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Annexin A3 Expression Increases in Hepatocytes and is Regulated by Hepatocyte Growth Factor in Rat Liver Regeneration

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Annexin (Anx) A3 increases and plays important roles in the signalling cascade in hepatocyte growth in cultured hepatocytes. However, no information is available on its expression and role in rat liver regeneration. In the present study, AnxA3 expression was investigated to determine whether it also plays a role in the signalling cascade in rat liver regeneration. AnxA3 protein and mRNA level both increase in liver after administration of carbon tetrachloride (CCl₄) or 70% partial hepatectomy. AnxA3 protein level increases in isolated parenchymal hepatocytes, but not in non-parenchymal liver cells, in these rat liver regeneration models. AnxA3 mRNA increases in hepatocytes after CCl₄ administration. Anti-hepatocyte growth factor antibody suppresses this increase in AnxA3 mRNA level. These results demonstrate that AnxA3 expression increases in hepatocytes through a hepatocyte growth factor-mediated pathway in rat liver regeneration models, suggesting that AnxA3 plays an important role in the signalling cascade in rat liver regeneration.

Key words: annexin A3, carbon tetrachloride, hepatocyte growth factor, parenchymal hepatocytes, partial hepatectomy.

Abbreviations: Anx, Annexin; CCl₄, carbon tetrachloride; HGF, hepatocyte growth factor.

Annexin (Anx) A3 is a member of the Anx family, which binds to phospholipids and membranes in a Ca²⁺-dependent manner (1–4). AnxA3 has been shown to have anti-coagulant and anti-phospholipase A₂ properties *in vitro* (5, 6), plus to promote Ca²⁺-dependent aggregation of isolated specific granules from human neutrophils (5, 6). Some reports describe its regulation and role in cultured cells (7–11); however, there are no reports describing these characteristics *in vivo*.

We recently reported that AnxA3 is expressed in cultured rat hepatocytes, but not in isolated hepatocytes and that inhibition of AnxA3 expression by RNA interference results in a significant inhibition of hepatocyte growth (10, 12, 13). These findings indicate that AnxA3 plays an important role in the signalling cascade in hepatocyte growth in cultured hepatocytes, although the mechanism remains to be elucidated. The significance of AnxA3 in hepatocyte growth is also supported by the finding that known stimulatory or inhibitory actions of various factors to hepatocyte growth correlated well with the increase or decrease in AnxA3 expression (14).

These findings indicate that AnxA3 increases and is likely to play an important role in the signalling cascade in rat liver regeneration. AnxA1 increases in rat and mouse liver regeneration models, e.g. after administration of carbon tetrachloride (CCl₄) and 70% partial hepatectomy (15, 16). Suppression of AnxA1 expression

using anti-sense technology inhibits proliferation in a mouse hepatocyte cell line (15). Therefore, AnxA1 is also likely to play an important role in the signalling cascade in rat liver regeneration.

In the present study, AnxA3 expression in rat liver regeneration models was investigated to explore the possibility that AnxA3 plays important roles in the signalling cascade in rat liver regeneration.

MATERIALS AND METHODS

Animals and Experimental Conditions—Adult male Wistar rats (180–200 g) were purchased from Japan SLC Co., Ltd. (Shizuoka, Japan) and used for all studies. They were maintained in a 12 h light/dark cycle, allowed food and water *ad libitum*. All animal care and procedures were approved by the institutional care committee and carried out in accordance with the guidelines established by the National Institute of Health.

For studies of liver regeneration after toxic injury, rats received CCl₄ intraperitoneally (2 ml/kg body weight of 50% solution of CCl₄ in olive oil). Control rats received olive oil intraperitoneally (1 ml/kg body weight of olive oil). Animals given CCl₄ or olive oil were sacrificed at 3–24 h after administration.

A 70% partial hepatectomy was performed according to Higgins and Anderson (17). In the sham operation, livers were exposed and manipulated but not removed. These procedures were performed under anaesthesia with Nembutal (Abbot, Chicago, IL, USA). Animals subjected to

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partial hepatectomy or sham operation were sacrificed at 2.5–20 h after the operation.

For infusion of anti-human hepatocyte growth factor (HGF) antibody, rats were intravenously injected with 0.2 ml goat anti-human HGF IgG (Sigma-Aldrich, St Louis, MO, USA) (1.25 mg/kg body weight) diluted in phosphate-buffered saline (PBS) through the tail vein, then received CCl₄ intraperitoneally, as described earlier. Control rats were injected with the same volume and amount of control goat IgG, and then received CCl₄ intraperitoneally in a similar manner. Parenchymal hepatocytes were prepared from the rats after 6 h, as described subsequently.

Preparation of Liver Lysate—The procedures were performed at low temperature, unless described otherwise. Liver was *in situ* perfused with PBS via the portal vein, then removed from the body. Liver was homogenized with a Potter-Elvehjem homogenizer in 4× (v/w) buffer A [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA and 2.5% (v/v) Triton-X 100] containing 1 mM benzylsulphonyl fluoride, 0.3 mM leupeptin and 0.5 mM aprotinin. The homogenate was shaken for 15 min at room temperature, then sonicated four times for 15 s each time. After centrifugation at 100,000g, the cytosolic fraction was stored at -70°C until use.

Cell Isolation—Parenchymal hepatocytes were isolated from rats by *in situ* perfusion of the liver with collagenase (18). Non-parenchymal liver cells were isolated from the supernatant of parenchymal cells by differential centrifugation, as described by Shimaoka *et al.* (19). In this article, hepatocytes are also referred to as parenchymal hepatocytes to distinguish between hepatocytes and non-parenchymal liver cells.

Preparation of Cell Lysate—Cell lysates were prepared by a modification of the reported by Römisch *et al.* (20). Procedures were performed at low temperature, unless described otherwise. Cells were resuspended in three volumes of buffer A containing 1/100 (v/v) protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). They were then shaken for 15 min at room temperature and sonicated four times for 15 s each time. After centrifugation at 100,000g, the cytosolic fraction was stored at -70°C until use.

Western Blot Analysis—An equal amount of cytosolic protein from each experiment was subjected to SDS-PAGE on a 10% gel and electroblotted to PVDF membrane (GVHP; Millipore, Bedford, MA, USA). After blocking the membrane with 5% skimmed milk, a western blot analysis was performed using rabbit anti-human Anx3 antibody serum (1: 5,250) (a gift from Drs F. Russo-Marie and C. Raguene-Nicol), mouse anti-human GAPDH monoclonal antibody (1: 5,000) (Abcam, Cambridge, UK), or rabbit anti-beta-actin polyclonal antibody (1: 500) (BioLegend, San Diego, CA, USA). Detection was performed using the ECL detection system (GE Health care Bioscience, Buckinghamshire, UK). Housekeeping protein, GAPDH and beta-actin, were selected based on results of preliminary studies. Intensity of each band was measured over a proportional range. A computer-assisted analyser was used to quantitatively analyse intensity, with intensity of the Anx3

band normalized to the intensity of the appropriate housekeeping protein. Protein amount from liver and cell lysate was measured using a previously described method (21), with bovine serum albumin used as a standard.

Total RNA Extraction and Real-Time Quantitative PCR—Total RNA was extracted from liver by a modification of guanidine thiocyanate-phenol-chloroform extraction method (22, 23). Total RNA was extracted from cells using Trizol® reagent (Invitrogen, Cergy Pontoise, France) in accordance with the manufacturer's protocol. Equal amounts of RNA (~1 µg) from each experiment were reverse-transcribed using a THERMOSCRIPT™ RT-PCR System (Invitrogen, Cergy Pontoise, France) and oligo(dT)₂₀ in a final volume of 40 µl, in accordance with the manufacturer's protocol. Subsequently, 2 µl of cDNA was used as templates for real-time PCR analysis using a LightCycler system (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. For Anx3 and 28S rRNA, the PCR programme consisted of 40 cycles of 10 s at 94°C, 10 s at 60°C and 12 s at 72°C. Primer sequences for Anx3 were 5' -CAA ATT CAC CGA GAT CCT GT-3' and 5' -TGC TGG AGT GCT GTA CGA AA-3' (14) and for 28S rRNA 5' -CCA GAG CGA AAG CAT TTG CCA-3' and 5' -GGC ATC ACA GAC CTG TTA TTG CTC-3' (14). Anx3 levels were normalized to the levels of 28S rRNA.

Statistical Analysis—Data were analysed using Student's *t*-test, and *P*-values <0.05 were considered to be statistically significant.

Immunohistochemical Examination—Serial liver sections cut at 3 µm thick from the paraformaldehyde-fixed and paraffin-embedded blocks. De-paraffinated and re-hydrated sections were heated for 5 min at 100°C in 10 mM citrate buffer (pH 6.0) followed by the treatment with 10 µg/ml Proteinase K (TAKARA BIO Inc., Shiga, Japan) for 5 min at room temperature. These activated sections were then subjected to blocking with 10% bovine serum albumin for 1 h at room temperature. After washing with PBS, sections were simultaneously incubated for 2 h with antibodies, e.g. anti-rat hepatic sinusoidal endothelial cells mouse IgG (SE-1, Immunobiological Laboratories Co., Ltd. Gunma, Japan) 1:20 and rabbit anti-human Anx3 antibody serum 1:200. The fluorescence-labelled secondary antibodies were AMCA-labelled sheep anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., PA, USA) 1:200 and FITC-labelled sheep anti-rabbit IgG (MP Biomedicals Inc., Ohio, USA) 1:200. The liver sections were thus mounted on a cover glass with a mounting medium, Vectashield (Vector Laboratories, CA, USA), and subjected to microscopic observation.

