

# Compensatory role of inducible annexin A2 for impaired biliary epithelial anion-exchange activity of inflammatory cholangiopathy

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The peribiliary inflammation of cholangiopathy affects the physiological properties of biliary epithelial cells (cholangiocyte), including bicarbonate-rich ductular secretion. We revealed the upregulation of annexin A2 (ANXA2) in cholangiocytes in primary biliary cirrhosis (PBC) by a proteomics approach and evaluated its physiological significance. Global protein expression profiles of a normal human cholangiocyte line (H69) in response to interferon- $\gamma$  (IFN $\gamma$ ) were obtained by two-dimensional electrophoresis followed by MALDI-TOF-MS. Histological expression patterns of the identified molecules in PBC liver were confirmed by immunostaining. H69 cells stably transfected with doxycyclin-inducible ANXA2 were subjected to physiological evaluation. Recovery of the intracellular pH after acute alkalinization was measured consecutively by a pH indicator with a specific inhibitor of anion exchanger (AE), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS). Protein kinase-C (PKC) activation was measured by PepTag Assay and immunoblotting. Twenty spots that included ANXA2 were identified as IFN $\gamma$ -responsive molecules. Cholangiocytes of PBC liver were decorated by the unique membranous overexpression of ANXA2. Apical ANXA2 of small ducts of PBC was directly correlated with the clinical cholestatic markers and transaminases. Controlled induction of ANXA2 resulted in significant increase of the DIDS-inhibitory fraction of AE activity of H69, which was accompanied by modulation of PKC activity. We, therefore, identified ANXA2 as an IFN $\gamma$ -inducible gene in cholangiocytes that could serve as a potential histological marker of inflammatory cholangiopathy, including PBC. We conclude that inducible ANXA2 expression in cholangiocytes may play a compensatory role for the impaired AE activity of cholangiocytes in PBC in terms of bicarbonate-rich ductular secretion and bile formation through modulation of the PKC activity.

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Primary biliary cirrhosis (PBC) is characterized by chronic bile duct injury and cholestasis in the early disease stage, and its etio-pathogenesis is still unrevealed. Chronic inflammatory cells, especially CD8<sup>+</sup> and CD4<sup>+</sup> lymphocytes that infiltrate around the bile ducts, are considered to be associated with the progression of bile duct injury and cholestasis in PBC through membranous death receptors and cytokine receptors.<sup>1–4</sup> Proinflammatory cytokines secreted by these inflammatory cells, such as interleukin (IL)-6, interferon- $\gamma$  (IFN $\gamma$ ), tumor necrosis factor- $\alpha$  and IL-1, would also cause cholestasis by means of impairment of the ductular secretion through their inhibitory effect on cyclic adenosine monophosphate (cAMP) formation, anion exchanger (AE) activity and cAMP-dependent Cl<sup>-</sup> efflux.<sup>5</sup> These findings

may be related to the manifestation of cholestasis in the initial disease stage without any evident features of chronic non-suppurative destructive cholangitis. The global profiling technique is regarded as a powerful methodology for the framing of a hypothesis. In the current study, we employed proteomics for the profiling of biliary epithelial cells, cholangiocytes, in terms of Th1 cytokine responses in order to screen for novel biomarkers of inflammatory cholangiopathy and to provide insight into the mechanism of its pathophysiology.

Annexin-A2 (ANXA2), one of the candidate molecules based on the results of proteomics applied to H69 cells, is a Ca<sup>2+</sup>- and acidic phospholipid-binding protein involved in many cellular processes. ANXA2, which can associate with

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caveolae, has been shown to form a lipid-protein complex and cholesteryl esters that seem to be involved in the internalization/transport of cholesteryl esters from caveolae to internal membranes.<sup>6-10</sup> ANXA2 is also required for transport of secretory vesicles in adipocytes and exocytic vesicles in polarized epithelial cells.<sup>11</sup> It has been investigated extensively in the field of cardiovascular system and has various roles, including coagulopathy.<sup>12</sup> In contrast to cardiovascular system, the role of ANXA2 in the hepato-biliary systems is not well understood. In terms of alcoholic liver diseases, an increase of parenchymal ANXA2 expression and a fibrinolytic role in the liver has been proposed.<sup>13</sup>

Apical expression of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> anion exchanger-2 (AE2: SLC4A2 as an official gene symbol) plays a pivotal role in the regulation of the intracellular pH (pH<sub>i</sub>)<sup>14</sup> in conjunction with the biliary HCO<sub>3</sub><sup>-</sup> secretion and bile formation by cholangiocytes.<sup>15</sup> Faint expression of AE2 in the cholangiocytes of PBC livers<sup>16</sup> may suggest some potential roles of AE in the development of PBC, which is further supported by cholangiopathy characterized by portal inflammation and bile duct injury observed in AE-deficient mice.<sup>17</sup> Therefore, in the current study, we focused on the pathophysiological relevance of ANXA2 to biliary excretion in terms of anion-exchange activity.

## MATERIALS AND METHODS

### Materials

The reagents for the cell culture, electrophoresis and mass spectrometry were purchased from Wako Pure Chemical Industries Ltd. (Japan), excluding Bio-Lyte<sup>®</sup>3/10 carrier ampholyte, ReadyStrip IPGStrips, PROTEAN IEF Cell, Tris/HCl (pH 8.8), ReadyGel J and flamingo gel stain (Bio-Rad, Hercules, CA, USA), Typhoon Variable Mode Imager, ImageMaster 2DPlatinum and Ettan SpotPicker (GE Healthcare, Piscataway, NJ, USA),  $\alpha$ -cyano-4-hydroxy-cinnamic acid, doxycycline (DOX), 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), IFN $\gamma$  (Pierce Endogen, Rockford, IL, USA), adenosine 5'-[ $\gamma$ thio]triphosphate tetralithium salt (ATP $\gamma$ S), 3-isobutyl-1-methylxanthine (IBMX), propionic acid and Krebs-Ringer bicarbonate (KRB) (Sigma-Aldrich, St Louis, MO, USA), Voyager-DE STR Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA), 3'-O-Acetyl-2',7'-bis(carboxyethyl)-4,5-carboxyfluorescein, diacetoxymethyl ester (BCECF-AM) (Molecular Probes, Eugene, OR, USA), FluoroskanAscent (MTX Lab Systems, Vienna, Virginia), phorbol-12-myristate-13-acetate (PMA), bisindolylmaleimide-I (Go6850, BIS) (Calbiochem, Darmstadt, Germany) and forskolin (Biomol, Plymouth Meeting, PA, USA). Anti-ANXA2 (monoclonal antibody (AB); BD Bioscience, San Jose, CA, USA), anti-AE2 (polyclonal AB; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cyclin-I and anti-Epacl (polyclonal AB; GeneTex Inc., San Antonio, TX, USA), anti-enolase-1 (monoclonal AB; Abnova Corporation, Taipei, Taiwan), anti-Hsp70 (monoclonal AB; Stressgen, Ann Arbor, MI, USA), anti- $\beta$ -actin (monoclonal

AB; Sigma), the phospho-protein kinase-C (PKC) Antibody Sampler kit (polyclonal AB; Cell Signaling, Danvers, MA, USA), N-Histofine Simple Stain MAXPO (NICHIREI BIOSCIENCES INC., Tokyo, Japan), VECTOR NovarRed (VECTOR, Burlingame, CA, USA) were used for immunoblotting and immunostaining. The Alexa<sup>-</sup>-labeled secondary antibodies for immunofluorescence were purchased from Molecular Probes. The materials for construction of expression vectors and transfection experiments were all purchased from Takara Bio Inc. (Japan), except for Tet-On System (Clontech, Mountain View, CA, USA), RNAeasy kit, ANXA2 siRNA, All Stars Negative Control siRNA (Qiagen, the Netherlands), pT7Blue T vector (Novagen Inc., Madison, WI, USA), TransIT<sup>®</sup>-LT1 Transfection Reagent (Mirus, Madison, WI, USA), G418 (Geneticin) and hygromycin (Invitrogen Corporation, Carlsbad, CA, USA). PepTag Assay for Non-Radioactive Detection of Protein Kinase C or cAMP-Dependent Protein Kinase (PepTag Assay) was purchased from Promega Corporation (Madison, WI, USA).

