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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 (CCl_4) 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 CCl_4 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; CCl_4, carbon tetrachloride; HGF, hepatocyte growth factor.

Annexin (Anx) A3 is a member of the Anx family, which binds to phospholipids and membranes in a Ca^{2+}-dependent manner (*1–4*). AnxA3 has been shown to have anti-coagulant and anti-phospholipase A_2 properties *in vitro* (*5*, *6*), plus to promote Ca^{2+}-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 tetracholoride (CCl_4) 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 libitium*. 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 CCl_4 intraperitoneally (2 ml/kg body weight of 50% solution of CCl_4 in olive oil). Control rats received olive oil intraperitoneally (1 ml/kg body weight of olive oil). Animals given CCl_4 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 CCl_4 intraperitoneally, as described earlier. Control rats were injected with the same volume and amount of control goat IgG, and then received CCl_4 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 100,000*g*, 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 100,000*g*, 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 AnxA3 antibody serum (1: 5,250) (a gift from Drs F. Russo-Marie and C. Ragueness-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 AnxA3 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 manufacture's protocol. Equal amounts of RNA (~1 μg) from each experiments 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 (Rosche Diagnostics, Tokyo, Japan) according to the manufacture's instructions. For AnxA3 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 AnxA3 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*). AnxA3 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, Immuno-Biological Laboratories Co., Ltd. Gunma, Japan) 1:20 and rabbit anti-human AnxA3 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

AnxA3 Expression in Liver Following CCl_4 Treatment—AnxA3 protein level increased ~3-fold at 6 h after administration of CCl_4 and this increased level was maintained to 24 h (Fig. 1). AnxA3 mRNA level started to increase at 3 h after administration, reaching an ~17-fold increase at 24 h (Fig. 2).

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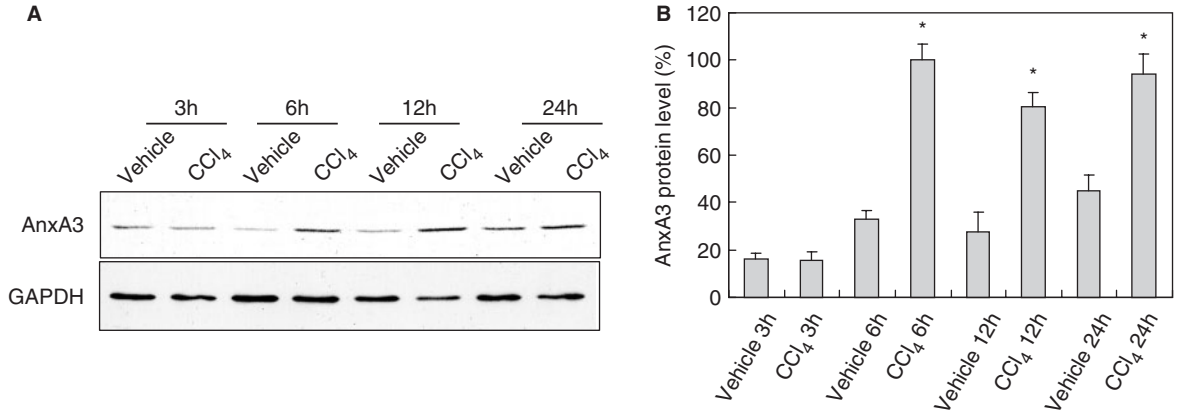

Fig. 1. **AnxA3 protein level in liver following treatment with CCl_4.** (A) Data shown are representative of western blot analysis results. Approximately 35 and 1.5 μg of protein were used for detection of AnxA3 and GAPDH, respectively. (B) Results are presented relative to the value produced by liver in rats at 6 h after CCl_4 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.

