individual sPLA₂s in tissues differ according to animal species and type of pathology. Although current studies using animal models have revealed the inducible expression of several sPLA₂s (particularly the group II subfamily sPLA₂s) during inflammation [1,2,29], their localization, dynamics and functions *in vivo*, particularly in humans, still remain elusive. To understand the expression and function of each sPLA₂ in pathological states in humans, we herein examined the expression of six sPLA₂s (IIA, IID, IIE, IIF, V and X) in human lung-derived cells and in human lungs with infectious pneumonia. Furthermore, we assessed by adenovirus- or lentivirus-mediated gene transfer the AA-releasing capacities of these sPLA₂s in various cells originating from this tissue.

MATERIALS AND METHODS

Materials

NHPFs (normal human pulmonary fibroblasts) and culture media and supplements for these cells were purchased from Bio-Whittaker (Walkersville, MD, U.S.A.). BEAS-2B cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% (v/v) FCS (fetal calf serum) as described previously [27]. Rabbit antisera specific for individual human sPLA₂s and pure recombinant human sPLA₂s were described previously [30]. Goat anti-human COX-1 and -2 antibodies and rabbit anti-human group IVA cPLA₂α antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit antibodies against cPGES [cytosolic PGE (prostaglandin E) synthase] and mPGES (microsomal PGE synthase-1 and -2) were described previously [31–33]. Goat anti-human GM130 antibody in the Organelle Sampler kit was obtained from BD Biosciences (San Diego, CA, U.S.A.). cDNAs for sPLA₂s and cPLA₂α were described previously [7,8,19]. The enzyme immunoassay kit for PGE₂ was purchased from Cayman Chemicals. Human IL-1β (interleukin-1β), TNFα (tumour necrosis factor α) and IFN-γ (interferon-γ) were obtained from Genzyme (Cambridge, MA, U.S.A.). LIPOFECTAMINE™ 2000, Opti-MEM medium, TRIzol® reagent, Geneticin, blasticidin and the pcDNA3.1 series of mammalian expression vectors were obtained from Invitrogen (Carlsbad, CA, U.S.A.). FITC-, Cy3- and horseradish peroxidase-conjugated anti-IgG antibodies were purchased from Zymed (San Francisco, CA, U.S.A.). Primers for RT (reverse transcriptase)-PCR were from Greiner Japan (Tokyo, Japan). Other reagents of analytical grade were from Wako Chemicals (Osaka, Japan).

RT-PCR

Synthesis of cDNAs was performed with 0.5 μg of total RNA obtained from cells and avian myeloblastosis virus RT, according to the manufacturer's instructions given with the RNA PCR kit (Takara Biomedicals, Osaka, Japan). Subsequent amplification of the cDNA fragments was performed with 0.5 μl of the reverse-transcribed mixture as a template with specific primers for each sPLA₂. For amplification of sPLA₂-IIA, -IID, -IIE, -IIF, -V and -X cDNAs, we used a set of oligonucleotide primers corresponding to the 5'- and 3'-nucleotide sequences of their open reading frames as follows: IIA sense, 5'-atgaagacctctactgttgcc-3' and antisense 5'-tcagcaacgagggtgctccctc-3'; IID sense, 5'-atggaactgcactgtgtgg-3' and antisense 5'-ctagcaccaggggtctgccc-3'; IIE sense, 5'-atgaaatctccccactgtctgg-3' and antisense 5'-tcagcaggcgccgggtggcccg-3'; IIF sense, 5'-atgaagaagtcttcaccgtgac-3' and antisense 5'-ctaggaggggcgccggggcgctg-3'; V sense, 5'-atgaaggcctccctccactggc-3' and antisense 5'-ggcctaggagcagaggatgttg-3'; X sense, 5'-atggggccgctactgtgtgcc-3' and antisense 5'-tcagtcacact-

tgggcgagtc-3'. The PCR conditions for sPLA₂-IIA, -IID, -IIE, -V and -X were 94 °C for 30 s and then 30–33 cycles of amplification at 94 °C for 5 s and 68 °C for 4 min, using the Advantage cDNA polymerase mixture (Clontech). The PCR conditions for sPLA₂-IIF were 94 °C for 30 s and then 30–33 cycles of amplification at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s, using *ExTaq* polymerase (Takara Biomedicals). The PCR products were analysed by 1% agarose gel electrophoresis with ethidium bromide.

Northern blotting

Equal amounts (~5 μg) of total RNA obtained from cells by use of TRIzol® reagent were applied to separate lanes of 1.2% (w/v) formaldehyde/agarose gels, electrophoresed and transferred on to Immobilon-N membranes (Millipore). The resulting blots were then probed with their respective cDNA probes that had been labelled with [³²P]dCTP (Amersham Biosciences) by random priming (Takara Biomedicals). Hybridization and subsequent membrane washing were performed as described previously [8].

SDS/PAGE/immunoblotting

Lysates from 10⁵ cultured cells in PBS were subjected to SDS/PAGE using 7.5% (for cPLA₂α and COXs), 12.5% (for PGESs) and 15% (for sPLA₂s) gels under reducing (for cPLA₂α, COXs and PGESs) and non-reducing (for sPLA₂s) conditions. For sPLA₂-IIF, SDS/PAGE was performed under both reducing and non-reducing conditions. The separated proteins were electroblotted on to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) with a semi-dry blotter (MilliBlot-SDE system; Millipore). After blocking with 3% (w/v) skimmed milk in PBS containing 0.05% Tween 20 (PBS-Tween), the membranes were probed with the respective antibodies (1:5000–1:10000 dilutions in PBS-Tween) for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-goat or -rabbit IgG (1:5000 dilution in PBS-Tween) for 2 h, and were visualized with the ECL® Western-blot system (NEN™ Life Science Products) as described in [8].