### Methods

#### Cell culture and Sample preparation for 2-dimensional electrophoresis

The H69 human cholangiocyte line was cultured in Dulbecco's Modified Eagle's Medium<sup>18</sup> with/without IFN $\gamma$  at a concentration of 1000 IU/ml for 24 h at 37°C in CO<sub>2</sub> incubators. Whole-cell lysates were prepared for two-dimensional electrophoresis (2DE) in the degenerate extraction buffer consisting of 8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS), 50 mM dithiothreitol (DTT) and 0.2% Bio-Lyte3/10 carrier ampholyte.

#### 2DGE and protein profiling

A 75-g weight of each sample was loaded on the ReadyStrip IPGStrips (7 cm, pH 5–8). Isoelectric Focusing was performed under conditions recommended by the manufacturer, briefly at 250 V for 20 min, then voltage gradually shifted to 4,000 V for 2 h, followed by 4,000 V for 10 h, finally 500 V for 24 h using PROTEAN IEF Cell. IPGStrips were equilibrated twice in sodium dodecyl sulfate (SDS)-containing buffers consisting of 6 M urea, 2% SDS, 0.05 M Tris/HCl (pH 8.8), 20% glycerol, DTT for the first step and iodoacetamide for the second step. Then the strips were placed onto the top of ReadyGel J (5–20%) for 2D SDS-PAGE. Separated proteins were visualized using the Flamingo fluorescent gel stain and the Typhoon Variable Mode Imager. Corresponding spots of the IFN $\gamma$ -stimulated and control samples were automatically determined by the ImageMaster 2DPlatinum ver. 5.0. Spots with fluorescence intensity that changed significantly in comparison with the control were automatically determined by the ImageMaster 2DPlatinum ver. 5.0 and excised by Ettan SpotPicker. After succeeding processes of in-gel digestion, desalting and mixture with the matrix ( $\alpha$ -cyano-4-hydroxy-cinnamic acid), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was

performed with the Voyager-DE STR Biospectrometry Workstation. Each unique mass spectrum was applied to database search for lining up of the candidates. Identical proteins were determined subsequently by the combination of probability, pI and molecular weight.

#### *Immunohistochemistry, immunoblotting and immunofluorescence analysis*

Under the approval of the institutional review board, liver specimens were obtained from 20 PBC patients, 20 chronic hepatitis-C (CHC) patients as disease controls and 10 donors for liver transplantation as normal controls. The usage of these specimens was approved by Institutional Review Board (2006-59) along with the written informed consent from the patients. The samples were fixed immediately in 4% paraformaldehyde (PFA) and embedded in paraffin. For immunohistochemistry, after overnight incubation with the specific antibodies and succeeding incubation with the corresponding peroxidase-labeled secondary antibodies, the expressions of the target molecules were visualized according to the manufacturer's protocol. Histological stages of PBC were determined according to Scheuer's criteria.<sup>19</sup> ANXA2 expression in cholangiocytes was classified into three grades and evaluated by two independent pathologists. Electrophoresis and immunoblotting were performed according to the standard protocol of chemiluminescence under degenerative condition. For immunofluorescence, the cells at subconfluency cultured in a glass-bottomed dish were fixed in 4% PFA for 1 h and permeabilized by 0.1% Triton-X. Fluorescence was observed by confocal microscopy (TE-2000; Nikon, Tokyo, Japan).

#### *Tet-On expression vectors and stable transfection*

The Tet-On vector for DOX inducible ANXA2 expression was constructed as follows:<sup>20</sup> The coding region of human ANXA2 was obtained by polymerase chain reaction (PCR) from cDNA derived from total RNA of H69 through reverse transcription and subsequently cloned into the pT7Blue T vector. The primers were designed based on the reference sequence (gi: 50845387). The cloned ANXA2 was inserted at *Sall*-*Bam*HI site of pTRE2Hyg (pTRE2-ANXA2). For preconditioning, H69 cells were stably transfected with pTet-On, which contains DOX-inducible *trans*-acting element, and subcloned after the 4 weeks of the G418 selection step. Suitable clones were selected by luciferase reporter assay with the DOX-inducible luciferase vector, pTRE2Hyg-Luc. The selected H69 clones were stably transfected with pTRE2-ANXA2 and subcloned through the hygromycin selection process. Final clones (H69-anxa2) were examined for the DOX-inducible ANXA2 synthesis by immunoblotting.

#### *Suppression of ANXA2's expression by siRNA*

Shortly before transfection, H69 cells were seeded at a concentration of  $2 \times 10^4$  cells per well in 96-well plates and  $2.5 \times 10^5$  cells per well in six-well plates. ANXA2 siRNA and

negative control siRNA were transfected at a concentration of 5 nM for 24 h. The amount of expression of ANXA2 was evaluated by western blotting for the determination of the suppressive efficacy of the siRNA.

#### *Measurement of AE activity*

The H69-anxa2 cells were assessed for their AE activity by measuring the recovery of  $\text{pH}_i$  from acute alkalization according to the established method.<sup>21</sup> In brief, for  $\text{pH}_i$  measurement, the cell culture medium was substituted with KRB and KRB-propionate buffers loaded with  $10 \mu\text{M}$  of BCECF-AM, and fluorescence intensity was consecutively measured by FluoroskanAscent at 488 nm for excitation and at 535 nm for emission. Rates of  $\text{pH}_i$  change ( $\text{dpH}_i/\text{dt}$ ) were compared under the following experimental conditions: preincubation with an AE inhibitor, DIDS, at a concentration of 1 mM, in the presence of the following agents under the indicated conditions of concentrations and incubation periods: PMA (PKC activator, 50 nM, 20 min), BIS (pan-PKC inhibitor,  $1 \mu\text{M}$ , 10 min), ATP $\gamma\text{S}$  ( $100 \mu\text{M}$ , 10 min), forskolin ( $100 \mu\text{M}$  15 min) with IBMX (0.5 mM, 20 min).

#### *PepTag Assay for non-radioactive detection of PKC activity*

For evaluation of PKC activity, confluent monolayers of H69 cells grown on dishes preincubated in PMA and/or ATP $\gamma\text{S}$  were rinsed twice in ice-cold phosphate-buffered saline and harvested by scraping on ice in  $500 \mu\text{l}$  of PKC extraction buffer according to the manufacturer's manual. The cells were lysed by mild sonication in the buffer and submitted to the PepTag Assay.