RESULTS

Anx3 Expression in Liver Following CCl₄ Treatment—Anx3 protein level increased ~3-fold at 6 h after administration of CCl₄ and this increased level was maintained to 24 h (Fig. 1). Anx3 mRNA level started to increase at 3 h after administration, reaching an ~17-fold increase at 24 h (Fig. 2).

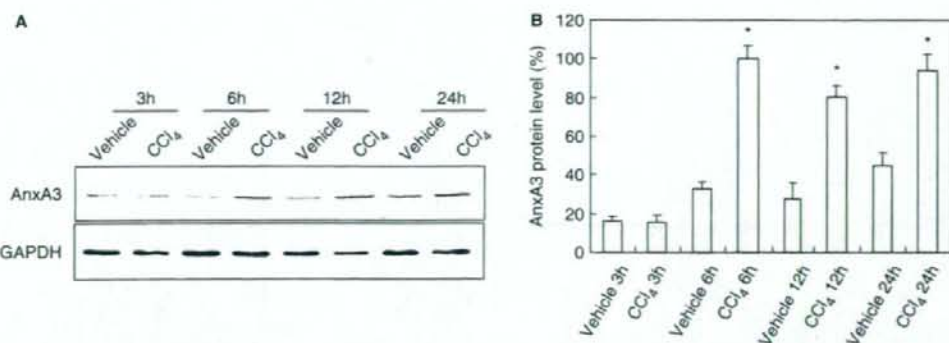


Fig. 1. AnxA3 protein level in liver following treatment with CCl₄. (A) Data shown are representative of western blot analysis results. Approximately 35 and 1.5 μg of protein were used for detection of AnxA3 and GAPDH, respectively. (B) Results are presented relative to the value produced by liver

in rats at 6 h after CCl₄ administration. AnxA3 protein levels were normalized to the housekeeping protein, GAPDH. Data are expressed as mean ± S.D. ($n = 4$ at each time point) * $P < 0.01$, compared to the value produced by liver in rats after olive oil administration.

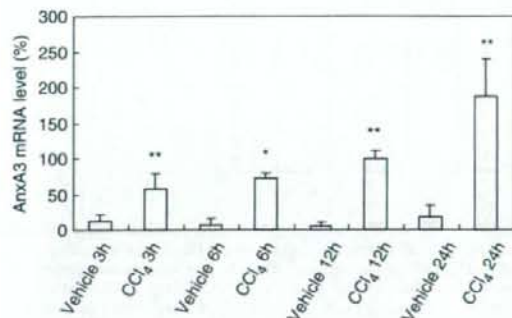


Fig. 2. AnxA3 mRNA level in liver following treatment with CCl₄. Results are presented relative to the value produced by liver in rats at 6 h after CCl₄ administration ($n = 4$ at each time point). AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Data are expressed as the mean ± SD ($n = 4$ at each time point) * $P < 0.01$, ** $P < 0.05$, compared to the value produced by liver in rats after olive oil administration.

AnxA3 Expression in Parenchymal Hepatocytes and Non-parenchymal Liver Cells Following CCl₄ Treatment—Parenchymal hepatocytes and/or non-parenchymal liver cells are involved in the increase of AnxA3 expression in liver following CCl₄ treatment. AnxA3 protein level increased ~5-fold in parenchymal hepatocytes at 6 h after CCl₄ treatment, but did not change in non-parenchymal liver cells (Fig. 3). AnxA3 mRNA level increased ~5-fold in parenchymal hepatocytes at 6 h after CCl₄ treatment; however, it did not change in non-parenchymal liver cells (Fig. 4).

AnxA3 Expression in Liver after Partial Hepatectomy—AnxA3 protein level started to increase at 5 h after partial hepatectomy, reaching a 1.6-fold increase at 20 h (Fig. 5). AnxA3 mRNA level increased to ~2,800-fold at 2.5 h, then began decreasing at 5 h, falling back to basal level at 20 h (Fig. 6).

AnxA3 Expression in Parenchymal Hepatocytes and Non-parenchymal Liver Cells After Partial Hepatectomy—AnxA3 protein level increased ~1.5-fold in isolated parenchymal hepatocytes at 6 h after partial hepatectomy, but did not change in non-parenchymal liver cells (Fig. 7). AnxA3 mRNA level decreased to ~80% in hepatocytes at 6 h after partial hepatectomy; however, AnxA3 mRNA did not change in non-parenchymal liver cells (Fig. 8).

AnxA3 Expression in Hepatic Sinusoidal Endothelial Cells—Non-parenchymal liver cells expressing AnxA3 were investigated by immunohistochemical staining. Hepatic sinusoidal endothelial cells were chosen as a candidate, as human umbilical vein endothelial cells express AnxA3 (20). AnxA3- and SE-1-positive cells were observed in normal rat liver section (Fig. 9 panel A and B, respectively), with localization of AnxA3-positive cells corresponding to SE-1-positive cells (Fig. 9, panel C).

Effect of Anti-HGF Antibody on AnxA3 mRNA Level in Hepatocytes Following CCl₄ Treatment—To investigate whether HGF is involved in the increase in AnxA3 mRNA level in hepatocytes following CCl₄ treatment, effect of anti-HGF antibody on mRNA level was investigated. Anti-HGF antibody decreased AnxA3 mRNA level to ~60% compared to control IgG (Fig. 10).

DISCUSSION

In the present study, we demonstrate that expression of AnxA3 increases in two rat liver regeneration models and in parenchymal hepatocytes, but not non-parenchymal liver cells. AnxA3 protein levels in the liver increased at 5 h and 6 h in partially hepatectomized rats and rats treated with CCl₄, respectively. DNA synthesis begins to change at ~16 and 24 h in partially hepatectomized rats and rats treated with CCl₄, respectively (24). AnxA3 plays an important role in the signalling cascade in hepatocyte growth for cultured rat hepatocytes (10), therefore is also likely to have the same role in rat liver regeneration.

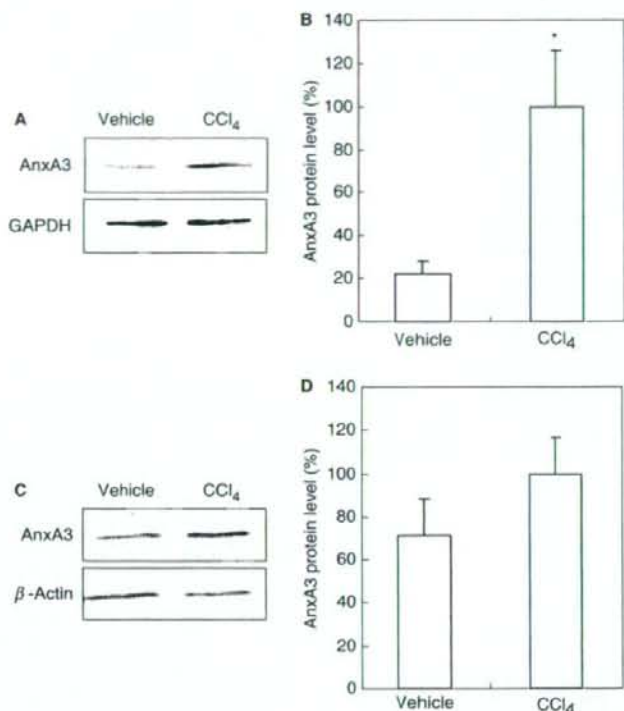


Fig. 3. AnxA3 protein level in parenchymal hepatocytes and non-parenchymal cells isolated from liver in rats following treatment with CCl₄. (A) Parenchymal hepatocytes and (C) non-parenchymal cells were isolated from liver in rats at 6 h after either CCl₄ or olive oil treatment. Data shown are representative western blot analysis results for parenchymal hepatocytes and non-parenchymal cells, respectively. Approximately 90 and 0.94 µg of protein was used for the detection of AnxA3 and GAPDH in parenchymal hepatocytes, respectively. Approximately 2.8 µg of protein was used for

detection of AnxA3 and beta-actin in non-parenchymal cells. Results for parenchymal hepatocytes (B) and non-parenchymal cell (D) are presented relative to parenchymal hepatocytes and non-parenchymal liver cells from rats at 6 h after CCl₄ administration, respectively. AnxA3 protein levels in parenchymal hepatocytes and non-parenchymal liver cells were normalized to housekeeping protein, GAPDH and beta-actin, respectively. Data are expressed as mean ± SD (n = 4) *P < 0.01, compared to the value for parenchymal hepatocytes or non-parenchymal liver cells from rats at 6 h after olive oil treatment.