Immunohistochemistry

Immunohistochemical staining of human tissue sections was performed as described previously [32,33]. Briefly, the tissue sections were incubated with Target Retrieval Solution (Dako) as required, incubated for 10 min with 3% (v/v) H₂O₂, washed three times with PBS for 5 min each, incubated with 5% skimmed milk for 30 min, washed three times with PBS-Tween for 5 min each, and incubated for 2 h with anti-human sPLA₂ antibodies at 1:200–1:500 dilutions in PBS. Then the sections were treated with a CSA system staining kit (Dako) with diaminobenzidine substrate. The cell type was identified from conventional haematoxylin and eosin staining of serial sections adjacent to the specimen used for immunohistochemistry. Studies on human tissue sections were approved by the ethical committee of our Universities.

Expression of PLA₂s by the adenovirus system

Adenovirus bearing individual PLA₂ cDNA was prepared with a ViraPower Adenovirus Expression System (Invitrogen) according to the manufacturer's instructions. Briefly, the full-length cDNAs for sPLA₂ and cPLA₂α, amplified by PCR with *Pyrobest* proof-reading polymerase (Takara Biomedicals), were subcloned into the pENTER/D-TOPO vector with a pENTER Directional TOPO Cloning kit (Invitrogen). After purification of the plasmids from the transformed Top10 competent cells (Invitrogen), the sequences of the cDNA inserts were verified with a *Taq* cycle sequencing kit (Takara Biomedicals) and an autofluorimetric DNA sequencer (310 Genetic Analyzer; Applied Biosystems). The

cDNA inserts were then transferred to the pAd/CMV/V5-DEST vector (Invitrogen) by means of the Gateway system using LR clonase (Invitrogen). After purification from the transformed Top10 cells, the plasmids were linearized by digestion with *PacI* (New England Biolabs) and then transfected into subconfluent 293A cells (Invitrogen) with LIPOFECTAMINETM 2000 in Opti-MEM medium. After 1–2 week of culture in RPMI 1640 containing 10 % FCS until most cells had floated, the culture medium and cells were harvested together, freeze–thawed twice, and centrifuged to obtain the adenovirus-enriched supernatants. Then, aliquots of the supernatants were added to fresh 293A cells, and the culture was continued for appropriate periods to amplify adenoviruses. After 2–4 cycles of amplification, the resulting adenovirus-containing supernatants were used as virus stocks. Viral titres were determined by the plaque-forming assay with 293A cells. As a control, the pAd/CMV/V5-GW/*lacZ* vector (Invitrogen) was transfected into 293A cells to produce *LacZ*-bearing adenovirus.

Aliquots of the adenovirus-containing media were added to BEAS-2B and NHPF cells grown in 24-well plates (Iwaki Glass, Tokyo, Japan). After culturing for 36–48 h, the media were replaced with fresh culture media with or without cytokines. After incubation for 12 h, the supernatants were subjected to sPLA₂ enzyme assay and PGE₂ enzyme immunoassay, and the cells were taken for Northern blotting and/or immunoblotting to assess the expression of PLA₂s and other related enzymes (COXs and PGESs). Replicate adenovirus-infected cells were incubated for 30 min with medium containing 1 M NaCl, and PLA₂ activities solubilized into the supernatants were measured [13]. In another set of experiments (time-course study), the cells were infected with adenoviruses in the presence of cytokines, and PGE₂ production was assessed after the incubation for appropriate periods.

Expression of PLA₂s by the lentivirus system

PLA₂ cDNAs were stably transfected into BEAS-2B and NHPF cells with a ViraPower lentiviral expression system (Invitrogen) according to the manufacturer's instructions. Briefly, PLA₂ cDNA inserts were amplified by PCR with the Advantage cDNA polymerase mixture and were subcloned into the pLenti6/V4-D-TOPO vector (Invitrogen). The resulting plasmid was transfected into 293FT cells (Invitrogen) with LIPOFECTAMINETM 2000 in Opti-MEM medium, and aliquots of the supernatants harvested for 3 days after transfection were then added to BEAS-2B and NHPF cells. The cells were cultured in the presence of blasticidin (30 and 5 µg/ml for BEAS-2B and NHPF respectively), and the surviving cells that expressed appropriate levels of individual PLA₂s were used in subsequent studies.

Co-expression of PLA₂s and COXs in BEAS-2B cells

COX-1 or -2 cDNA in pCDNA3.1/neo(+) was transfected into BEAS-2B cells with LIPOFECTAMINETM 2000, and Geneticin-resistant clones were selected by limiting dilution and subsequent screening by Western blotting as described previously [33]. Then, each PLA₂ cDNA was transfected into the COX-1 or -2 stable transfectants using the lentivirus system, and the double transfectants were selected by culture with blasticidin, as described above.

Measurement of sPLA₂ activity

sPLA₂ activity was assayed by measuring the amounts of radio-labelled linoleic acid released from the substrate 1-palmitoyl-2-[¹⁴C]linoleoyl-phosphatidylethanolamine (Amersham Biosciences) as described previously [7,8]. For rough quantification of individual sPLA₂s, their enzymic activities released from the cells into the culture supernatants were compared with respective

pure recombinant sPLA₂s (provided by Dr M. H. Gelb, University of Washington, Seattle, WA, U.S.A.) that were diluted with cell culture medium.

AA release assay

Cells grown to near confluency in 48-well plates (Iwaki Glass) were incubated overnight with 0.1 µCi/ml [³H]AA (Amersham Biosciences). After three washes with fresh medium, 100 µl of culture medium with or without cytokines was added to each well, and the radioactivities released into the supernatants after incubation for appropriate periods were measured. The percentage release was calculated using the formula $[S/(S + P)] \times 100$, where *S* and *P* are the radioactivities measured in the supernatant and cell pellet respectively as described previously [7,8].