PKCs are matured by phosphorylation of multiple serine/threonine sites and further activated by diacylglycerol or its analogues. PepTag Assay is designed for quantification of the actual whole PKC activity with the usage of fluorescent peptide as the specific substrate for PKCs. Activated forms of PKCs change the net charge of the substrate from positive to negative, thereby allowing the phosphorylated and non-phosphorylated substrate to be separated by agarose gel electrophoresis. Cell lysates with the equivalent protein concentration were incubated with the PKC reaction mixture at  $30^\circ\text{C}$  for 30 min. The reactions were stopped by placing the tubes on a  $95^\circ\text{C}$  heating block. After adding 80% glycerol, the samples were electrophoresed in 0.8% agarose gel for 15 min at 100 V and visualized by UV light.

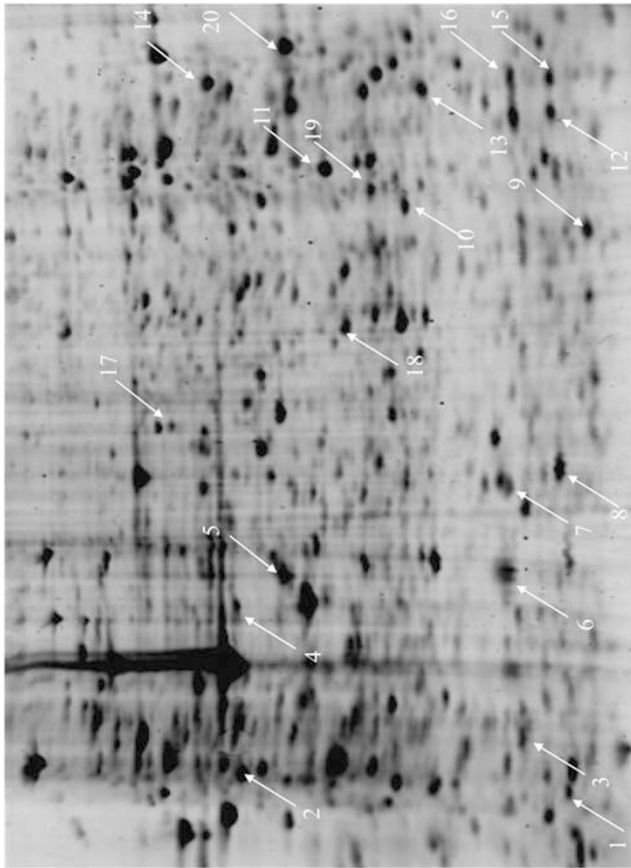
#### *Statistics*

Results were analyzed by paired *t*-test, Mann-Whitney's *U*-test, Kruskal-Wallis test with Bonferroni correction or Spearman's correlation. *P* values less than 0.05 were considered statistically significant.

## RESULTS

### Proteomics

Three hundred and seventy spots among the 1400 spots detected by 2DGE matched between IFN $\gamma$ -treated and



**Figure 1** A representative 2DGE image for expression profiling of H69 cells treated with IFN $\gamma$  is shown. The fluorescence intensities of the 20 spots indicated by the arrows changed significantly in comparison with the control. The intensities for the 16 spots numbered 1 to 16 increased and the four spots numbered 17 to 20 decreased, respectively. Paired *t*-test was applied to triplicate data sets analysis.

IFN $\gamma$ -untreated groups of the H69 cells. The fluorescence intensities of 16 spots (numbers 1 to 16) treated with IFN $\gamma$  were significantly increased compared with that of the untreated spots, and the fluorescence intensities of four spots (numbers 17 to 20) were significantly decreased (Figure 1). Proteins identified by MALDI-TOF-MS and peptide mass fingerprinting (PMF) are listed in Table 1.

### Cellular and Subcellular ANXA2 Expression of the PBC Liver

Among these inflammation-related molecules, we focused on the IFN $\gamma$ -inducible molecules as candidates of cholangiopathy. Five of 16 molecules were evaluated for expression in the liver specimens by immunohistochemistry (Figures 2 and 3). ANXA2 showed a clear and unique expression pattern in the liver, which was characterized by almost exclusive expression in the cholangiocytes of normal livers as well as parenchymal expression especially in the periportal area of PBC or CHC liver with infiltration of inflammatory cells. In accordance with the *in vitro* results, the expression of ANXA2

in cholangiocytes was augmented in PBC liver that was accompanied by peribiliary inflammation (Figure 3). Other molecules detected by TOF-MS were evaluated with regard to cyclin-I, EPAC1, enolase-1 and HSP70 by immunohistochemistry (Figure 2). Cyclin-I was expressed almost exclusively in hepatocytes both in PBC and normal liver. EPAC1 and enolase-1 were expressed equally in biliary epithelial cells of PBC and normal liver. The expression of HSP70 was not clear by the usual immunohistochemistry in these liver specimens. ANXA2 was subcellularly distributed mainly membranously and partly in the cytosol of cholangiocytes in the PBC liver (Figure 3e–g). Especially, its apical expression was unique and other physiological roles of this molecule other than those coagulation-related were speculated. With regard to PBC livers, subcellular localization of ANXA2 to the apical membrane of cholangiocyte was positively correlated with the clinical markers of cholestasis and liver injury such as the serum concentration of alkaline phosphatase,  $\gamma$ -glutamyltranspeptidase, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total cholesterol (Table 2). Thereby, its expression was likely to be related to the cholestatic status.

### Effect of Controlled Induction of Anxa2 to AE Activity

To investigate the significance of ANXA2 expression in cholangiocytes, we constructed systems for the controlled induction of ANXA2 in H69 cells under the Tet-On system. We speculated that the apical localization of ANXA2 in correlation to cholestasis might have a close relation to the AE activity, which was involved in bile formation by the apical domain of cholangiocytes. AE activity was assessed in the ordinary manner, by measurement of DIDS-sensitive intracellular pH recovery from acute intracellular alkalization (Figure 4). Induction of ANXA2 under DOX *trans*-activating promoter led to a 2.5–3fold increase of the pH<sub>i</sub> recovery, which was sensitive to DIDS (Figure 4a). IFN $\gamma$  and ATP $\gamma$ S supplemented in the culture media of the cells attenuated the AE activity, which was partly restored by induction of ANXA2 (Figure 4b, c, d). In contrast, PMA also reduced the AE activity, which was not rescued by ANXA2 expression. BIS and forskolin did not alter the AE activity, regardless of the status of ANXA2. Therefore, ANXA2 was considered to have contributed to the restoration of impaired AE activity probably through some mechanisms that involve PKC. In contrast, ANXA2 expression and pH<sub>i</sub> recovery were significantly decreased simultaneously against the negative control by ANXA2 siRNA to 46.9% and 57.3%, respectively (Figure 4e–g).