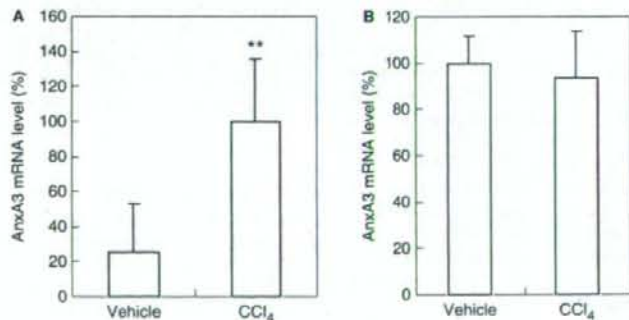


Fig. 4. AnxA3 mRNA level in parenchymal hepatocytes and non-parenchymal cells isolated from livers in rats following treatment with CCl₄. (A) Parenchymal hepatocytes and (B) non-parenchymal liver cells were isolated from liver in rats at 6 h after either CCl₄ or olive oil treatment. AnxA3 levels were normalized to the housekeeping gene, 28S rRNA.

Results for parenchymal hepatocytes and non-parenchymal liver cells are presented relative to hepatocytes and non-parenchymal cells from rats at 6 h after CCl₄ treatment, respectively. Data are expressed as the mean ± SD (n = 4) **P < 0.05, compared to parenchymal hepatocytes and non-parenchymal liver cells from liver in rats at 6 h after olive oil treatment.

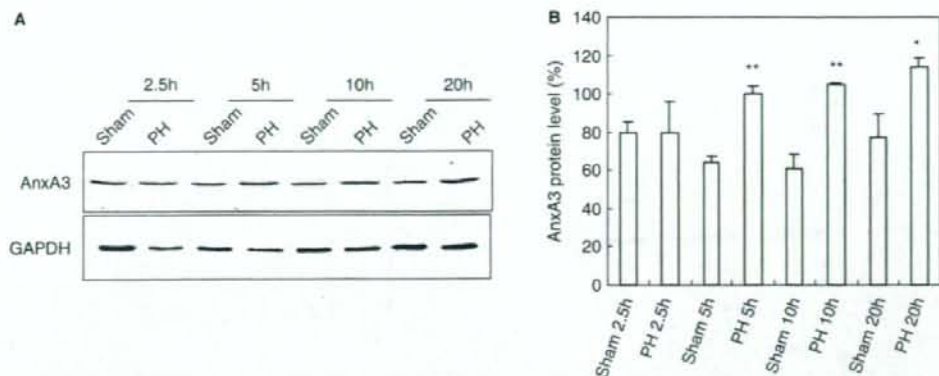


Fig. 5. AnxA3 protein level in liver after partial hepatectomy. (A) Data shown are representative of western blot analysis results. Approximately 35 and 1.5 μ g of protein were used for detection of AnxA3 and GAPDH, respectively. (B) Results are presented relative to the values for liver in

rats at 5h after partial hepatectomy. AnxA3 protein levels were normalized to levels of housekeeping protein, GAPDH. Data are expressed as mean \pm SD ($n=4$ at each time point) * $P<0.01$, ** $P<0.05$, compared to the value produced by liver in rats after sham operation.

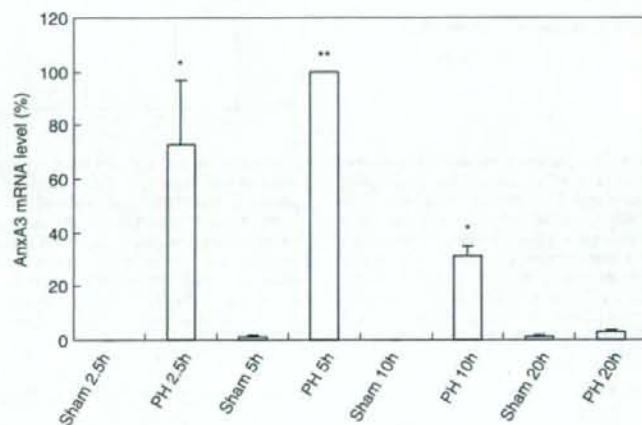


Fig. 6. AnxA3 mRNA level in liver after partial hepatectomy. Results are presented relative to the value produced by liver in rats at 5h after partial hepatectomy. AnxA3 mRNA

levels were normalized to housekeeping gene, 28S rRNA. Data are expressed as mean \pm SD ($n=4$ at each time point) * $P<0.01$, ** $P<0.05$, compared to after sham operation.

Extent of increase in AnxA3 protein level was lower than in AnxA3 mRNA level in rat liver regeneration models, suggesting that AnxA3 protein, for which synthesis is enhanced, degrades rapidly in these conditions. Several proteases are induced or activated in rat liver regeneration (25–31). Therefore, AnxA3 may be rapidly degraded by some of these proteases, resulting in the relatively low level of increase in AnxA3 protein expression compared to mRNA expression.

AnxA3 in the liver from rats at 24h after CCl₄ treatment was investigated using immunohistochemical analysis, to determine whether proliferating cells are AnxA3-positive parenchymal cells. AnxA3 was not detected in parenchymal hepatocytes, but was detected

in non-parenchymal liver cells (data not shown). This failure of detection in parenchymal hepatocytes may be because expression of AnxA3 in these cells is too low to detect compared to non-parenchymal liver cells.

AnxA3 protein level increased in hepatocytes after partial hepatectomy; however, AnxA3 mRNA level after sham operation was even higher than after partial hepatectomy, inconsistent with the results for AnxA3 protein level. AnxA3 protein levels did, however, correlate with AnxA3 mRNA levels in cultured rat hepatocytes (14). AnxA3 mRNA was undetectable in hepatocytes from normal rats that were not sham operated (10, 12). Therefore, sham operation may induce some signal that leads to an increase in AnxA3

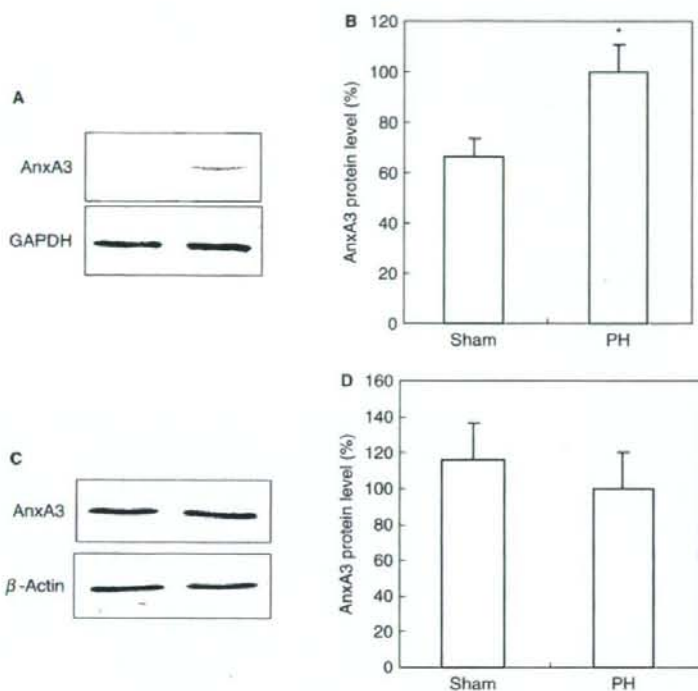


Fig. 7. AnxA3 protein level in parenchymal hepatocytes and non-parenchymal liver cells after hepatectomy. (A) Parenchymal hepatocytes and (C) non-parenchymal liver cells were isolated at 5 h after partial hepatectomy or sham operation. Data shown are representative of western blot analysis results for parenchymal hepatocytes and non-parenchymal liver cells, respectively. Approximately 90 and 2.8 μ g of protein were used for detection of AnxA3 and GAPDH in parenchymal hepatocytes, respectively. Approximately 2.8 μ g of protein was used for detection of AnxA3 and beta-actin in non-parenchymal

liver cells. AnxA3 protein levels in parenchymal hepatocytes and non-parenchymal liver cells were normalized to housekeeping proteins GAPDH and beta-actin, respectively. Results for parenchymal hepatocytes (B) and non-parenchymal liver cells (D) are presented relative to the value produced by parenchymal hepatocytes and non-parenchymal liver cells from rats at 5 h after partial hepatectomy, respectively. Data are expressed as mean \pm SD ($n=4$) * $P < 0.01$, compared to parenchymal hepatocytes and non-parenchymal liver cell from rats at 5 h after sham operation.