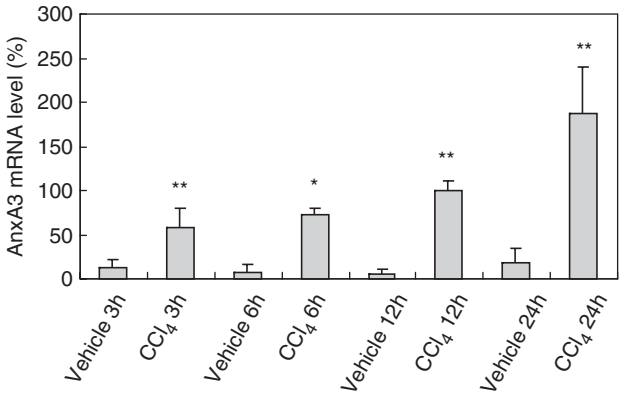

Fig. 2. **AnxA3 mRNA level in liver following treatment with CCl_4.** Results are presented relative to the value produced by liver in rats at 6 h after CCl_4 administration ($n = 4$ at each time point). AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Data are expressed as the mean ± 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 CCl_4 Treatment—Parenchymal hepatocytes and/or non-parenchymal liver cells are involved in the increase of AnxA3 expression in liver following CCl_4 treatment. AnxA3 protein level increased ~5-fold in parenchymal hepatocytes at 6 h after CCl_4 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 CCl_4 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 CCl_4 Treatment—To investigate whether HGF is involved in the increase in AnxA3 mRNA level in hepatocytes following CCl_4 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 CCl_4, respectively. DNA synthesis begins to change at ~16 and 24 h in partially hepatectomized rats and rats treated with CCl_4, 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.

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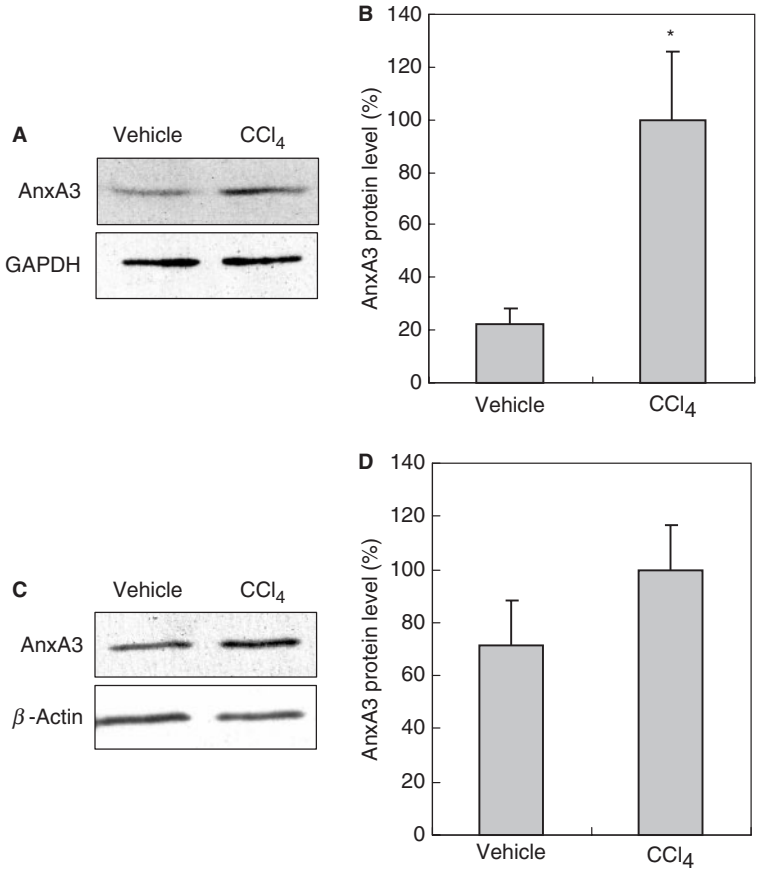

Fig. 3. **AnxA3 protein level in parenchymal hepatocytes and non-parenchymal cells isolated from liver in rats following treatment with CCl_4.** (A) Parenchymal hepatocytes and (C) non-parenchymal cells were isolated from liver in rats at 6 h after either CCl_4 or olive oil treatment. Data shown are 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 CCl_4 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.

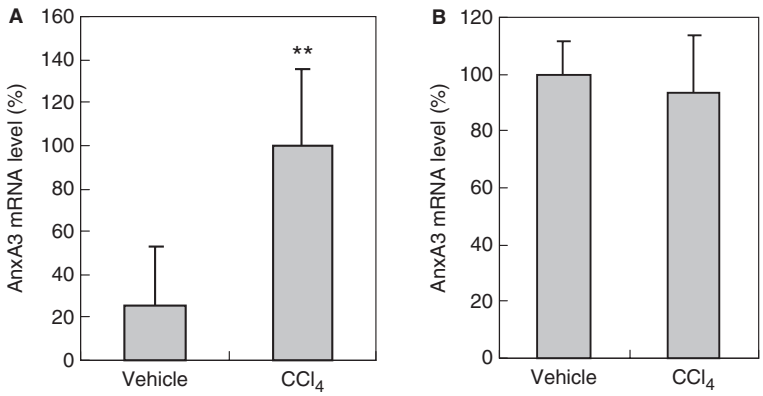

Fig. 4. **AnxA3 mRNA level in parenchymal hepatocytes and non-parenchymal cells isolated from livers in rats following treatment with CCl_4.** (A) Parenchymal hepatocytes and (B) non-parenchymal liver cells were isolated from liver in rats at 6 h after either CCl_4 or olive oil treatment. AnxA3 levels 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 CCl_4 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.