### AE2 Expression in H69 Cells

The AE activity is mainly assumed to occur by AE2 expression in cholangiocytes.<sup>15,22</sup> To test the hypothetical ANXA2–AE2 pathway, we evaluated the AE2 status in H69. Immunofluorescence and immunoblotting revealed that incubation with IFN $\gamma$  or induction of ANXA2 did not alter

**Table 1 Expression profiling by MALDI-TOF-MS and peptide-mass finger printing**

Spot number	Gene name	GenBank accession number	Gene ontology
1	DGCR8	gi: 50415809	Transcription
2	HSP70	gi: 4529893	Anti apoptosis
3	Amilorid-sensitive cation channel 3	gi: 9998946	Transport
4	STAT6	gi: 58737047	Transcription
5	Axonormal dynein heavy chain-7	gi: 17864092	Trafficking
6	Tryptophanyl-tRNA synthetase	gi:47419918	Negative control of cell proliferation.
7	Lines homolog	gi: 95007028	Transcription
9	EPAC1	gi: 32171400	Cell proliferation
10	Tyrosine-protein kinase BTK isoform52	gi: 63147587	Apoptosis
11	Annexin A2	gi: 18088908	
12	Elastin microfibril interfacter-3	gi: 71681796	Cell adhesion
13	Chondroitin sulfate glucuronyltransferase	gi: 48717495	Transferase
14	Enolase-1	gi: 45035371	Negative control of cell proliferation.
15	Cyclin-I	gi: 54696900	Cyclin family
16	CLCA homolog	gi: 4572289	Transport
17	HNRPH1	gi: 48145673	Transcription
18	C-type lectin superfamily-4	gi: 38348296	Cell adhesion
19	Triosephosphate isomerase-1	gi: 17389815	Fatty-acid biosynthetase
20	Myo-inositol monophospatase-A3	gi: 17389522	Hydrolase

Abbreviations: MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

either the amount of expression or subcellular localization, that is, to the cell-surface membrane, of AE2 in polarized H69 cells (Figure 5a and b). Therefore, it was speculated that inducible ANXA2 might restore the AE activity that was impaired under the inflammatory conditions through mechanisms other than changes in the expression or the subcellular localization in biliary epithelial cells or cholangiocytes.

#### AE Activity, ANXA2 and Involvement of PKC Pathway

The AE activity was previously reported to be affected by PKC through phosphorylation of the serine/threonine residue of AE.<sup>23</sup> Similar to PKC, ANXA2 is a calcium-dependent and membrane-associated molecule, which led us to hypothesize that PKC might be involved in the restoration of AE activity by ANXA2. Actually, PMA decreased the AE activity in H69 cells (Figure 4c), in concordance with the redistribution of AE2 protein from the plasma membrane to the intracellular space (Figure 5b). Consistent with our hypothesis, the induction of ANXA2 reduced the PKC activity, as revealed by PepTag Assay (Figure 6a) and immunoblotting (Figure 6b and c). The extracellular ATP level unexpectedly decreased the AE activity and the induction of ANXA2 partially recovered the diminished AE activity (Figure 4d), which was accompanied by reduction of PKC activity (Figure 6a). Especially, reduction of matured nPKC was

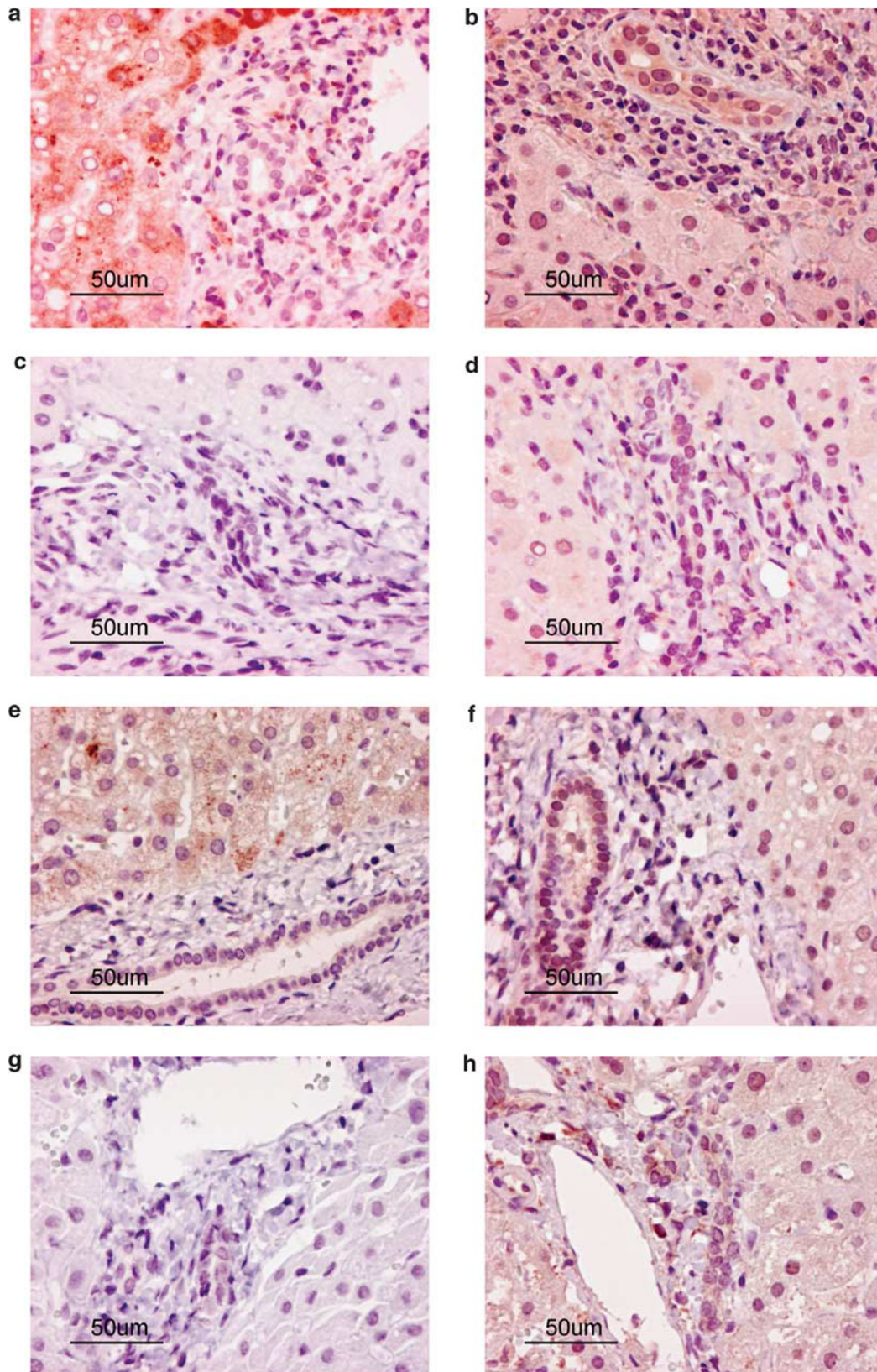
evident compared with that of the other subtypes of PKC (Figure 6b, c).