Confocal laser microscopy

Cells grown in glass-bottomed dishes (Matsunami Glass, Tokyo, Japan) precoated with 10 µg/ml fibronectin (Sigma) were fixed with 3 % (w/v) paraformaldehyde for 30 min in PBS. Cells were infected with adenoviruses bearing PLA₂s for 36 h and then stimulated with IL-1β before fixation, as required for the experiments. After three washes with PBS, the fixed cells were sequentially treated with 1 % (w/v) BSA (for blocking) containing 0.5 % (w/v) saponin (for permeabilization) in PBS for 1 h, with anti-sPLA₂ antibodies (1:200–1:500 dilutions) for 1 h in PBS containing 1 % albumin, and then with FITC-conjugated anti-rabbit IgG (1:200 dilution) for 1 h in PBS containing 1 % albumin, with three washes with PBS at each interval. For double immunostaining, cells stained with anti-sPLA₂ antibodies were incubated with goat anti-GM130 antibody (1:250 dilution) for 2 h, followed by incubation with Cy3-conjugated anti-goat IgG antibody (1:100 dilution) for 2 h. After six washes with PBS, the fluorescent signal was visualized with a laser scanning confocal microscope (IX70; Olympus, Tokyo, Japan) as described previously [7].

RESULTS

Expression of sPLA₂s and PGE₂-biosynthetic enzymes in cultured cells derived from human lung

We examined by semi-quantitative RT-PCR the expression of six sPLA₂s (IIA, IID, IIE, IIF, V and X) in cultured cells derived from human lung, including the human bronchial epithelial cell line BEAS-2B and human lung fibroblast NHPFs. In BEAS-2B cells, sPLA₂-IIA was barely detectable under all culture conditions (Figure 1A, left panel). Expression of sPLA₂-IID and -V was undetectable in BEAS-2B cells under normal culture conditions, but was markedly induced after stimulation with IL-1β, whereas TNFα or IFN-γ showed minimal effect (Figure 1A, left panel). In NHPF, sPLA₂-IIA and -IID were induced after cytokine stimulation, with IFN-γ exhibiting a more potent effect than IL-1β or TNFα (Figure 1A, right panel). sPLA₂-V and -X were clearly detected in NHPF even under normal culture conditions, and expression was not increased appreciably in response to cytokines (Figure 1A, right panel). No expression of sPLA₂-IIE and -IIF was found in either cell type (Figure 1A).

The expression of related enzymes involved in PGE₂ biosynthesis in these cells was also examined by immunoblotting. In BEAS-2B cells, the expressions of cPLA₂α, COX-1, -2 and mPGES-1 were not detected even after cytokine stimulation (Figure 1B, left panel) as described previously [33]. Consistent with poor expression of these enzymes, PGE₂ production by cytokine-stimulated BEAS-2B cells was negligible (Figure 1C, left panel). Constitutive expression of cPGES and mPGES-2 was seen

Figure 1 Expression of endogenous sPLA $_2$ s and other PGE $_2$ -biosynthetic enzymes in cultured human lung-derived cells

(A) Expression of sPLA $_2$ s in BEAS-2B and NHPF with or without stimulation for 24 h with cytokines, as assessed by RT-PCR (30 cycles). (B) Expression of various PGE $_2$ -biosynthetic enzymes in these cells with or without stimulation for the indicated periods with cytokines, as assessed by immunoblotting. (C) Production of PGE $_2$ in these cells with or without stimulation for the indicated periods with cytokines. Representative results of 3–4 independent experiments are shown.

in BEAS-2B cells (Figure 1B, left panel). In NHPF, all enzymes were detectable by immunoblotting, and each displayed distinct cytokine dependence (Figure 1B, right panel). Thus, cPLA $_2\alpha$ was constitutively expressed and modestly increased in response to all cytokines, whereas COX-1 expression was constitutive under all culture conditions. Expression of COX-2 was markedly induced by IL-1 β over 6–24 h, induced to a lesser extent by TNF α , and not appreciably induced by IFN- γ . Expression of mPGES-1 was also strongly inducible, increasing continuously over 24 h on treatment with IL-1 β and TNF α , but was only modestly increased by IFN- γ . cPGES was constitutively expressed and was increased significantly by IFN- γ over 6–24 h and by

TNF α over 12–24 h. Expression of mPGES-2, which appeared as two bands [33], was modestly increased by IFN- γ over 6–24 h. Delayed PGE $_2$ production was elicited by IL-1 β and to a lesser extent by TNF α , but not appreciably by IFN- γ , in NHPF (Figure 1C, right panel), thus being correlated with the induction of COX-2.

Effects of sPLA $_2$ s on AA metabolism in human bronchial epithelial cells

To assess the contribution of sPLA $_2$ s to AA metabolism in cells of human lung origin, we transfected these enzymes into BEAS-2B

Figure 2 AA metabolism in human bronchial epithelial BEAS-2B cells transfected with various PLA₂s by the lentivirus system

(A, B) Individual PLA₂s stably expressed in BEAS-2B cells by the lentivirus system were detected by Northern blotting (A) and immunoblotting (B). Dark arrows indicate the bands for individual sPLA₂s, and grey arrows may correspond to their unprocessed forms. Molecular masses are indicated in kDa. (C) Immunocytochemistry of sPLA₂s expressed in BEAS-2B cells. Fluorescent signals for individual sPLA₂s were visualized with specific antibodies ($\times 200$ magnification) (panel a) or signals for GM130, a Golgi marker ($\times 400$ magnification) (panel b). In panel c, the sPLA₂-IIA signal was unaffected by treatment of the transfectants with 1 mg/ml heparin ($\times 100$ magnification). Parental cells exhibited minimal staining for sPLA₂s. Other sPLA₂s displayed similar staining patterns (results not shown). (D) sPLA₂ activities in the supernatant and membrane-associated fractions of the transfectants. In the latter fraction, sPLA₂s were solubilized by treatment of the cells with a medium containing 1 M NaCl. Values show the amounts of sPLA₂s estimated by comparison of the activities between the samples and the authentic sPLA₂ standards. (E) [³H]AA release by BEAS-2B cells stably transfected with individual PLA₂s. The cells were incubated for 4 h with or without TNF α in culture medium. (F) PGE₂ production by BEAS-2B cells stably transfected with PLA₂s in combination with either of the two COX isoenzymes. The cells were incubated for 4 h with TNF α in a medium containing 10% FCS. Inset: expression of COX-1 and -2 proteins in the transfectants, as assessed by immunoblotting. Representative results of 3–5 independent experiments are shown in (A–D), and values are means $\pm$ S.E.M. for four independent experiments in (E, F).

cells by means of the lentivirus- or adenovirus-mediated gene transfer system. As described below, this method allowed us to control the expression levels of sPLA₂s to low ng/ml orders, which are within the range of reported concentrations of sPLA₂-IIA at inflamed sites [1,2].