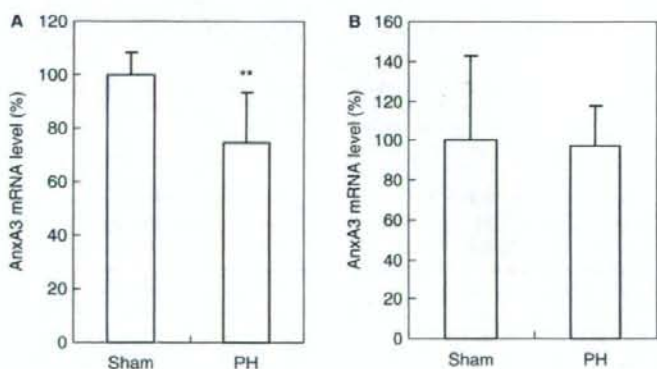


Fig. 8. AnxA3 mRNA level in parenchymal hepatocytes and non-parenchymal liver cells after partial hepatectomy. (A) Parenchymal hepatocytes and (B) non-parenchymal liver cells were isolated from liver in rats at 5 h after either partial hepatectomy or sham operation. AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Results for

parenchymal hepatocytes and non-parenchymal liver cells are presented relative to parenchymal hepatocytes and non-parenchymal liver cells from rats at 5 h after partial hepatectomy, respectively. Data are expressed as mean \pm SD ($n=4$) ** $P < 0.05$, compared to parenchymal hepatocytes and non-parenchymal liver cells from rats at 5 h after sham operation.

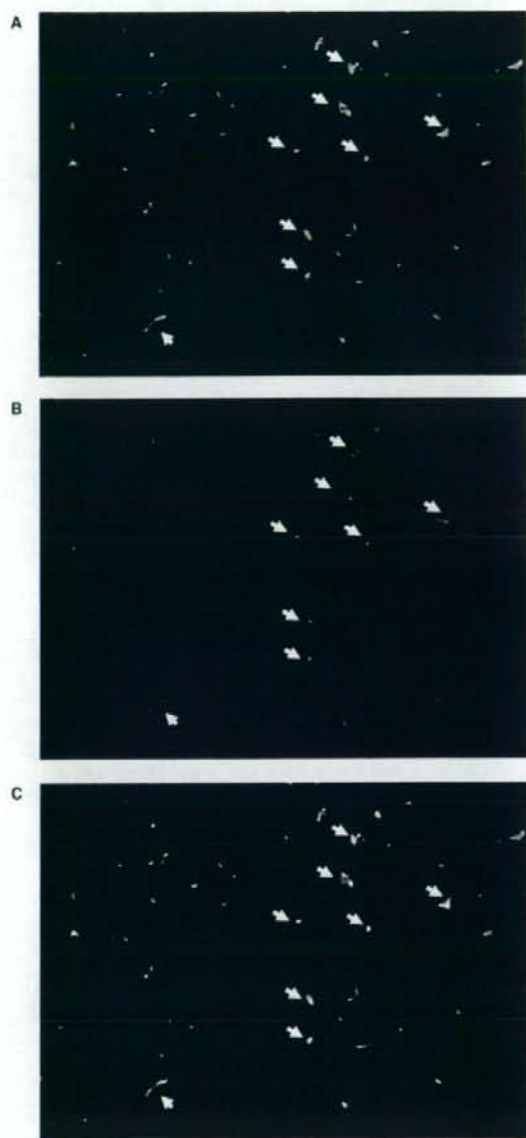


Fig. 9. AnxA3 expression in hepatic sinusoidal endothelial cells in normal rat liver. (A) AnxA3-positive cells; (B) SE-1-positive cells; (C) Merged image of AnxA3- and SE-1-positive cells. In (A-C), arrows show examples of positive immunoreactive cells.

mRNA level only in hepatocyte isolation procedures, including perfusion with collagenase at 37°C. This possibility may be supported by the finding that AnxA3 mRNA level is greatly enhanced in the liver from rats after partial hepatectomy, compared to after sham

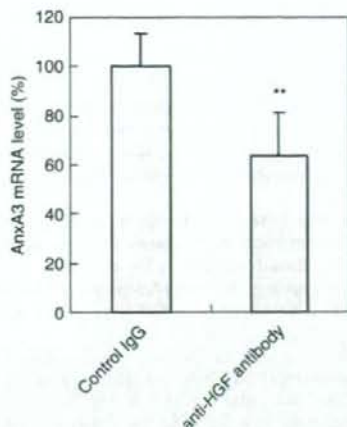


Fig. 10. Effect of anti-HGF antibody on AnxA3 mRNA level in parenchymal hepatocytes following treatment with CCl₄. Hepatocytes were isolated from liver in rats at 6h following treatment with either anti-HGF IgG or control IgG, then CCl₄. AnxA3 levels were normalized to housekeeping gene, 28S rRNA. Results are presented relative to the value produced by hepatocyte isolated from liver in rats at 6h following treatment with control IgG, then CCl₄. Data are expressed as mean ± SD (n = 4) **P < 0.05, compared to hepatocytes from rats at 6h following treatment with control IgG, then CCl₄.

operation in analysis using total RNA directly extracted from liver perfused with cold PBS.

Increase in AnxA3 mRNA level was inhibited by anti-HGF antibody in hepatocytes from rats at 6h after CCl₄ administration, indicating that HGF is involved in increasing AnxA3 mRNA expression in hepatocytes. Consistent with this finding, HGF increased AnxA3 mRNA level in hepatocytes cultured on Matrigel (14), on which hepatocytes maintain functions similar to those within a normal animal (32). HGF protein needs to increase in blood within 6h at the latest after CCl₄ administration for HGF to increase AnxA3 mRNA level. This was indicated by the finding that HGF protein dramatically rises in the plasma at 2h after partial hepatectomy and CCl₄ administration (33).

Effect of anti-HGF antibody on AnxA3 protein level was investigated; however, reproducible results were not obtained for AnxA3 and GAPDH protein levels in the experiments using control IgG and anti-HGF IgG antibodies. Also, there was a decreased recovery of total protein compared to the parenchymal hepatocytes isolated from liver in rats without these treatments. As administration of IgG was performed only *via* tail vein in this experiment, this procedure may be a factor in this variation. It is likely that the increases in fluid pressure to liver cause liver injury followed by enhancement of protein degradation by some proteases. This is supported by the finding that alanine transaminase transiently elevates in serum from rats after administration of PBS *via* the tail vein (34). However, strict control of fluid pressure is difficult in practice. Therefore, variation in

these sequential cascades may result in no reproducible results.

AnxA3 was demonstrated to be expressed in non-parenchymal liver cells, although proteins levels do not change in the liver regeneration models. Further immunohistochemical analysis showed co-localization of AnxA3-positive and SE-1-positive cells indicating that AnxA3 is expressed in hepatic sinusoidal endothelial cells.

In conclusion, the results of this study demonstrate that AnxA3 expression increases in hepatocytes through an HGF-mediated pathway in rat liver regeneration models, suggesting that AnxA3 plays an important role in the signalling cascade in rat liver regeneration.

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Full Paper

**The Novel Compounds That Activate Farnesoid X Receptor:
the Diversity of Their Effects on Gene Expression**

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Abstract. Farnesoid X receptor (FXR) controls the expression of critical genes in bile acid and cholesterol homeostasis. To study FXR and to develop a regulator of cholesterol, some non-steroidal and steroidal ligands have been found in addition to endogenous ligands for FXR. In this study, we discovered five bile acid derivatives (methyl cholate, methyl deoxycholate, 5 β -cholanic acid, 5 β -cholanic acid-7 α ,12 α -diol, and NIHS700) and two natural products (marchantin A and marchantin E) that activated FXR in the reporter assay. These compounds activated FXR to a high level comparable to the most potent endogenous bile acid, chenodeoxycholic acid, although it was not predicted from their structures; five of them were similar to the lower potency bile acids, and two were structurally much different from bile acids. The elevation levels of reporter gene expression by some of the screened compounds were varied in Cos-7, HepG2, HuH-7, and Caco-2 cells. These compounds also controlled the expression of genes regulated by FXR, and some of the compounds regulated these genes in a cell-type-specific and/or gene-selective fashion. Therefore, molecular design of the compounds can cause selective modulation of the expression of FXR target genes.

Keywords: farnesoid X receptor (FXR), reporter assay, ginkgolic acid, marchantin, cell-type-specific modulation

Introduction

The farnesoid X receptor (FXR, *NR1H4*) is a member of the nuclear-receptor superfamily. Nuclear receptors are ligand-activated transcription factors that are involved in a variety of physiological, developmental, and toxicological processes. The nuclear-receptor superfamily includes receptors for thyroid and steroid hormones, retinoids, and vitamin D, as well as receptors for unknown ligands. These receptors share a highly conserved DNA-binding domain and a discrete ligand-binding domain; and they bind to hormone response

elements (HRE) on the DNA during the formation of homodimers, heterodimers, or monomers. The ligand-binding to nuclear receptors leads to a conformational change of these receptors and the recruitment of coactivator complexes, resulting in transcriptional activation (1). Their ligand-dependent activity makes nuclear receptors good pharmacological targets.