#### DISCUSSION

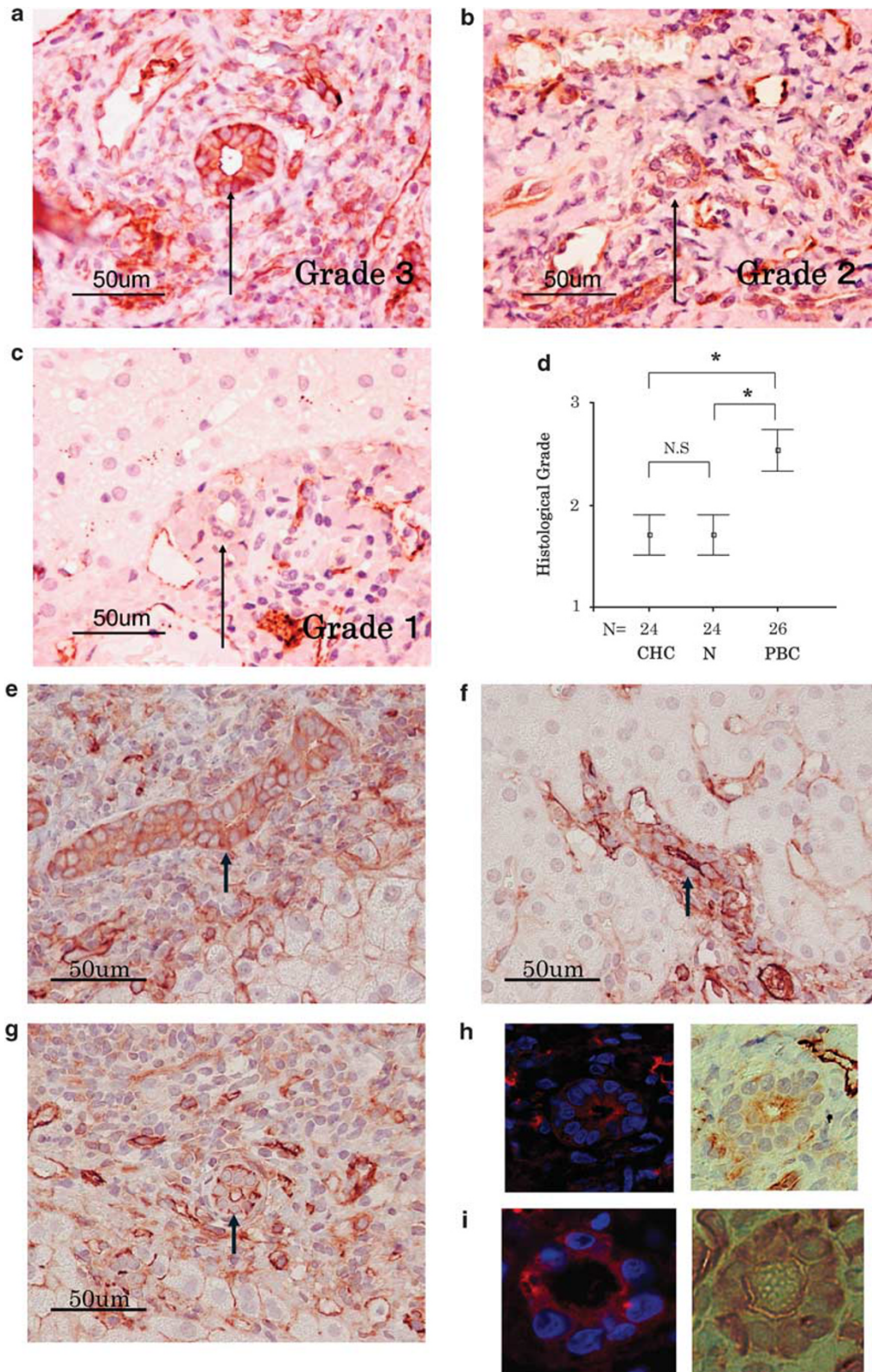
In the current research, we proposed 20 molecules as candidates, which might play roles in the development of cholangiopathy.<sup>24–37</sup>

The heat-shock 70-kDa protein (HSP70) is a member of HSPs, which are molecular chaperones that control protein folding and prevent aggregation.<sup>38</sup> Axonemal dynein heavy chain-7 is a force-generating protein of respiratory cilia<sup>39</sup> and dynein is supposed to facilitate the trafficking of AQP1-containing vesicles in cholangiocytes.<sup>40</sup> The exchange factor directly activated by cAMP-1 (EPAC1) is a guanine nucleotide-exchange factor and a second messenger of cAMP that leads to cell proliferation.<sup>41</sup> Enolase-1, together with its role in glycolysis, plays a role in various processes such as growth control, hypoxia tolerance and allergic responses, and may also function in the fibrinolytic system as a receptor and activator of plasminogen on the cell surface.<sup>42</sup> The expression of enolase-1 is assumed to be involved in cholangiocytes destruction and relevant to the disease progression in PBC.<sup>43</sup>

ANXA2 is a membranous acidic phospholipid-binding protein that contains putative binding sites for Ca<sup>2+</sup>, and is involved in many cellular processes.<sup>44,45</sup> It undergoes Ca<sup>2+</sup>-mediated membrane bridging and has been demonstrated to



**Figure 2** Four candidate molecules were evaluated for expression in the human liver specimen, stained with anti-cyclin-I (a, e), anti-Epac1 (b, f), anti-HSP70 (c, g) and anti-enolase-1 (d, h) ABs. (a-d) PBC liver and (e-h) normal liver.



**Figure 3** Human cholangiocytes expressed ANXA2. Representative images for the grading of expression of ANXA2 in cholangiocytes are shown ((a) grade 3, (b) grade 2 and (c) grade 1). Expression of ANXA2 was rather strong in the cholangiocytes of PBC liver as compared with that in the CHC or normal liver (d), according to the grading. Subcellular localization of ANXA2 was evaluated by immunohistochemistry in cholangiocytes of PBC livers. A representative staining pattern of cholangiocytes of PBC livers with predominant cytosolic (e) and membranous staining: apical (f) and basolateral staining (g) are shown. Immunofluorescence images also clearly demonstrate the subcellular localization of ANXA2 (h, i). Data sets were analyzed by Kruskal–Wallis test and Mann–Whitney *U*-test with Bonferroni correction; \* = *P* < 0.05.

**Table 2 Correlation between clinical features and subcellular localization of ANXA2 in cholangiocytes**

	Ductules (<20 μm)			Interlobular ducts (20–100 μm)			Septal bile ducts (>100 μm)		
	Apical	Basolateral	Cytosol	Apical	Basolateral	Cytosol	Apical	Basolateral	Cytosol
Stage	—	—	—	—	—	—	—	—	—
Age	—	—	—	—	—	—	—	—	—
T-Bil	—	—	—	—	—	—	—	—	—
ALP	—	—	—	—	—	—	rs = 0.805*	—	—
GGT	—	—	—	rs = 0.866*	—	—	rs = 0.866*	—	—
AST	—	—	—	rs = 0.805*	—	—	rs = 0.805*	—	—
ALT	—	—	—	rs = 0.805*	—	—	rs = 0.805*	—	—
T-CHO	—	—	—	—	—	—	rs = 0.805*	—	—
TG	—	—	—	—	—	—	—	—	—
PLT	—	—	—	—	—	—	—	—	—
PT(INR)	—	—	—	—	—	—	—	—	—
IgM	—	—	—	—	—	—	—	—	—
AMA-M2	—	—	—	—	—	—	—	—	—
Hyaluronan	—	—	—	—	—	—	—	—	—

Abbreviations: —, not significant; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMA, anti mitochondria antibody; AST, aspartate aminotransferase; GGT,  $\gamma$ -glutamyl transferase; IgM, immunoglobulin M; PLT, platelet; PT, prothrombin time; T-Bil, serum concentration of total bilirubin; T-CHO, total cholesterol; TG, triglyceride.