The expression of individual sPLA₂s as well as cPLA₂α, which was used as a positive control for increased AA metabolism (see below), in the obtained stable transfectants was assessed by

Northern blotting (Figure 2A) and immunoblotting (Figure 2B). On immunoblotting, sPLA₂-IIA, -IID, -IIF and -V each gave a 14–18 kDa band (Figure 2B), revealing the strict specificity of each antibody. Both sPLA₂-IIE and -X appeared as a doublet band with closely similar molecular masses (Figure 2B). The upper band of sPLA₂-X may correspond to its unprocessed form bearing an N-terminal propeptide [23]. As we used a synthetic sPLA₂-IIE cDNA construct in which the cDNA encompassing the sPLA₂-IIE mature

protein is flanked by an oligonucleotide encoding the sPLA₂-IIA signal peptide, cleavage of the artificial signal peptide might not be complete in BEAS-2B cells.

Confocal laser microscopic immunocytochemical staining of these transfectants with anti-sPLA₂ antibodies revealed that all sPLA₂s were mainly localized in the Golgi apparatus, as verified by colocalization with the Golgi marker GM130 (Figure 2C, panels a and b). Weak sPLA₂ signals were also diffusely distributed in the ER (endoplasmic reticulum). Treatment of the transfectants with heparin did not appreciably alter the location and signal intensity of sPLA₂s (Figure 2C, panel c), implying that the HSPG-dependent internalization of sPLA₂s, which occurs in several other cell types [7,8,20,22], does not take place appreciably in BEAS-2B cells. Thus it is probable that the staining of the Golgi and ER reflects *de novo* produced sPLA₂s entering the secretory pathway.

When sPLA₂ activities in the culture supernatant and membrane-bound (1 M NaCl-solubilized) fractions of the transfectants were measured, >90% of the activity of sPLA₂-IIA was distributed in the latter fraction, whereas sPLA₂-IIF and -X were predominantly recovered from the former fraction (Figure 2D). The activities of sPLA₂-V (Figure 2D) and -IID (results not shown) were equally distributed in the supernatant and membrane-bound fractions. The activity of sPLA₂-IIE was very low (most probably because of its intrinsic weak activity [18]) but significant, and approx. 80% of the activity was detected in the supernatants (results not shown). The existence of significant pools of the cationic sPLA₂s (IIA, IID and V) in the NaCl-solubilized fractions suggests their association with non-internalized HSPGs or certain anionic components on cell surfaces. On the basis of comparison of enzymic activities with those of pure recombinant sPLA₂s diluted in culture medium, the levels of sPLA₂s produced by these transfectants were estimated to be 30–60 ng/ml (Figure 2D).

As shown in Figure 2(E), spontaneous [³H]AA release from [³H]AA-prelabelled cells was significantly increased in cells transfected with sPLA₂-IIF, -V and -X, but not in those transfected with sPLA₂-IIA, -IID and -IIE, compared with control cells. Stimulation of these sPLA₂ transfectants with TNF α (Figure 2E) or with other cytokines (results not shown) did not further increase [³H]AA release. As a positive control, transfection with cPLA₂ α markedly increased [³H]AA release, which was further augmented in TNF α -stimulated cells (Figure 2E). [³H]AA release elicited by sPLA₂-IIF, -V and -X was similar to that elicited by cPLA₂ α in the absence of TNF α , and after stimulation with TNF α [³H]AA release by cPLA₂ α was approx. 2-fold higher than that by sPLA₂-IIF, -V and -X.

To assess PLA₂-COX coupling leading to PGE₂ generation, these enzymes were co-transfected into BEAS-2B cells. Appropriate expression of each enzyme in the double transfectants was verified by Northern blotting (results not shown) and by immunoblotting (expression of COXs is shown in Figure 2F, inset). As shown in Figure 2(F), expression of COX-2, but not COX-1, alone in BEAS-2B cells slightly increased TNF α -stimulated PGE₂ production, and expression of COX-2, but not COX-1, in combination with sPLA₂-V or -X resulted in significant increase in PGE₂ production over that of COX-2 alone. In contrast, the combination of COX-2 with sPLA₂-IIA or -IIE showed no augmentative effect on PGE₂ production (Figure 2F). Co-expression of COX-2 and cPLA₂ α led to a marked increase in PGE₂ production, which reached an approx. 2-fold higher level than that observed in cells co-transfected with COX-2 and sPLA₂-V or -X. Although COX-2 induction by PLA₂s could occur in several cell types [7,8,21], overexpression of sPLA₂s and cPLA₂ α did not affect the expression of endogenous COX-2 in BEAS-2B cells (results not shown).

Intriguingly, in contrast with sPLA₂s, which failed to couple with COX-1, cPLA₂ α was capable of producing PGE₂ through COX-1 as efficiently as through COX-2 in this experimental setting (Figure 2F). Although COX-1 has been believed to mediate mainly immediate PG generation [34,35], our present results indicate that COX-1-dependent delayed PG generation can occur, supporting recent *in vivo* evidence that both COX-1 and -2 can participate in lung pathology and colon carcinogenesis [36,37]. More importantly, different profiles of COX-1 coupling between cPLA₂ α and sPLA₂s suggests that these enzymes may act on different phospholipid pools in bronchial epithelial cells. In the light of the different AA concentration sensitivity of the two COX enzymes [35], the present results agree with a recently proposed model that the AA released by cPLA₂ α around the perinuclear membrane can be supplied efficiently to adjacent COXs, whereas AA released at the plasma membrane surface by sPLA₂s does not reach a threshold intracellular concentration sufficient for COX-1-directed catalysis [38].