FXR is a receptor for bile acids such as chenodeoxycholic acid (CDCA), deoxycholic acid, cholic acid, and their conjugates. Bile acids are synthesized in the liver and secreted into the intestine, where their physical properties facilitate the absorption of fats and vitamins through micelle formation. Cholesterol disposal from the liver is also dependent on the bile acid composition of the secreted bile. FXR is activated by bile acids and controls the expression of critical genes in bile

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acid and cholesterol homeostasis such as the bile salt export pump (*BSEP*), small heterodimer partner (*SHP*), *CYP7A1*, ileal bile acid-binding protein (*IBABP*), and phospholipid transfer protein (*PLTP*) (2–4). FXR plays a critical role in lipid metabolism since FXR-null mice showed elevated serum cholesterol and triglyceride levels (5), and an FXR agonist has been shown to reduce serum triglyceride levels (6). Moreover, an FXR agonist has been reported to confer hepato-protection in a rat model of cholestasis (7). Recently, FXR has also been reported to mediate glucose metabolism and to protect the intestinal mucosa from bacterial overgrowth and inflammatory insults (8, 9). Therefore, the development of FXR agonists might prove useful for the treatment of a wide variety of diseases, including diabetes, cholesterol gallstones, and hepatic and intestinal toxicity.

In addition to bile acids, some compounds whose structures are much different from bile acids (e.g., GW4064) and several selective modulators (e.g., guggulsterone and AGN34) that regulate a subset of FXR-specific genes have been identified as FXR ligands (10, 11). These compounds other than bile acids are useful for analysis of the role of FXR in lipid and glucose metabolism because they may not have the FXR-independent property of bile acids (e.g., dietary lipid absorption) and are not metabolized to form harmful lithocholic acid. On the other hand, selective ligands have been studied in detail regarding the other nuclear receptors (e.g., selective estrogen-receptor modulators (SERMs)). These compounds exhibit variable effects (e.g., function as agonists or antagonists) depending on the cells and tissues, and they have been used in therapy (e.g., tamoxifen and raloxifen). Because FXR has been found to play many roles in addition to lipid metabolism, selective FXR modulators might be useful for therapy.

We previously reported the reporter assay system of FXR, RAR, and RXR using green fluorescent protein derivatives (12). We screened a compound library (NIHS library containing about 700 compounds) and found five bile acid derivatives (methyl cholate, methyl deoxycholate, 5 β -cholanic acid, 5 β -cholanic acid-7 α ,12 α -diol, and NIHS700) and two natural products (marchantin A and marchantin E) as FXR activators. Concerning these seven compounds and ginkgolic acids that we previously showed as FXR activators, we investigated the FXR activation by reporter assay in four types of cells and the expression of the genes regulated by FXR. These compounds activated FXR comparably to the most potent bile acid, and some controlled the expression of genes regulated by FXR in a cell-type-specific and/or gene-selective fashion.

Materials and Methods

Chemicals

Methyl cholate, methyl deoxycholate, 5 β -cholanic acid, and 5 β -cholanic acid-7 α ,12 α -diol were purchased from Steraloids, Inc. (Newport, RI, USA). NIHS700 was provided from Research Foundation Itsuu Laboratory (Tokyo). Ginkgolic acid 15:1 was purified *Ginkgo biloba* L. var. *diptera* as described previously (12). Ginkgolic acid 17:1 was purchased from Nagara Science (Gifu). Chenodeoxycholic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cholic acid, deoxycholic acid and lithocholic acid were purchased from Wako (Osaka).

Isolation of marchantins A and E from *Marchantia paleacea* var. *diptera*

Fresh material (6.67 kg) of *Marchantia paleacea* var. *diptera* collected in Tokushima, Japan in 1993 was extracted with MeOH (10 L) for 1 month at room temperature. The extract was filtered and evaporated *in vacuo* to afford a brown residue (176.0 g), which was subjected repeatedly to column chromatography (CC) on silica gel (*n*-hexane-EtOAc, gradient) and Sephadex-LH-20 (CHCl₃-MeOH = 1:1) to afford marchantin A (79.5 g) and marchantin E (8.34 g) (13, 14).

Plasmid construction

The construction of plasmids for the reporter assay using green fluorescent protein (GFP) derivatives has been described in a previous report (12). For expression of FXR and RXR α , the ORF region of human FXR or human RXR α (accession number U68233, X52773) was inserted into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). For reporter plasmids, the FXR response element (4 copies of DR1: ggatccaaactgaGGGTCAgTGACCC aagtgaactgaGGGTCAgTGACCCaagtgaactcaact gagGGTCAgTGACCCaagtgaactgaGGGTCAgTGACCCaagtgaactct), the 3' region (201 bp) of cytomegalovirus (CMV201) promoter, and enhanced yellow fluorescent protein (EYFP) were ligated. As an internal control plasmid, the luciferase gene of pGL3-Control Vector (Promega, Madison, WI, USA) was replaced with enhanced cyan fluorescent protein (ECFP).

For a reporter assay using luciferase, FXRE was inserted into the *Mlu*I and *Bgl*II sites of pGL3-Control Vector, and the SV40 promoter was replaced with minimal CMV promoter. The pRL-CMV vector (Promega) was used as an internal control vector.

The reporter assay using GFP derivatives

A monkey kidney cell line, COS-7, was kept in DMEM (Sigma-Aldrich) with penicillin (100 unit/ml),

streptomycin (100 µg/ml), and 10% FBS. Transfections were performed using Effectene transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The ratio of the reporter plasmid, FXR expression plasmid, RXR expression plasmid, and the internal control plasmid was 4:1:1:1. The culture medium was replaced with DMEM without phenol red (GIBCO, Carlsbad, CA, USA) supplemented with 10% charcoal-treated FBS (Hyclone, Logan, UT, USA) when the transfections were performed. At 15 h after transfection, the cells were treated with trypsin-EDTA (GIBCO) and divided among wells of a black 96-well plate with 100 µl of the culture medium. At 6 h after division among wells, the cells were treated with chemicals. After 40-h incubation, the medium was eliminated by decantation, the cells were washed twice with PBS, and the wells were filled with 200 µl PBS. Fluorescence was detected using a microplate reader (ARVO; Perkin Elmer, Fremont, CA, USA). The fluorescence of EYFP was detected with an excitation filter of 485 nm and an emission filter of 545 nm, and that of ECFP was detected with filters of 420 nm and 486 nm (Perkin Elmer), respectively. The autofluorescence in COS-7 cells was subtracted from each of the detected fluorescences, and the EYFP/ECFP ratio was calculated using the resulting values.

The reporter assay using luciferase

The human hepatocyte cell line HepG2 was kept in MEM (Sigma-Aldrich) with penicillin (100 unit/ml), streptomycin (100 µg/ml), and 10% FBS. The cells were transfected with 3 times more plasmids than recommended with salmon sperm DNA (200 ng for 1 well of a 6-well plate) using Effectene transfection reagent (Qiagen). In contrast, the human hepatocyte cell line HuH-7 was kept in DMEM with penicillin (100 unit/ml), streptomycin (100 µg/ml), and 10% FBS; and the human intestinal cell line Caco-2 was kept in DMEM with penicillin (100 unit/ml), streptomycin (100 µg/ml), 10% FBS, and 100 µM MEM Non-Essential Amino Acids Solution (GIBCO). HuH-7 and Caco-2 cells were transfected with plasmids using Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. The ratio of the reporter plasmid using luciferase, the FXR expression plasmid, RXR expression plasmid, and the internal control plasmid using *renilla* luciferase was 4:1:1:1. FBS of the culture medium was replaced with charcoal-treated FBS (Hyclone) when the transfections were performed. The following manipulations were the same as those used in the reporter assay with GFP derivatives. After 40-h treatment with the compounds, measurement of luciferase and *renilla* luciferase was performed with

the Dual-Glo™ Luciferase Assay System (Promega) according to the manufacturer's instructions.

TaqMan primers and probes

Oligonucleotide primers and probes for human *BSEP*, *SHP*, and *CYP7A1* were synthesized by Applied Biosystems (Foster City, CA, USA). These sequences (5' to 3') were as follows: Human *BSEP*, forward primer (GGGCCATTGTACGAGATCCTAA), probe (6FAM-TCTTGCTACTAGATGAAGCCACTTCTGCCTTAGA-TAMRA) and reverse primer (TGCACCGTCTTT CACTTTCTG); Human *SHP*, forward primer (GGTG CAGTGGCTTCAATGC), probe (6FAM-TCTGGAG CCTGGAGCTTAGCCCCA-TAMRA), and reverse primer (GGTTGAAGAGGATGGTCCCTTT); Human *CYP7A1*, forward primer (GAGAAGGCAAACGGGT-GAAC), probe (6FAM-TGGATTAATCCATACCTG GGCTGTGCTCT-TAMRA), and reverse primer (GGT ATGACAAGGGATTGTGATGA). The primers and probe for 18S rRNA were also purchased from Applied Biosystems.