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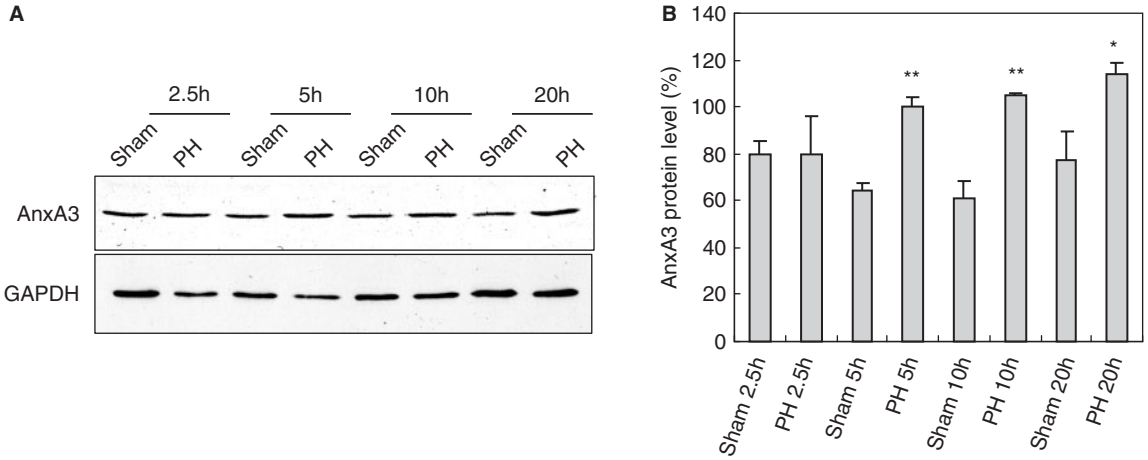

Fig. 5. **AnxA3 protein level in liver after partial hepatectomy.** (A) Data shown are representative of western blot analysis results. Approximately 35 and 1.5 μg of protein were used for detection of AnxA3 and GAPDH, respectively. (B) Results are presented relative to the values for liver in rats at 5 h after partial hepatectomy. AnxA3 protein levels were normalized to levels of housekeeping protein, GAPDH. Data are expressed as mean ± 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.

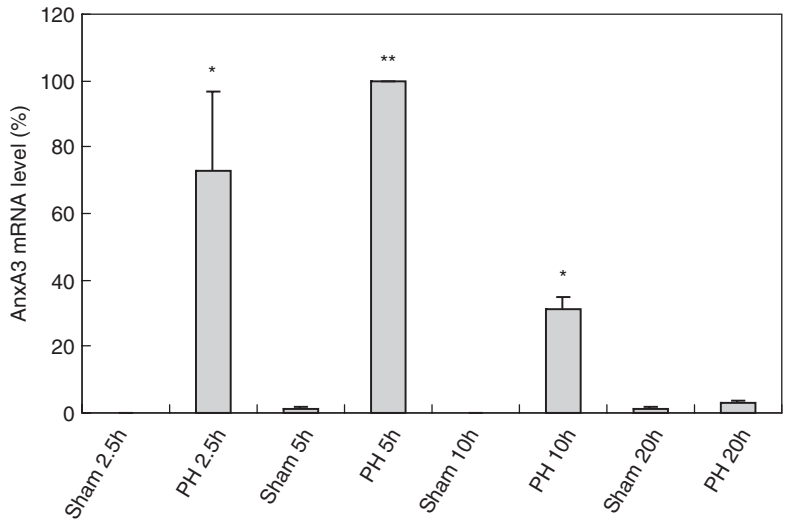

Fig. 6. **AnxA3 mRNA level in liver after partial hepatectomy.** Results are presented relative to the value produced by liver in rats at 5 h after partial hepatectomy. AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Data are expressed as mean ± SD ($n = 4$ at each time point) $^{*}P < 0.01$, $^{**}P < 0.05$, 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 24 h after CCl_4 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