As an alternative approach, we transfected PLA₂s into COX-2-expressing BEAS-2B cells by adenovirus-mediated gene transfer. Expression of adenovirus-transferred PLA₂s in BEAS-2B cells was verified by Northern blotting (Figure 3A, inset) and by immunoblotting (results not shown). Immunocytochemical staining revealed that nearly 100% of cells were sPLA₂-positive after 2 days of adenovirus infection, and that these enzymes were distributed mainly in the Golgi and ER (results not shown). The concentrations of sPLA₂s released from cells infected with sPLA₂-bearing adenoviruses were comparable with those achieved by the lentivirus-based method, as assessed by their enzymic activities (results not shown). As shown in Figure 3(A), sPLA₂-IIF, -V and -X increased PGE₂ production, whereas sPLA₂-IIA and -IID exhibited only modest effects. Adenoviral expression of cPLA₂ α resulted in 2–3-fold more PGE₂ generation compared with the expressions of sPLA₂-IIF, -V and -X (Figure 3A). As shown in Figure 3(B), increased production of PGE₂ by sPLA₂-X and -IIF occurred kinetically in parallel with the induction of these enzymes after adenovirus infection, whereas the expression of sPLA₂-IID exhibited a minimal effect at any time point.

Effects of sPLA₂s on AA metabolism in NHPF

Similar virus-mediated PLA₂ transfection experiments were conducted with NHPF. By the lentivirus method, we obtained NHPF clones stably expressing sPLA₂-IIA, -IID, -V, -IIE and -X, as assessed by Northern blotting (Figure 4A). On immunoblotting, sPLA₂-IIA, -IID, -V and -IIE each gave a 14–18 kDa immunoreactive band, whereas sPLA₂-X appeared as a doublet band that may correspond to the pro- and mature forms of this enzyme (Figure 4B). Immunocytochemical staining revealed that these sPLA₂s were enriched in the perinuclear Golgi apparatus (Figure 4C, panels a and b), and this staining pattern was unaffected by treatment of the cells with heparin (Figure 4C, panel c). As compared with other sPLA₂s, a large portion of sPLA₂-X was distributed diffusely in the ER (Figure 4C, panels a and b). Although the reason for this subtle difference in subcellular localization among distinct sPLA₂ enzymes is not known, one possibility is that the unprocessed form of sPLA₂-X is retained in the ER of these cells. As assessed by enzymic activities, approx. 50% of sPLA₂-IIA and -V were membrane-bound (1M NaCl-solubilized), whereas >90% of sPLA₂-X was recovered from the supernatants (Figure 4D), suggesting again the association of significant pools of the cationic sPLA₂s with non-internalized HSPGs or some other anionic components on cell surfaces. The levels of sPLA₂s produced by the transfectants reached 10–30 ng/ml, as estimated from their enzyme activities in comparison with those of the

Figure 3 AA metabolism in BEAS-2B cells transfected with various PLA₂s by the adenovirus system

(A) BEAS-2B cells stably expressing COX-2 were infected with adenoviruses for various PLA₂s or control (LacZ) [multiplicity of infection (MOI) = 10] for 36 h and stimulated for 12 h with TNF α in culture medium, and the supernatants were then taken for PGE₂ assay. Values are means $\pm$ S.E.M. for three independent experiments. * P < 0.05 compared with the control. (B) Time-course study of adenovirus infection. BEAS-2B cells stably expressing COX-2 were stimulated for the indicated periods with TNF α in the presence of adenoviruses bearing various sPLA₂s. The supernatants and cells were harvested at each time point to quantify the levels of PGE₂ and sPLA₂ expression respectively. A representative result of three independent infection experiments is shown. Inset: expression of individual PLA₂s was assessed by Northern blotting.

authentic standard sPLA₂s diluted in culture medium (Figure 4D).

The AA release assay using [³H]AA-prelabelled cells demonstrated that sPLA₂-V and -X, but not sPLA₂-IIA, -IID and -IIE, were capable of increasing spontaneous [³H]AA release (Figure 4E). Stimulation of the cells with cytokines (e.g. IL-1 β and TNF α) did not further increase [³H]AA release by these sPLA₂s (results not shown). In agreement with the AA-releasing profiles (Figure 4E) and endogenous COX-2 and mPGES-1 expression (Figure 1B), there were marked increases in PGE₂ synthesis by cells expressing sPLA₂-V or -X after stimulation with IL-1 β or, to a lesser extent, with TNF α , but not appreciably with IFN- γ (Figure 4F), suggesting that the AA released by sPLA₂-V or -X can be supplied to endogenous COX-2/mPGES-1 induced by these cytokines for PGE₂ synthesis. In contrast, sPLA₂-IIA and -IID failed to augment PGE₂ production significantly under any condition (Figure 4F). As for BEAS-2B cells, sPLA₂ overexpression did not affect endogenous COX-2 expression (results not shown).

To confirm these observations further, we next introduced PLA₂s (sPLA₂-IIA, -IID, -IIF, -V and -X as well as cPLA₂α) into NHPF by the adenovirus method. The expression of individual PLA₂s in NHPF 36 h after infection with PLA₂-bearing adenoviruses was verified by Northern blotting (Figure 4G, inset). The amounts of individual sPLA₂s produced by the adenovirus-infected NHPF ranged from 0.5 to 60 ng/ml according to the adenovirus dose added, as assessed by their enzymic activities (results not shown). Interestingly, sPLA₂-IIF gave a 34 kDa band under reducing conditions and 34 and > 50 kDa bands under non-reducing conditions on immunoblotting (Figure 4B). This suggests that sPLA₂-IIF undergoes some post-translational modifications (such as glycosylation or homodimer formation through a free cysteine in the long C-terminal extension) in a cell type-specific manner, a possibility that is under investigation (S. Masuda, M. Murakami, S. Shimbara and I. Kudo, unpublished work). Furthermore, sPLA₂-IIF displayed a unique staining in the cytoplasmic aggregates as well as in the Golgi (Figure 4C, panel d). As in the case of sPLA₂-X, sPLA₂-IIF was mostly released into the supernatants, but was not associated with cell surfaces (Figure 4D).