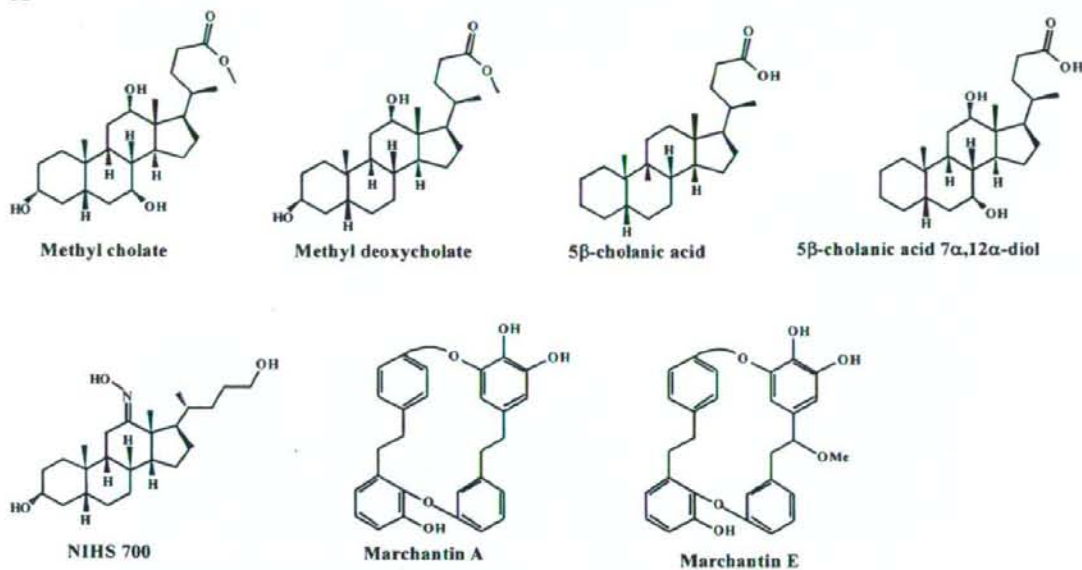
RNA isolation and real-time quantitative PCR

The culture medium of HepG2, HuH-7, and Caco-2 cells was replaced with the medium supplemented with 10% charcoal-treated FBS (Hyclone) at 24 h before treatment with the compounds. The cells were treated with the tested chemicals for 24 h, and total RNA was then prepared using the RNeasy purification system according to the manufacturer's instructions (Qiagen). Reverse transcription reactions and TaqMan-PCRs were performed using the High-Capacity cDNA Archive Kit (Applied Biosystems) and the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Sequence-specific amplification was quantified with the ABI Prism 7700 sequence detection system (Applied Biosystems), and values were normalized to 18S rRNA.

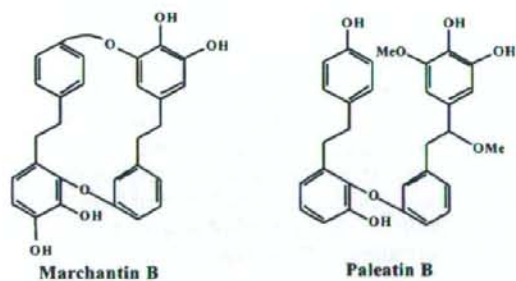
Results

We found seven compounds that activate FXR (the chemical structures of which are shown in Fig. 1A) using the reporter assay system described by Suzuki et al. (12). In the reporter assay system, two fluorescent proteins, EYFP and ECFP, were used for detection of FXR activation and as an internal control, respectively. The activation of FXR by the seven compounds and some endogenous ligands [i.e., cholic acid (CA), deoxycholic acid (DCA), lithocholic acid (LCA), and chenodeoxycholic acid (CDCA)] is shown as the increased ratio of EYFP/ECFP fluorescence intensity in the upper panel of Fig. 2A. As a control, the reporter vector

A



B



C

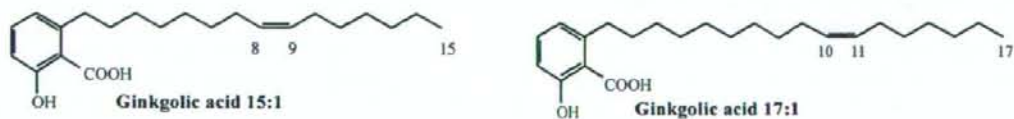


Fig. 1. Structure of the compounds that activated FXR and their related compounds. A: Structure of the compounds that activated FXR as determined by the reporter assay. B: The compounds similar to marchantin A and marchantin E. C: Ginkgolic acid 15:1 and ginkgolic acid 17:1 that highly activated FXR described in Ref. 12.

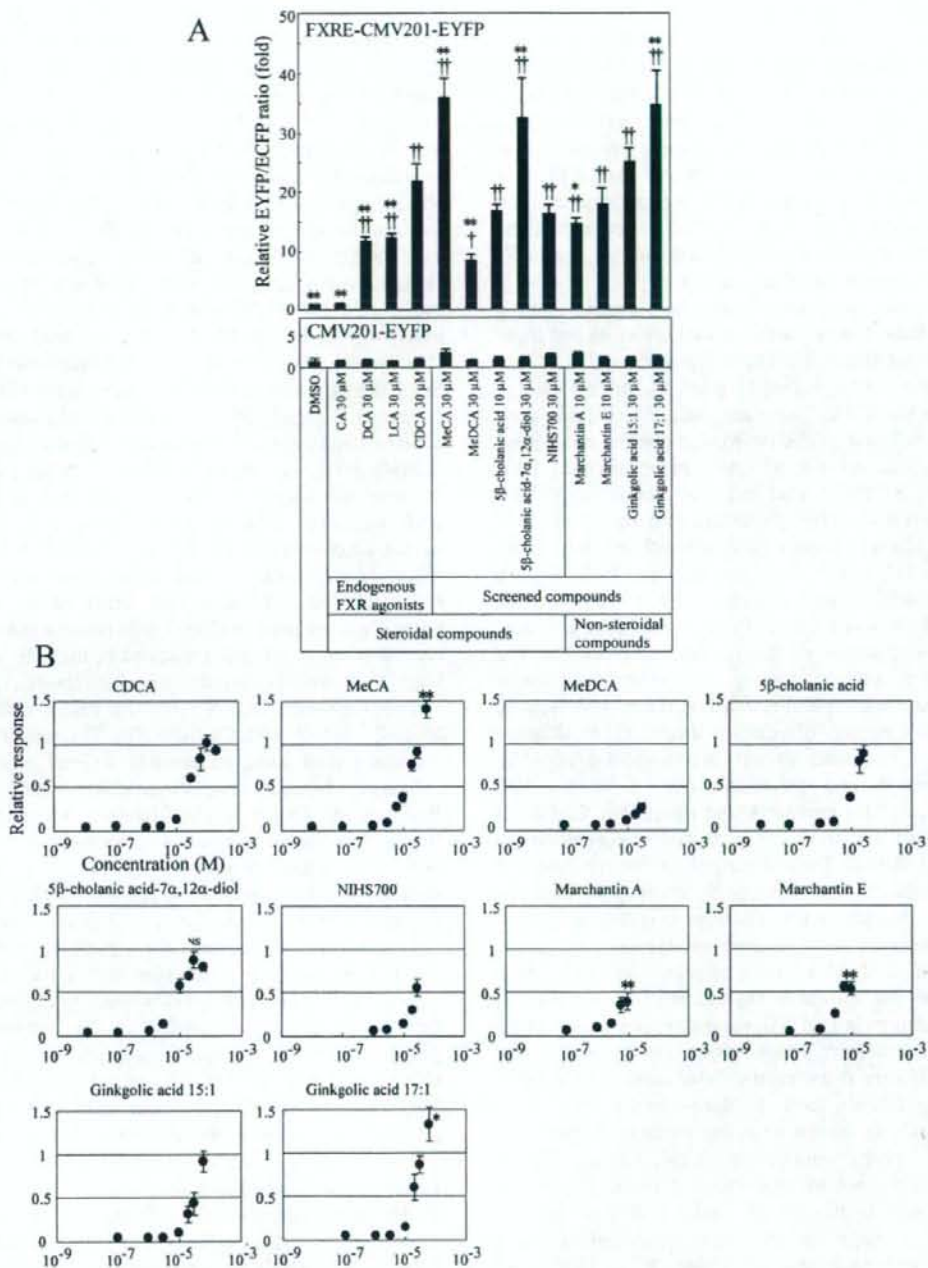


Fig. 2. Response in the reporter expression. **A:** The activation of FXR. COS-7 cells were transfected with the reporter plasmid containing FXRE, the expression plasmids of FXR and RXR α , and the internal control plasmid. The transfected cells were treated with each compound. All values are means \pm S.D., $n = 4$. $!P < 0.05$ vs DMSO, $!P < 0.01$ vs DMSO, $*P < 0.05$ vs CDCA (30 μ M), $**P < 0.01$ vs CDCA (30 μ M), according to Dunnett's test. **B:** Dose-response analyses on the reporter assay of FXR. The response was shown as a ratio, compared with that by CDCA at 100 μ M. All values are means \pm S.D., $n = 4$. $*P < 0.05$, $**P < 0.01$; NS, not a significant difference, compared with CDCA at 100 μ M according to Student's t -test.

without FXR response element was used in place of the reporter vector to determine the response by the unexpected factors (the change in the transcriptional efficiency unrelated to FXR, the self-fluorescence of the tested chemicals, and so forth) (lower panel of Fig. 2A). Moreover, it was confirmed that these seven compounds activated the expression of the reporter gene EYFP via FXR because the induction of EYFP expression required cotransfection of an FXR expression vector and the compounds did not activate RXR homodimer and RAR-RXR heterodimer (data not shown).