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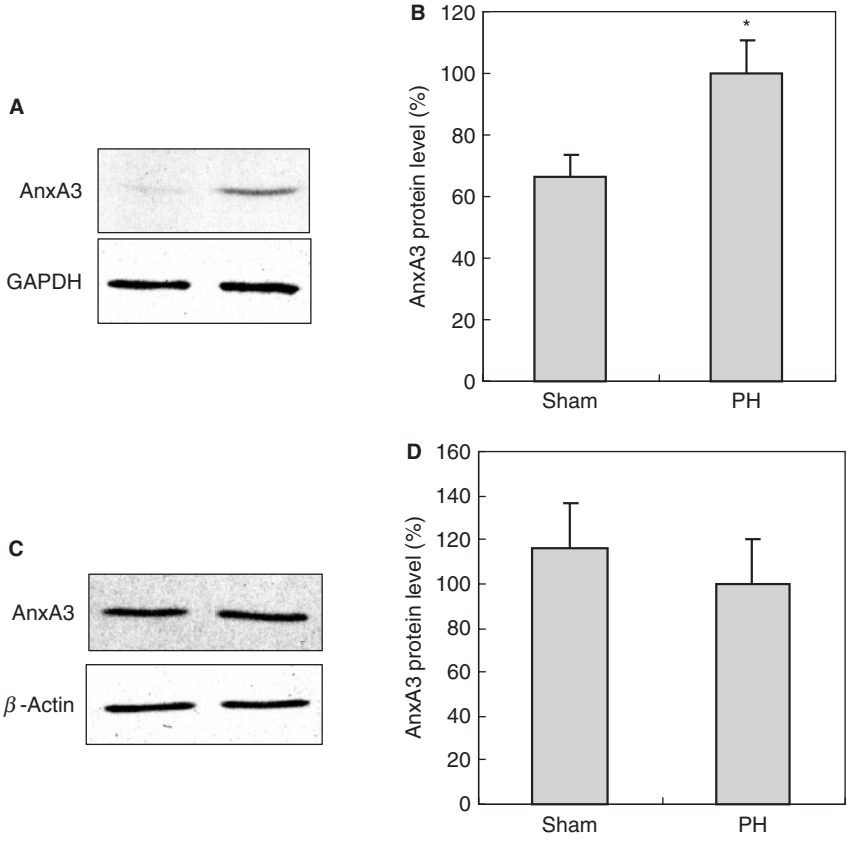

Fig. 7. **AnxA3 protein level in parenchymal hepatocytes and non-parenchymal liver cells after hepatectomy.** (A) Parenchymal hepatocytes and (C) non-parenchymal liver cells were isolated at 5 h after partial hepatectomy or sham operation. Data shown are representative of western blot analysis results for parenchymal hepatocytes and non-parenchymal liver cells, respectively. Approximately 90 and 2.8 μg of protein were used for 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 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 ± SD ($n = 4$) $^{*}P < 0.01$, compared to parenchymal hepatocytes and non-parenchymal liver cell from rats at 5 h after sham operation.

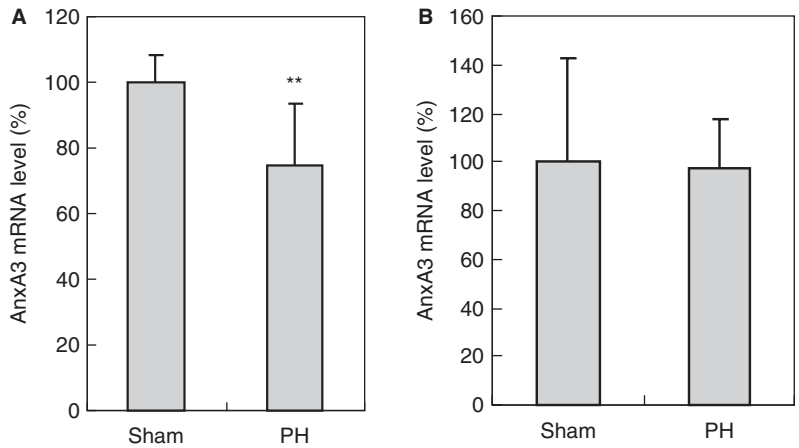

Fig. 8. **AnxA3 mRNA level in parenchymal hepatocytes and non-parenchymal liver cells after partial hepatectomy.** (A) Parenchymal hepatocytes and (B) non-parenchymal liver cells were isolated from liver in rats at 5 h after either partial hepatectomy or sham operation. AnxA3 mRNA levels were normalized 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 ± SD ($n = 4$) $^{**}P < 0.05$, compared to parenchymal hepatocytes and non-parenchymal liver cells from rats at 5 h after sham operation.