As shown in Figure 4(G), IL- β -stimulated PGE₂ generation was augmented significantly in NHPF transfected with sPLA₂-IIF, -V and -X, but not with sPLA₂-IIA and -IID, in a manner dependent on adenovirus doses (i.e. the amounts of PLA₂s produced). As expected, PGE₂ production was markedly increased when NHPFs were infected with adenovirus bearing cPLA₂α (Figure 4G). Augmented PGE₂ production by sPLA₂-X (Figure 4H), -V and -IIF (results not shown) depends on its catalytic activity, since cells infected with adenovirus bearing a catalytically inactive mutant Gly³⁰ $\rightarrow$ Ser, in which Gly³⁰ in the Ca²⁺-binding loop was replaced with serine [39], failed to increase PGE₂ production (Figure 4H).

Expression of sPLA₂s in lungs of humans with infectious pneumonia

We next performed immunohistochemistry with antibodies specific for individual enzymes to examine the localization of these sPLA₂s in human lung tissues. Immunohistochemistry was performed on eight autopsied cases, including five cases with infectious pneumonia (bronchial pneumonia) in the age group 62–78 years and three cases with normal structure (control) in the age group 61–76 years, all of which were obtained by autopsy within 3 h *post mortem*. These lung tissues were fixed with 10% neutral buffered formalin and embedded in paraffin, and thin sections (4 μ m thickness) were used for immunohistochemistry. We observed at least five tissues in each case, and typical results are shown in Figure 5.

Although the expression and potential functions of sPLA₂-IIA have been demonstrated in the lungs of rats, rabbits and guinea pigs [14,40], we found in the present study that the expression of sPLA₂-IIA in human lungs affected by pneumonia was restricted to the pulmonary arterial walls and bronchial chondrocytes (Figure 5A, panels a, b and d). In the arterial walls, sPLA₂-IIA immunostaining was observed in VSMC (vascular smooth muscle cells) of the intima and media (Figure 5A, panel d). There was no obvious staining of sPLA₂-IIA in the bronchial and alveolar epithelium and alveolar macrophages (Figure 5A, panels a and c). In contrast, sPLA₂-V was highly expressed in bronchial epithelial cells, alveolar macrophages and interstitial fibroblasts (Figure 5B, panels a–c). sPLA₂-X was also widely detected in alveolar and bronchial epithelial cells as well as in alveolar macrophages (Figure 5C, panels a–c). sPLA₂-IID was located in bronchial

Figure 4 AA metabolism by various PLA₂s in NHPF

(A, B) Individual sPLA₂s stably expressed in NHPF cells by the lentivirus system were detected by Northern blotting (A) and immunoblotting under non-reducing conditions (B). Cells infected with adenovirus for sPLA₂-IIF were also subjected to immunoblotting under both reducing [2-mercaptoethanol (+)] and non-reducing [2-mercaptoethanol (-)] conditions (B) MOI, multiplicity of infection. (C) Confocal laser microscopic analyses of sPLA₂s expressed in NHPF. Fluorescent signals for individual sPLA₂s stably transfected into NHPF by the lentivirus method were visualized with specific antibodies ($\times 100$ magnification) (panel a). Parental cells showed no appreciable staining (panel a). Magnified views ($\times 400$) of the staining of sPLA₂-IIA and -X are shown in panel b. sPLA₂-IIA was mainly located in the Golgi apparatus (arrows), which was verified by double immunostaining with the Golgi marker GM130 (results not shown), whereas sPLA₂-X was also distributed in the ER (light blue arrow). Signal for sPLA₂-IIA was detected equally in cells before and after treatment with 1 mg/ml heparin ($\times 100$) (panel c). Signal for sPLA₂-IIF, which was transfected into NHPF by the adenovirus method, was found in the Golgi as well as in the cytosolic aggregates (arrowheads, panel d). (D) sPLA₂ activities in the supernatant (S) and membrane-associated (C) fractions of the transfectants obtained by the lentivirus (for sPLA₂-IIA, -V and -X) or adenovirus (for sPLA₂-IIF) method. In the latter fraction, sPLA₂s were solubilized from cell surfaces by treatment with medium containing 1 M NaCl. (E) [³H]AA release from NHPF stably transfected with individual PLA₂s after incubation for 4 h in culture medium. Addition of cytokines did not augment [³H]AA release by sPLA₂s (results not shown). (F) PGE₂ production by NHPF stably transfected with PLA₂s after incubation for 4 h with or without various cytokines. (G) PGE₂ production in NHPF transfected with various PLA₂s by means of the adenovirus system. The cells were infected for 36 h with various doses of adenoviruses bearing PLA₂s or control (LacZ) and then stimulated for 12 h with IL-1 β . Inset: expression of individual PLA₂s was

Figure 5 Immunohistochemical localization of sPLA₂s in human lungs affected by pneumonia