The seven compounds could be separated into 2 groups: those that contained steroid skeletons and those that did not (Fig. 1A). The compounds in the former group were methyl cholate (MeCA), methyl deoxycholate (MeDCA), 5 β -cholic acid, 5 β -cholic acid-7 α ,12 α -diol, and NIHS700. Methyl cholate and methyl deoxycholate are methyl esters of endogenous FXR ligands (i.e., cholic acid and deoxycholic acid). FXR was also activated by 5 β -cholic acid and 5 β -cholic acid-7 α ,12 α -diol, whose structures differed only in the 3 α -hydroxyl group from endogenous FXR ligands (i.e., lithocholic acid and cholic acid). The structure of NIHS700 was different from that of lithocholic acid at the substituents of the 11- and 24-positions. The compounds in the latter group, non-steroidal compounds, were marchantin A and marchantin E that were isolated from the liverwort *Marchantia* species (13). Although these two compounds strongly activated FXR (Fig. 2A), marchantin B and paleatin B (shown in Fig. 1B), analogues of marchantin A and marchantin E, did not cause such activation, even at higher concentrations (data not shown). The difference between marchantin A and marchantin B is only one hydroxyl group, and that between marchantin E and paleatin B is ring-opening or not. Along with the seven compounds shown in Fig. 1A, ginkgolic acid 15:1 and ginkgolic acid 17:1 (their structures are shown in Fig. 1C and the activation of FXR is shown in Fig. 2A), the major constituents of the crude extracts from ginkgo leaves that highly activated FXR (12) were also studied in detail in the present study.

In Fig. 2B, we show the dose-dependency of these compounds as compared to the maximal response by the most potent endogenous ligand, CDCA. All the compounds were hydrophobic and could not be dissolved at over 30–60 μ M, each, in culture medium. Moreover, responses by some compounds even at soluble concentrations (Marchantin E at 30 μ M and MeDCA at 60 μ M) could not be measured due to their toxicity. Therefore, we could not obtain the data at higher concentrations. Although the value of treatment with 30 μ M 5 β -cholic acid is shown in Fig. 2B, this compound slightly separated out from the solution at

this concentration. Therefore, the concentration applied in the other experiments was limited to 10 μ M. Since the EC₅₀ values of 5 β -cholic acid-7 α ,12 α -diol, marchantin A, and marchantin E could be estimated to be 3–10, 3–6, and 3–6 μ M, respectively, the values were lower than that of CDCA (approximately 30 μ M). Maximal activation by 5 β -cholic acid-7 α ,12 α -diol was comparable to that by CDCA, but those by the marchantins were lower ($P < 0.01$). The EC₅₀ and maximal values of MeCA, MeDCA, 5 β -cholic acid, NIHS700, and ginkgolic acids could not be assessed because of low solubility or toxicity. However, activations of FXR by 60 μ M of MeCA and ginkgolic acid 17:1 were higher than maximal activation by CDCA. Since the EC₅₀ values of the 6 compounds could not be estimated, we analyzed activations by all the compounds at 30 or 10 μ M in the following experiments.

Since FXR was primarily localized in the liver and intestine, we determined that the compounds also act as FXR activators in cultured hepatoma and intestinal cancer cells (HepG2, HuH-7, and Caco-2 cells). The reporter assay using GFP derivatives could be applied to these cell lines. However, the signal of the internal control was less than in COS-7 cells because the internal control plasmid was not replicated in the cells without large T antigen. We therefore used luciferase (LUC) and *renilla* luciferase (RLUC) for the reporter assay in HepG2, HuH-7, and Caco-2 cells. The extent of the induction with some compounds differed among the cells on the basis of the activation extent by endogenous ligands (Figs. 2A and 3). The differences are enumerated below. Reporter expression was strongly induced by MeCA to higher level than by CDCA in COS-7, moderately induced in HuH-7, and only weakly induced to a lower level than by CDCA in HepG2. Marchantins (A and E) scarcely induced the expression in HepG2. MeDCA induced the expression higher than LCA in HepG2 ($P < 0.05$, according to Student's *t*-test), but lower than LCA in COS-7 ($P < 0.01$). The induction by ginkgolic acid 17:1 in HepG2 was lower than that by CDCA, but higher in COS-7. Moreover, the induction by NIHS700 in Caco-2 was lower than that by LCA ($P < 0.05$, according to Student's *t*-test), but higher in COS-7 ($P < 0.01$). These compounds seemed to activate FXR in a cell-type-specific fashion.

We then examined the effects of the screened compounds on the expression of genes regulated by FXR. FXR controls the expression of critical genes in bile acid and cholesterol homeostasis (2–4). In this experiment, we detected the expression of three genes, bile salt export pump (*BSEP*), small heterodimer partner (*SHP*), and *CYP7A1*. By FXR activation, the expression of *BSEP* and *SHP* genes are directly upregulated, and

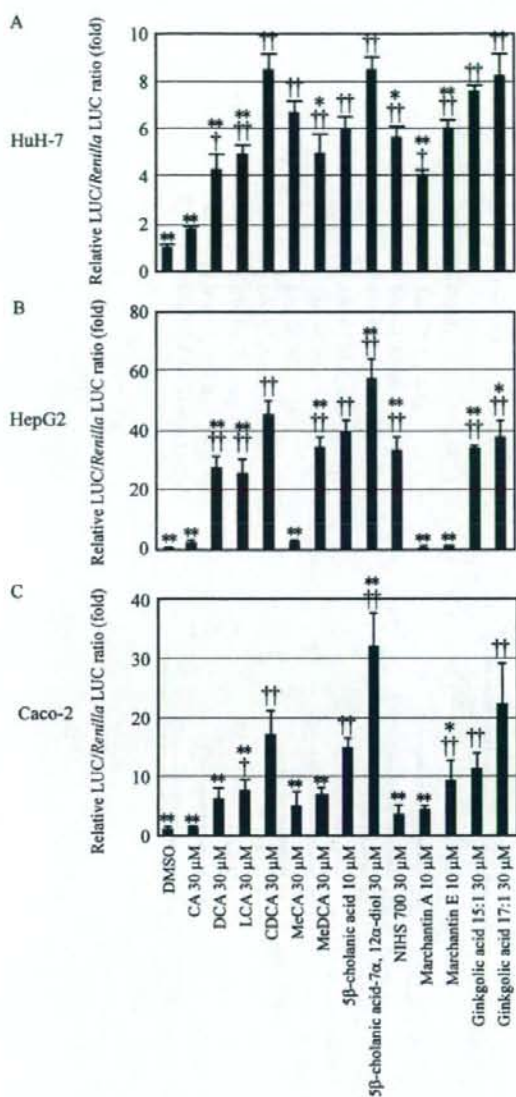


Fig. 3. The difference of FXR-activation in the reporter assay. A: The activation of FXR in HuH-7 cells is shown. HuH-7 cells were transfected with the reporter plasmid containing FXRE and luciferase, the expression plasmids of FXR and RXR α , and the internal control plasmid containing *renilla* luciferase. The transfected cells were treated with each compound. Data are shown as the means \pm S.D. derived from four wells. $^{\dagger}P < 0.05$ vs DMSO, $^{\dagger\dagger}P < 0.01$ vs DMSO, $^*P < 0.05$ vs CDCA (30 μ M), $^{**}P < 0.01$ vs CDCA (30 μ M), according to Dunnett's test. The activation of FXR in HepG2 and Caco-2 cells is shown in the middle and lower panel, respectively. Except for the cell type, all manipulations were the same as those shown above. B: HepG2 cells, $n = 4$. C: Caco-2 cells, $n = 4 - 5$.

that of *CYP7A1* gene is indirectly down-regulated. HepG2 cells without transfection of artificial genes were treated with the compounds, and the amount of the mRNAs was measured by real-time quantitative PCR. The expression of the genes in HepG2 cells is shown in Fig. 4 (A - C). The compounds other than MeCA, 5 β -cholanic acid, marchantin A, and marchantin E significantly induced the expression of *SHP* mRNA ($P < 0.05$ vs DMSO, according to Dunnett's test). Because *BSEP* was expressed only at low levels in HepG2 cells, Fig. 4C showed scattered results. However, the change in *BSEP* expression was similar to the change in *SHP* expression. On the other hand, the reduction levels of *CYP7A1* mRNA by some compounds differed from the change of *SHP* mRNA levels. The reduction in *CYP7A1* mRNA accumulation by MeDCA was lower than that by CDCA, although the compound induced *SHP* mRNA accumulation to a higher level than CDCA. Moreover, marchantin A and E reduced *CYP7A1* mRNA accumulation ($P < 0.05$ vs DMSO), although the regulation of *SHP* mRNA accumulation was not detected.

Moreover, regulation of gene expression in a cell-type-specific fashion was also detected (Fig. 4D). The induction of *BSEP* expression by MeCA in HuH-7 cells was higher than that of *BSEP* and *SHP* expression in HepG2 cells, and that by MeDCA was lower. These results indicate that the screened compounds could regulate the expression of critical genes in bile acid and cholesterol homeostasis and suggested that MeCA, MeDCA, marchantin A, and marchantin E possibly regulate expression of the genes in a cell-type-specific and/or gene-species selective fashion.