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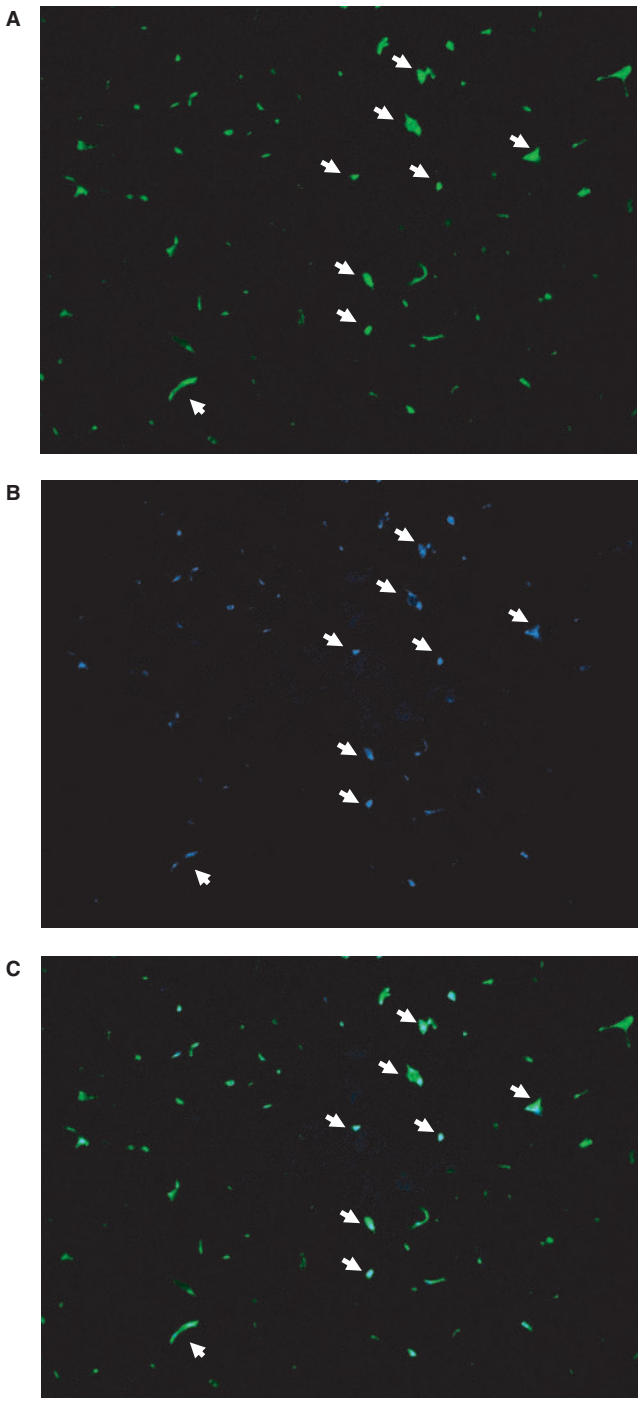

Fig. 9. **AnxA3 expression in hepatic sinusoidal endothelial cells in normal rat liver.** (A) AnxA3-positive cells; (B) SE-1-positive cells; (C) Merged image of AnxA3- and SE-1-positive cells. In (A–C), arrows show examples of positive immunoreactive cells.

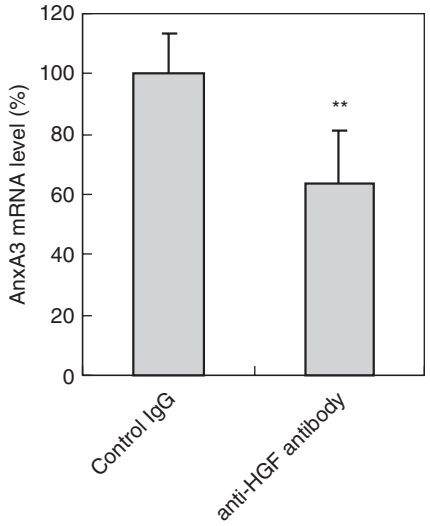

Fig. 10. **Effect of anti-HGF antibody on AnxA3 mRNA level in parenchymal hepatocytes following treatment with CCl_4.** Hepatocytes were isolated from liver in rats at 6 h following treatment with either anti-HGF IgG or control IgG, then CCl_4. AnxA3 levels were normalized to housekeeping gene, 28S rRNA. Results are presented relative to the value produced by hepatocyte isolated from liver in rats at 6 h following treatment with control IgG, then CCl_4. Data are expressed as mean ± SD ($n = 4$) $^{**}P < 0.05$, compared to hepatocytes from rats at 6 h following treatment with control IgG, then CCl_4.

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 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 6 h after CCl_4 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 6 h at the latest after CCl_4 administration for HGF to increase AnxA3 mRNA level. This was indicated by the finding that HGF protein dramatically rises in the plasma at 2 h after partial hepatectomy and CCl_4 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

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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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