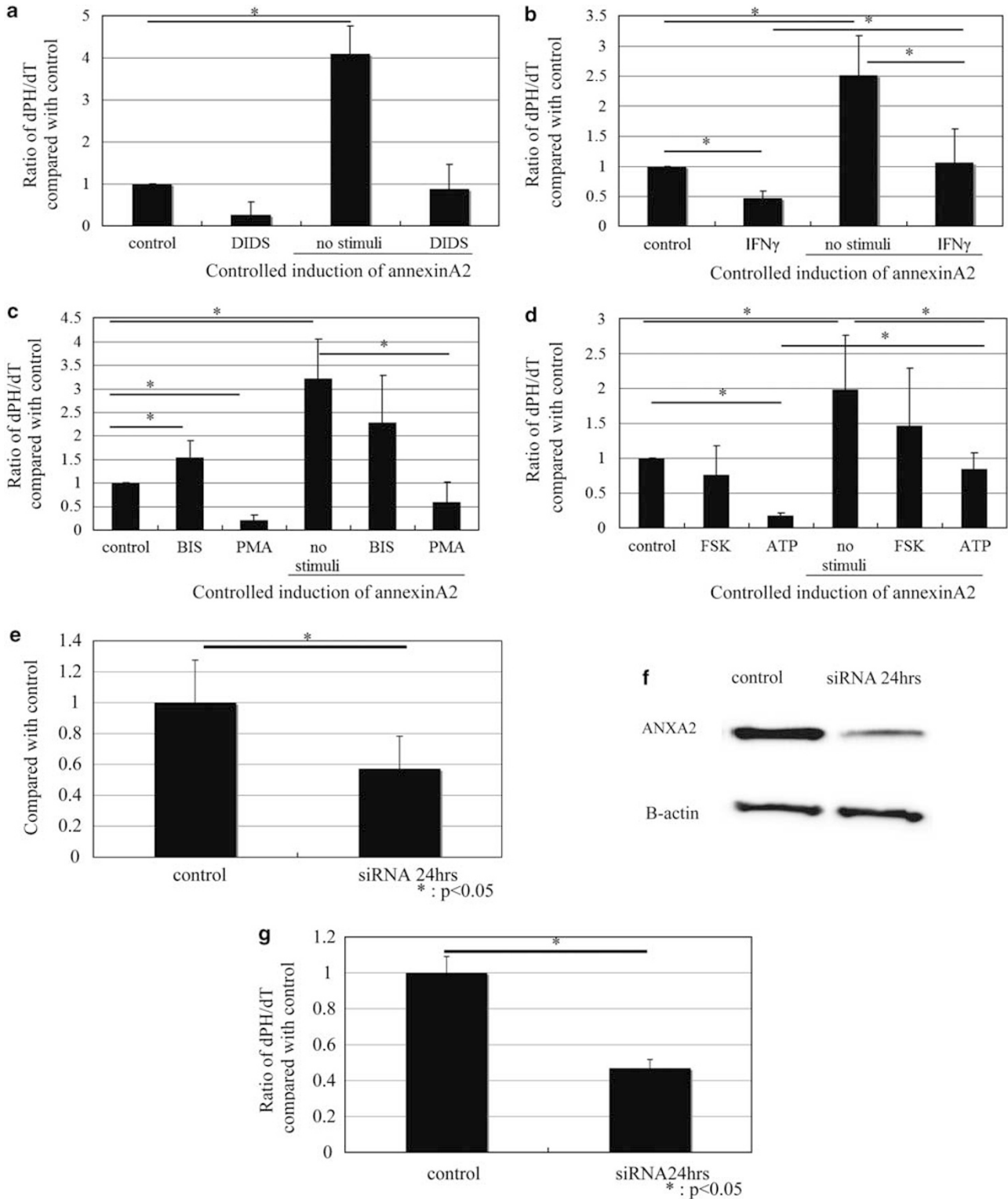
Data sets were analyzed by Spearman's correlation.

\*P-values less than 0.05 were considered statistically significant. Ductules, interlobular ducts and septal bile ducts were defined as biliary ducts less than 20 μm, from 20 to 100 μm and more than 100 μm in diameter, respectively. Serum markers were measured by a usual method.

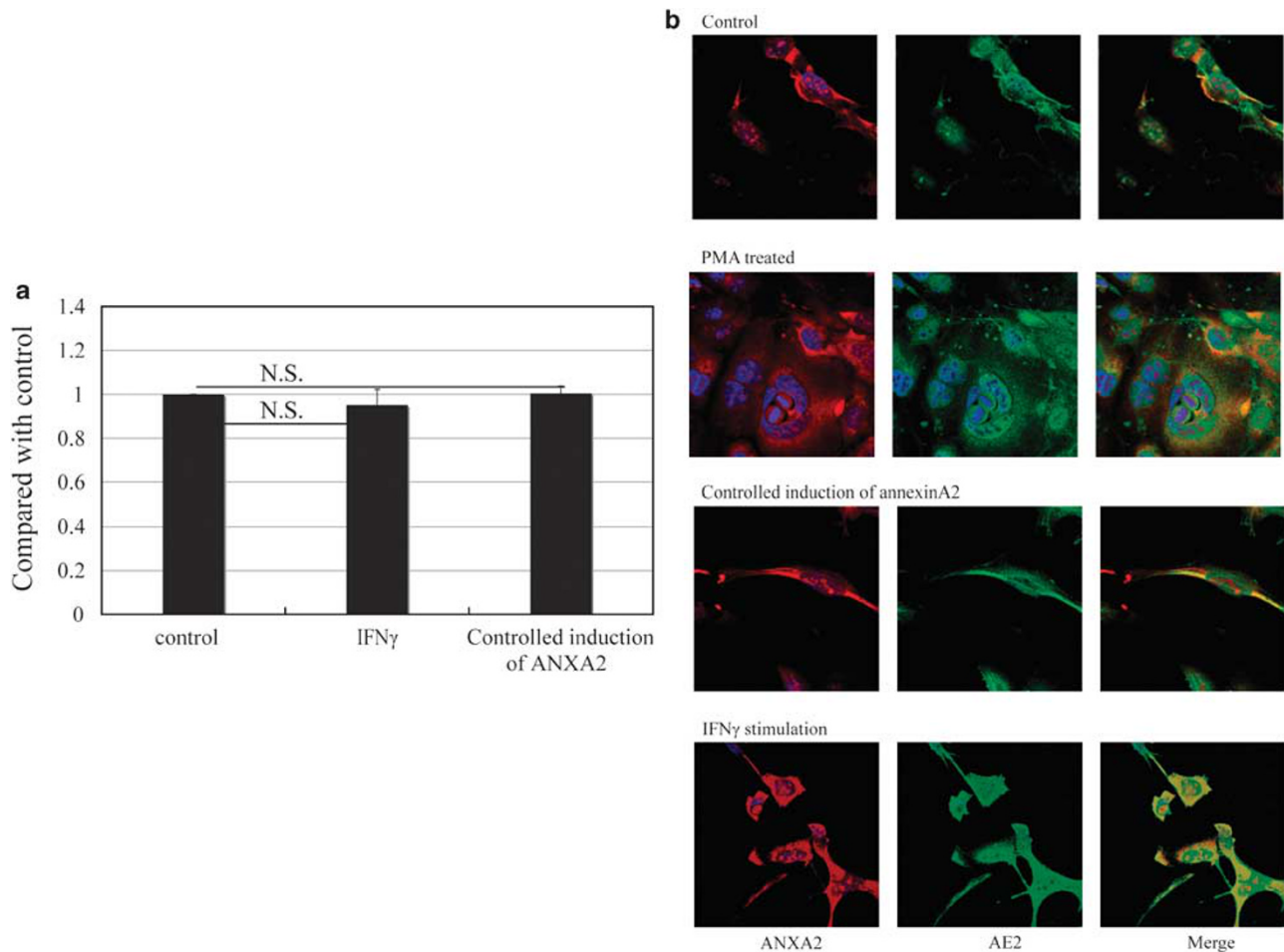
be involved in an H<sup>+</sup>-mediated mechanism.<sup>45</sup> In addition, ANXA2 binds tissue plasminogen activator (tPA) as well as its substrate, plasminogen, and accelerates the catalytic efficiency of plasmin generation *in vitro*.<sup>46,47</sup> Through conformational changes of ANXA2 that are caused by several factors, for example, binding of Ca<sup>2+</sup>, pH or the annexin itself, ANXA2 acquires high binding affinity to phospholipids, facilitating its relocation to the nuclear and plasma membranes. ANXA2 was discovered as an inhibitory molecule of phospholipase-A2<sup>48</sup> and recognized later as a major cellular substrate of the tyrosine kinase encoded by the SRC oncogene. Currently, various potential roles of this molecule have been proposed, which can be classified from two aspects, that is, unrelated intracellular and extracellular roles. Cell-surface ANXA2 functions as a co-receptor or platform for tPA that enables plasminogen to mediate the generation of plasmin, which leads to fibrinolysis, extracellular matrix degradation and cellular migration.<sup>49</sup> In terms of alcoholic liver diseases, increased parenchymal expression of ANXA2 and its fibrinolytic role were proposed.<sup>13</sup> We found expression of ANXA2 not only in the parenchymal hepatocytes of PBC and CHC liver, but also in the cholangiocytes of PBC. The subcellular localization of ANXA2 to the basolateral cell membrane of cholangiocytes or hepatocytes may be related to the fibrinolytic role of ANXA2 in the injured liver as a