(A) Staining of sPLA₂-IIA. Bronchial chondrocytes (panels a and b, green arrow) and arterial VSMC (panels a and d, pink arrows) were positively stained, whereas alveolar macrophages (panel c, yellow arrows) and bronchial epithelial cells (panel a) were negative. Areas with no or mild inflammation showed no sPLA₂-IIA staining (panel e). (B) Staining of sPLA₂-V. Bronchial epithelial cells (panels a and b, red arrowheads), interstitial fibroblasts (panel b, light blue arrowhead) and alveolar macrophages (panel c, yellow arrowheads) were intensely positive, whereas arterial VSMC were not stained (panel a). Although sPLA₂-V staining was weak in areas with no or mild inflammation (panel d), scattered expression was found in bronchial epithelial cells (panel e). (C) Staining of sPLA₂-X. Alveolar epithelial cells, macrophages (panels a and b) and bronchial epithelial cells (panel c) were positively stained for sPLA₂-X. sPLA₂-X was also detected in the interstitium beneath the bronchial epithelium, but not in VSMC, in the control lungs (panel d). Scattered staining of sPLA₂-X was also evident in the alveolar epithelium of the control lungs (panel e). (D) Staining of sPLA₂-IID. Bronchial, but not alveolar, epithelial cells (panels a and b) as well as alveolar macrophages (panels b and c), were positively stained. Mildly inflamed lesions were not stained (panel d). No appreciable staining for sPLA₂-IIE and -IIF was observed (results not shown).

epithelial cells and alveolar macrophages, although it was poorly detected in alveolar epithelial cells (Figure 5D, panels a–c). No obvious staining of sPLA₂-V (Figure 5B), -X (Figure 5C) and -IID (Figure 5D) was observed in VSMC, which contrasts with VSMC localization of sPLA₂-IIA (Figure 5A). Control lungs with no or mild inflammation generally yielded no or weak staining for sPLA₂-IIA (Figure 5A, panel e) and -IID (Figure 5D, panel d). Similarly, the expression of sPLA₂-V in the control lungs was scarce, except that the scattered immunoreactivity was detected in the bronchial epithelium (Figure 5B, panels d and e). The alveolar epithelium and interstitial tissues beneath the bronchial epithelium in control lungs showed positive staining for sPLA₂-X (Figure 5C, panels d and e). This suggests the constitutive expression of sPLA₂-X (relative to the marked inducibility of the group II subfamily sPLA₂s) in human lungs, even though its staining was more obvious in the inflamed lungs. Staining for sPLA₂-IIE and -IIF was negligible throughout the tissues, in spite of severe inflammation (results not shown).

DISCUSSION

Accumulation of sPLA₂-IIA in inflamed tissues has been demonstrated in several animal models of inflammation and in human

inflammatory diseases such as rheumatoid arthritis, sepsis and Crohn's disease [1,2,6]. Although recent studies have provided evidence that the transcripts for various sPLA₂s are also induced during inflammatory responses in experimental animal models [1,2], the expression, cellular origins and functions of each sPLA₂ in human pathologic tissues still remain obscure. In the present study, we examined the localization of six sPLA₂s in the lungs of humans suffering from pneumonia by immunohistochemistry. Furthermore, we evaluated the potential contribution of these sPLA₂s to AA metabolism in cultured cells originating from human lung by taking advantage of virus-mediated sPLA₂ transfer. Our results reveal that various sPLA₂s are expressed in human inflamed lungs, in which they exhibit unique cellular distributions, that there are cell-type-specific machineries for expression of individual sPLA₂s in human lung-derived cells with or without particular stimuli, and that the abilities of individual sPLA₂s to augment AA metabolism differ in these cells.

On the basis of studies using animal models of lung disorder, it has long been thought that sPLA₂-IIA is induced and participates in lung pathology [14,40]. In fact, fibroblasts and macrophages derived from rat and guinea pig lungs express sPLA₂-IIA in response to proinflammatory stimuli [14,40]. However, we show in the present study that the expression of sPLA₂-IIA in human

assessed by Northern blotting. (H) Adenoviruses for WT (wild-type) sPLA₂-X, its catalytically inactive mutant (Gly³⁰ → Ser) and control were infected (MOI = 10) into NHPF, and PLA₂ activity in the supernatants (upper panel) and PGE₂ production after stimulation for 12 h with IL-1 β (lower panel) were assessed. Inset: expressions of WT and mutant sPLA₂-X were verified by Northern blotting. Representative results of 3–5 independent experiments are shown in (A–D) and (H) and values are means $\pm$ S.E.M. for four independent experiments in (E–G).

lungs affected by pneumonia is restricted to pulmonary VSMC and bronchial chondrocytes (Figure 5A). It is unlikely that the lack of sPLA₂-IIA immunoreactivity in the airway epithelium and alveolar macrophages is due to technical failures, since it is intensely detected in rheumatoid arthritic joints [40a], intestinal Paneth cells (S. Masuda, M. Murakami, T. Ishii, T. Ishikawa and I. Kudo, unpublished work) and prostate epithelium [40b], in which sPLA₂-IIA has been shown to be expressed [41–43]. Location of sPLA₂-IIA in the pulmonary VSMC is consistent with the immunohistochemical staining of VSMC in human atherosclerotic foci [12,13], and our immunohistochemical analyses also reveal that this enzyme is commonly expressed in VSMC in a variety of human tissues [40a]. Location of sPLA₂-IIA in bronchial chondrocytes is reminiscent of its presence in cartilage chondrocytes in rheumatoid arthritis joints (S. Masuda, M. Murakami, K. Komiyama, T. Ishii, T. Ishikawa and I. Kudo, unpublished work), indicating, together with the *in vitro* evidence that cultured chondrocytes express sPLA₂-IIA in response to cytokines [44], that this cell type is another common source of sPLA₂-IIA in inflamed human tissues. However, we do not rule out the possibility that sPLA₂-IIA is induced in cells other than pulmonary VSMC and bronchial chondrocytes in different types of human lung disease, such as those accompanying T_H response, since induction of sPLA₂-IIA occurs in IFN- γ -stimulated NHPF (Figure 1A).