Discussion

In this paper, we found five steroidal compounds and two non-steroidal compounds that activate FXR. These compounds possess some properties that differ from those of CDCA.

First, two of the steroidal compounds, 5 β -cholanic acid and 5 β -cholanic acid-7 α ,12 α -diol, were found to be effective ligands for FXR in a reporter assay and in quantitative real-time PCR. The crystal structure of the FXR ligand binding domain was analyzed as a complex with 3-deoxyCDCA and revealed that the 3-hydroxyl group was not responsible for the activation of FXR (15). However, FXR was activated to lower levels by lithocholic acid and cholic acid, whose structures were different only in the 3 α -hydroxyl group from 5 β -cholanic acid and 5 β -cholanic acid-7 α ,12 α -diol. In this case, elimination of the 3-hydroxyl group increased the potency of the activation.

The other three steroidal compounds were MeCA,

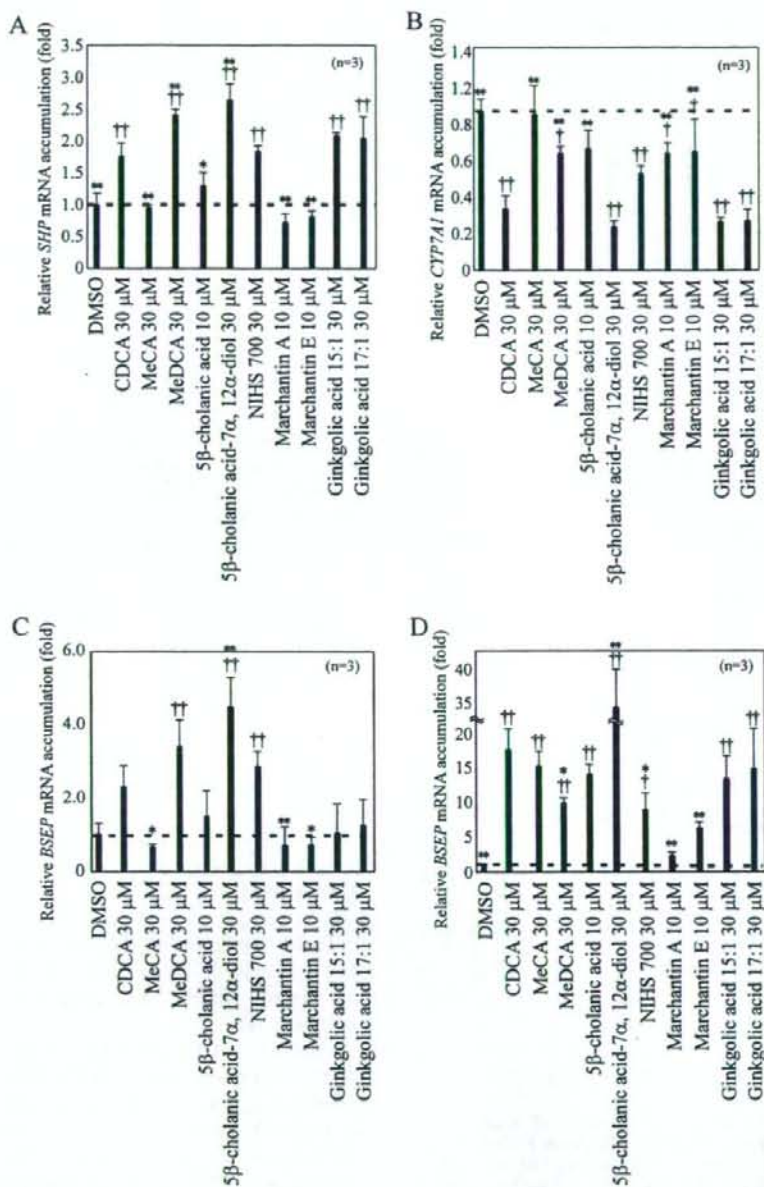


Fig. 4. Expression of *SHP*, *CYP7A1*, and *BSEP* genes in cells treated with the compounds. HepG2 cells were treated with each compound for 24 h. Accumulation of *SHP* (A), *CYP7A1* (B), and *BSEP* (C) mRNA in HepG2 cells was detected by real-time quantitative PCR. D: Accumulation of *BSEP* mRNA in HuH-7 cells treated with each compound for 24 h. All values are means \pm S.D., $n = 3$. $^{\dagger}P < 0.05$ vs DMSO, $^{\dagger\dagger}P < 0.01$ vs DMSO, $^*P < 0.05$ vs CDCA (30 μ M), $^{**}P < 0.01$ vs CDCA (30 μ M), according to Dunnett's test.

MeDCA, and NIHS700. Although the structures of these compounds were similar to those of the endogenous

ligands, the slight modification of bile acids caused different regulation of FXR. It has been suggested that

the binding of bile acids with a slightly different structure (CA, DCA, UDCA, and CDCA) resulted in different FXR conformations, which in turn differentially regulated expression of individual FXR targets (16). Therefore, these compounds may also produce different FXR conformations than that produced by CDCA. In fact, MeDCA showed different properties in the regulation of the genes as compared to CDCA. DCA, CA, and UDCA have been reported to partially increase *BSEP* expression, but repress *CYP7A* mRNA with nearly equal effects as CDCA (16). Adversely, MeDCA strongly induced *BSEP* mRNA, but only weakly reduced *CYP7A1* mRNA (Fig. 4: B and C). It is possible that the *CYP7A1* expression was also influenced by other factors such as c-jun N-terminal kinase and xenobiotic receptor (17, 18). However, since MeDCA possibly induces the export of bile acids from hepatocytes without disturbing catabolization from cholesterol to bile acids, they might effectively improve the disorder of cholesterol and bile acids. NIH700 seemed to have properties similar to those of MeDCA, although statistically significant differences could not be observed by Dunnett's test. Therefore, further analyses about MeDCA and NIH700 are needed for effective regulation of FXR.

Since MeCA and MeDCA are methyl esters of endogenous FXR ligands, there is a question about whether these compounds are hydrolyzed and act as CA or DCA. Considering that MeDCA showed different properties in the gene expression as compared to DCA, these compounds did not seem to act as hydrolyzed forms.

The compounds screened in this study contained several non-steroidal chemicals. Marchantin A was isolated from the liverwort *Marchantia* species, as its major component (13). It shows antifungal, antimicrobial, cytotoxic, muscle-relaxing, and 5-lipoxygenase, cyclooxygenase, and calmodulin inhibitor activities [reviewed in Asakawa et al. (19)]. The compounds with a slight structural change from marchantin A and E (i.e., marchantin B and paleatin B) did not activate FXR. It may be interesting to examine the mechanism of binding to FXR and that of the subsequent regulation of the target genes. Since these two compounds and ginkgolic acids have a much different structure from bile acids, the structural change of FXR in response to these compounds might be different from that in response to bile acids, which might result in different regulation pattern of the genes of bile acid homeostasis. In fact, marchantin A and marchantin E activated FXR diversely in each cell, although ginkgolic acids did not show FXR activation in a cell-type specific or gene-selective fashion. Bile acids function in the dietary lipid absorption and were reported to activate mitogen-activated protein kinase

pathways without FXR (17, 20). The non-steroidal compounds were thought not to have these functions, although it has not been clear whether these compounds have effects other than FXR activation. Moreover, the non-steroidal compounds are not metabolized to form harmful lithocholic acid (21, 22). Therefore, it is possible that these compounds can be used for studying the pharmacology of FXR.

As described above, some compounds demonstrated properties of cell-type-specific and/or gene-selective modulators. Until now, several compounds have been found to be cell-type-specific and/or gene-selective modulators of FXR (10, 11, 16). Although the mechanism of selective FXR modulation remains to be elucidated, it was suggested that differences in coregulator recruitment play a critical role in cell-type-specific and promoter-specific regulation by other nuclear receptors (23, 24). We therefore investigated the binding capacity of FXR with receptor-interacting domains of the activator for thyroid hormone and retinoid receptors (ACTR), vitamin D-interacting protein 205 (DRIP205), glucocorticoid receptor-interacting protein (GRIP), receptor-interacting protein 140 (RIP140), and steroid receptor coactivator-1 (SRC-1) with a mammalian two-hybrid assay. However, we could not detect enough obvious differences to explain the cell-type (and/or gene) specific modulation (data not shown).

There are the other possibilities about cell-type-specific modulation, although the gene specific modulation could not be explained. First, the compounds are metabolized in cultured cells and their metabolites bind to FXR as ligands. In case that the metabolism varies with cell-type, FXR is differentially-activated. Second, the compounds are inactivated by metabolism in some cells. Third, permeability of the compounds is different according to cell-type. Since all the compounds tested were hydrophobic and separated out in culture medium at lower concentrations as compared to CDCA, the third possibility is unlikely. To reveal the mechanism of selective FXR modulation and to produce a synthetic selective modulator, farther analyses are required.

Finally, FXR has pleiotropic therapeutic potential, but a simple FXR agonist will have undesired side-effects (reviewed in refs. 25 and 26). The compounds discussed in the present paper appear to be useful for studying FXR modulation leading to selective FXR modulation for therapy.

Acknowledgments

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