consequence of the activation of plasmin, leading to remodeling of the injured bile ducts. We speculated that the correlation between apical ANXA2 expression and serum cholestatic markers might indicate a unique role of this molecule in terms of the cholestasis or bile juice production of cholangiocytes. Bicarbonate secretion is one of the crucial roles of cholangiocytes, which is mediated by AE2 of the apical domain of cholangiocytes. Therefore, we focused on the putative role of ANXA2 in terms of bicarbonate secretion of the impaired cholangiocytes. There were several lines of indirect evidence that supported our hypothesis. ANXA2 is implicated in the exocytosis, endocytosis, organization of lipid-raft-like membrane constituents and ion-channel regulation.<sup>45</sup> With regard to anion transport, ANXA2 serves as a Cl<sup>-</sup> channel regulator in vascular epithelial cells<sup>50</sup> and as an essential molecule in the CFTR function in bronchial epithelial cells through formation of ANXA2/CFTR complex.<sup>51</sup> The controlled induction of ANXA2 led to an unexpected increase of the pH<sub>i</sub> recovery that was DIDS-sensitive and was not affected by the CFTR inhibitor (data not shown). Moreover, induction of ANXA2 restored the AE activity impaired by IFN $\gamma$ . Faint expression of AE2 in the cholangiocytes of the PBC livers was supported by the immunohistochemistry (data not shown), as mentioned before.<sup>16</sup> Therefore, a compensatory role for the cholestasis of





**Figure 4** ANXA2 affected the AE activity of H69 cells. H69-anxa2 was established as described under section Materials and Methods. AE activity was assessed by measurement of the DIDS-sensitive pH<sub>i</sub> recovery from acute intracellular alkalization. The induction of ANXA2 upregulated the AE activity (a). Preincubation with IFN $\gamma$  at a concentration of 1000 IU/ml for 24 h caused downregulation of AE activity of H69 cells, which was partially recovered by induction of ANXA2 (b). PMA (950 nM), a pan-PKC activator, suppressed AE activity and 1  $\mu$ M of BIS, a pan-PKC inhibitor, increased AE activity significantly. The part of the AE activity augmented by induction of ANXA2 was negated by PMA (c). ATP, but not forskolin, attenuated the AE activity of H69 cells, which was partially rescued by controlled induction of ANXA2 (d). ANXA2 expression was significantly reduced to 46.9% as compared with that in the negative control by ANXA2 siRNA, as evaluated by immunoblotting and densitometry in triplicate (e, f). pH<sub>i</sub> recovery was attenuated in the same manner by ANXA2 siRNA to 57.3% (g). Data sets were analyzed by Mann-Whitney U-test and P values less than 0.05 were considered statistically significant; \* = P < 0.05.