Our immunohistochemical analyses indicate that the major sPLA₂s expressed in human airway epithelium are sPLA₂-V and -X (Figures 5B and 5C). Expression of these two enzymes in the airway epithelium is already evident in lung areas with no or mild inflammation, and the expression of sPLA₂-V, in particular, is markedly increased at severely inflamed sites. In support of this finding, Seeds et al. [45] have recently reported that the transcripts for these two sPLA₂s, but not sPLA₂-IIA, are detected in normal human lung epithelial cells by means of *in situ* hybridization, and Hanasaki et al. [23] have demonstrated by immunohistochemistry that sPLA₂-X is distributed in human alveolar epithelial cells. Severely inflamed regions are enriched in haematopoietic cells, including alveolar macrophages and granulocytes, which are strikingly positive for sPLA₂-V and -X. Expression of sPLA₂-V and -X, but not -IIA, in isolated human neutrophils [30] also supports our results. sPLA₂-V is also expressed in interstitial fibroblasts in inflamed lungs (Figure 5B). In addition, sPLA₂-IID is located in bronchial epithelial cells and alveolar macrophages in the inflamed lungs (Figure 5D), consistent with a recent report that this enzyme is detected in the airway epithelium and infiltrating eosinophils of asthmatic patients [46].

To assess the possible contribution of sPLA₂s to the production of eicosanoids, which exert both detrimental and beneficial effects on lung pathogenesis as shown by studies using knockout mice for cPLA₂ α [47], COXs [48] and 5-lipoxygenase [49], we examined the expression and AA-releasing and PGE₂-biosynthetic functions of sPLA₂s in two types of cultured human lung-derived cells: the bronchial epithelial cell line BEAS-2B and NHPFs. The expression profiles of sPLA₂s endogenously expressed in these cultured cells with or without cytokine stimulation (Figure 1A) are similar to, even if not entirely identical with, those expressed in the respective cells in human lungs with pneumonia (Figure 5). The observed difference is probably because the supplementary factors required for proper expression of individual sPLA₂s for *in vivo* microenvironments and *in vitro* culture are different. It is also conceivable that, since BEAS-2B cells are transformed with adenovirus-SV40 hybrid, an intrinsic feature of this cell line may not be entirely identical with normal bronchial epithelial cells in terms of the expression of sPLA₂s and other eicosanoid-biosynthetic enzymes.

Irrespective of these limitations, our virus-mediated gene transfer study revealed that sPLA₂-V and -X have the capacity to augment PGE₂ synthesis when they are overexpressed in these two cell types (Figures 2–4). The observed sPLA₂ selectivity, along with several other lines of evidence, suggests that the action of sPLA₂s on airway epithelial cells and fibroblasts occurs through the external plasma membrane pathway, which depends on the ability of sPLA₂s to interact with the PC-rich outer leaflet of the plasma membrane [8,20,23,24]. Indeed, 10–50 ng/ml sPLA₂-V and -X can induce AA release when added exogenously to various cells [20,23,24]. Alternatively, sPLA₂-V and -X may act on a putative PC-rich microdomain in the Golgi membrane (in which sPLA₂s are enriched) before secretion [9]. Although we have not tested the effects of sPLA₂-V and -X on human alveolar macrophages in which these two enzymes are highly expressed (Figures 5B and 5C), several previous studies using macrophages or macrophage-like cell lines have demonstrated increased AA release and eicosanoid production by these two sPLA₂s [21,23]. Moreover, Arm and co-workers [50] have recently shown that peritoneal macrophages from sPLA₂-V-deficient mice produce less eicosanoids when compared with those from wild-type mice. In contrast, failure of sPLA₂-IIA and -IID to increase AA metabolism suggests that the HSPG-shuttling pathway does not occur in bronchial epithelial cells and lung fibroblasts. This conclusion is supported by the observation that the intracellular localization of sPLA₂-IIA was unaffected by treatment with heparin (Figures 2C and 4C), which blocks HSPG-dependent internalization of heparin-binding sPLA₂s into several cell types [7,8]. In this context, the functions of sPLA₂-IID, which is expressed (Figure 5D) but is unable to augment PGE₂ biosynthesis in bronchial epithelial cells (Figures 2–4), might be associated with events unrelated to eicosanoid production, although the possibility that some other untested stimuli can trigger sPLA₂-IID-mediated eicosanoid production should be considered.

The present study also provides new insight into a unique property of sPLA₂-IIF. Although the expression of sPLA₂-IIF in human lungs with pneumonia is negligible (Figure 5F), this enzyme can augment PGE₂ synthesis efficiently when transfected into human lung-derived cells (Figures 2–4). This finding substantiates the previous proposal (as evidenced by a study using HEK-293 cells [19]) that the potency of sPLA₂-IIF to mobilize cellular AA is similar or next to that of sPLA₂-V and -X and is superior to that of sPLA₂-IIA. Unexpectedly, we found that this enzyme exists in higher-molecular-mass forms compared with the predicted size and is located in unusual cytoplasmic aggregates in NHPF (Figure 5C). Formation of these aggregates appears to be cell-type-specific, since it was not observed in HEK-293 [19] and BEAS-2B (results not shown) transfectants. Although the identity of the higher-molecular-mass forms, the mechanism for the formation of the cytoplasmic aggregates and their physiological relevance are currently unknown, this unique feature might be related to an unexplored regulatory mechanism for this enzyme that needs to be addressed.

In summary, our tissue expression and functional studies suggest that sPLA₂-V and -X may participate in human lung pathology. During altered pathological states, lipid mediator production, bacterial killing [10,11] and surfactant hydrolysis [14,50] by sPLA₂s may co-ordinately promote inflammatory reactions and participate in the processes that lead to lung tissue injury or repair. Although the concentrations of individual sPLA₂s in human lungs affected by pneumonia are currently unknown, several biological fluids, such as tears, seminal plasma and rheumatoid synovial fluid, often contain sPLA₂-IIA in μ g/ml levels [1,2]. Thus, the local concentrations of sPLA₂-V and -X, and possibly of other sPLA₂s, in human lungs in various pathological states could

reach levels that are achieved by our transfection strategy. It is also theoretically possible that sPLA₂-V and -X secreted from the airway epithelium and alveolar macrophages may act on neighbouring leukocytes to promote transcellular eicosanoid synthesis. Nevertheless, beyond species-associated differences in the expression profiles of individual sPLA₂s, more insights into their roles in pathophysiology will be clarified by forthcoming studies using sPLA₂ gene-manipulated mice.

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