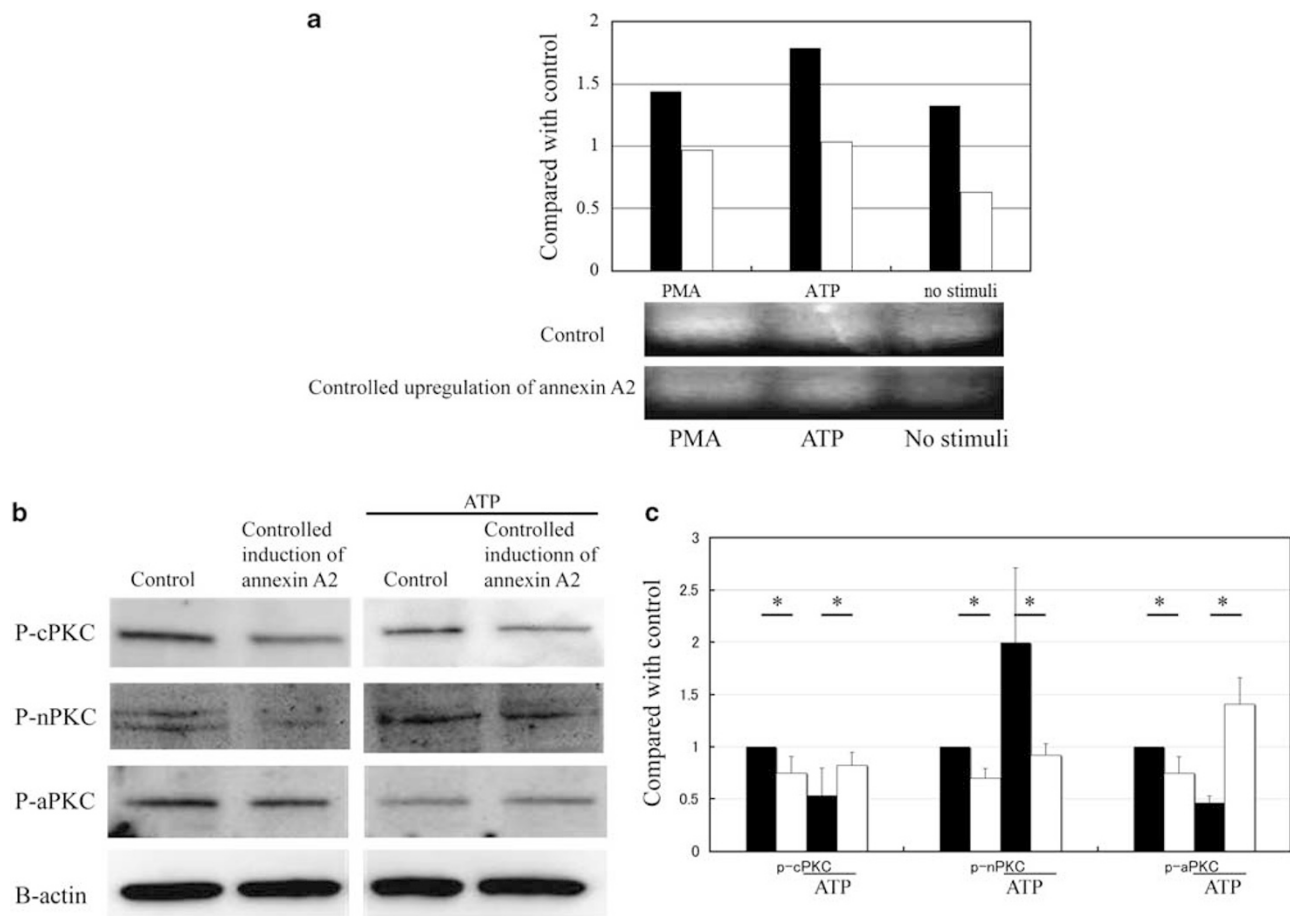


**Figure 5** The AE2 status in H69 cells with the Tet-On vector of ANXA2 was confirmed by immunoblotting (**a**) and immunofluorescence (**b**). AE2 expression was not affected by IFN $\gamma$ , which was constant even under DOX (**a**, **b**). Nuclear redistribution of ANXA2 from peripheral membrane and cytosol, or from cytosolic relocation of AE2 from peripheral membrane by PMA, is indicated by red and yellow arrows, respectively (**b**). Data sets were analyzed with Mann-Whitney *U*-test. NS = not significant.

ANXA2 was postulated for the bile formation through the modulation of the AE activity that colocalized to the apical membrane of cholangiocytes together with ANXA2.

Our investigation clarified, at least, some part of the underlying mechanism of the modulation of AE activity. PKC activator relocated ANXA2 and AE2 from the membrane compartment to the cytosol and nucleus in H69 cells, which would explain the mechanism of the AE activity attenuated by PKC activation. Methyl- $\beta$ -cyclodextrin, which extracts cholesterol from cell membranes,<sup>52</sup> totally abolished the AE activity regardless of the ANXA2 status (data not shown). Therefore, the AE activity was likely to be dependent on the microdomain, lipid rafts. AE2 that uptakes Cl<sup>-</sup> from the outer cell space and excretes HCO<sub>3</sub><sup>-</sup> is a dimerized molecule that exists in the lipid rafts.<sup>53</sup> Through modulation of the affinity of ANXA2 for the phospholipids under the existence of intracellular cytosolic Ca<sup>2+</sup>, the accessibility of ANXA2 to AE of the microdomain may become possible.

Extracellular ATP causes rapid increase of the intracellular Ca<sup>2+</sup> concentration and subsequent activation of membrane Cl<sup>-</sup> channels through purinergic receptors in the process of bile formation by cholangiocytes.<sup>54-57</sup> In the present study, we demonstrated the decrease of AE activity by the extracellular ATP and partial recovery of the AE activity by the induction of ANXA2. The phosphorylation status of the amino-acid residue of ANXA2 also modulates its binding affinity to the membrane. For example, the tyrosine phosphorylation of ANXA2 by pp60src decreases the binding of the protein to phospholipid vesicles at low Ca<sup>2+</sup> concentrations, whereas serine phosphorylation of ANXA2 by PKC inhibits its ability to aggregate phospholipid vesicles.<sup>58</sup> Extracellular ATP enhances both phosphorylation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) and its translocation to the plasma membrane, through binding to P2Y receptors.<sup>59</sup> Activated PDK1 by phosphorylation further matures the downstream PKC by phosphorylation.



**Figure 6** PMA and ATP increased the PKC activity of H69 cells shown as an increase of phospho-peptide, as evaluated by PepTag assay (a). Decreased induction of ANXA2 suppressed PKC activity. Phosphorylation of PKC subtypes as the matured form evaluated by immunoblotting (b) and the results of densitometry (c) are shown. P-PKC $\alpha/\beta$  (as cPKC), p-PKC $\delta/\theta$  (as nPKC), and p-PKC $\lambda/\zeta$  (as aPKC) all decreased in association with induction of ANXA2. Extracellular ATP increased the level of p-nPKC. ANXA2 decreased the level of p-nPKC and increased that of p-aPKC, paradoxically, together with ATP level. White bar, H69 lysates preincubated with DOX and black bar, without DOX. Data sets were analyzed with Mann-Whitney U-test. \* =  $P < 0.05$ .

ANXA2 can bind directly and specifically to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>), which is the source of diacylglycerol, a classical PKC activator, and inositol trisphosphate.<sup>60</sup> With the PepTag Assay, we found that the ability of all PKC subtypes for phosphorylation of the substrate was attenuated by induction of ANXA2. The PKC activity might be directly modulated by the binding ability of ANXA2 to PtdIns(4,5)P<sub>2</sub>. The expressions of the matured types of conventional, novel and atypical PKCs were all decreased by ANXA2. Extracellular ATP upregulated the matured form of novel PKC, that is, subtype  $\theta/\delta$ , in contrast to conventional PKC, that is, subtype  $\alpha/\beta$  (subtype  $\gamma$  was not tested) and atypical PKC, that is, subtype  $\lambda/\zeta$ , which remained constant regardless of the supplementation of ATP. ANXA2 specifically suppressed the part of the matured form of novel PKC that was increased by ATP. These findings indicate that ANXA2 could affect the maturation of whole PKC through modulating the phosphorylation status, which would affect the downstream signaling of cholangiocytes, including bicarbonate secretion.

The contradictory results of extracellular ATP on the AE activity, forskolin and CFTR inhibitor on the AE activity observed in the current study may be explained by the imbalance of the reaction caused by the extracellular ATP stimulation, such as the Ca<sup>2+</sup> release from the ER, which leads to augmentation of AE activity and PKC maturation, which may counteract intracellular Ca<sup>2+</sup> increase along with weak expression of CFTR in H69 cell speculated by the data mining of microarray (data not shown). Another possible weakness of our conclusion is that it was based on the results of the measurements of intracellular pH, which might miss the actual bicarbonate secretion into the lumen by cholangiocytes. In the current study, we aimed at revealing the physiological interactions of molecules in human-derived samples under limited accessibility, which might also lead to results with certain limitations. Thus, we think that several lines of evidence related to AE2 activity should be reconciled for a better comprehension of biliary physiology and pathophysiology.

In conclusion, ANXA2 expressed in the biliary epithelial cells might be a histological marker of cholangiopathy, including PBC. ANXA2 would have a compensatory role for bicarbonate secretion through modulating the AE activity of human cholangiocytes under pathophysiological conditions.

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#### DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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