EXPERIMENTAL PROCEDURES

Cell Culture—The hMSCs derived from bone marrow [Lonza (Cambrex), Walkersville, Maryland, USA] were cultured in mesenchymal stem cell growth medium (MSCGM) [Lonza (Cambrex) #PT-3001; mesenchymal stem cell basal medium supplemented with mesenchymal cell growth supplement, L-glutamine and penicillin/streptomycin] at 37 C in CO_2 (5%) incubator. Cells were passaged according to the manufacturer's protocol with slight modification using trypsin–EDTA solution [Lonza (Cambrex) #CC-3232]. Lot numbers of the hMSC batches were as follows: #4F1127, #4F0312, #5F0138, #4F1560, #4F0591 and #4F0760. Informed consent was obtained in Poietics human mesenchymal stem cell systems [Lonza (Cambrex)]. All differentiation procedures were performed according to Lonza (Cambrex) protocol with slight modification.

Osteogenic Differentiation—The hMSCs were plated onto 12-well plates and 24 h later, the medium was changed to MSCGM (as control) or osteogenic induction medium (OIM) [Lonza (Cambrex) #PT-3002; differentiation basal medium containing dexamethasone, ascorbate, mesenchymal cell growth supplement, L-glutamine, penicillin/streptomycin and β-glycerophosphate]. Medium was changed every 3–4 days and cells were differentiated for 21 days.

Calcium Deposition Assay—Calcium deposition was measured using the Stanbio Total Calcium Liquicolor® kit (Stanbio Laboratory, Boerne, Texas, USA; #0150-250) according to the manufacturer's protocol (Cambrex, Stanbio Laboratory). Briefly, the cells cultured on 12-well plates for 22 days (osteogenic-induced for 21 days) were rinsed with phosphate buffered saline (PBS) without calcium and magnesium [Lonza (Cambrex) #17-516Q] and harvested in 0.5 N HCl (600 μl). Calcium was extracted from the cells by shaking the tubes for approximately 20 h at 4 C. Lysates were centrifuged at 500*g* for 2 min at 4 C and 20 μl of the supernatant was used for the assay. Absorption at 560 nm was measured to detect the Ca-ortho-cresolphthalein complexone (OCPC) complex using an EnVision 2103 multilabel reader (PerkinElmer, Waltham, Massachusetts, USA). Calcium deposition was adjusted with the total protein concentration of the samples. Cells harvested in 0.5 N HCl were centrifuged at 15,000 rpm for 10 min at 4 C. The pellet was washed once with PBS without calcium and magnesium, and resuspended in 100 μl of 0.1 N NaOH/0.1% SDS. After overnight incubation at 37 C, the lysate was centrifuged at 15,000 rpm for 10 min at room temperature, and the supernatant was quantitated using the DC protein assay (Bio-Rad Laboratories, Hercules, California, USA) according to the manufacturer's protocol. Absorbance at 620 nm was measured using the EnVision 2103 multilabel reader (PerkinElmer). The standard curve was obtained using bovine serum albumin.

Adipogenic Differentiation—The cells were plated onto a 24 well-plate at 2.1 x 10^4/cm^2, and cultured in MSCGM for 5–6 days. After cells reach confluence, medium was changed to MSCGM (as control) or adipogenic induction medium (AIM) [Lonza (Cambrex) #PT-3004; induction basal medium supplemented with recombinant human insulin, L-glutamine, mesenchymal stem cell growth supplement, penicillin/streptomycin, dexamethasone, indomethacin and IBMX (3-Isobutyl-1-methylxanthine)]. Medium was changed after 3 days into adipogenic maintenance medium (maintenance basal medium supplemented with recombinant human insulin, L-glutamine, penicillin/streptomycin and mesenchymal stem cell growth supplement). After three complete cycles of induction/maintenance, the cells were cultured for 7 more days in adipogenic maintenance medium, replacing the medium every 2–3 days.

Oil Red O staining—The cells were rinsed with 500 μl of PBS and fixed with 10% neutral buffered formalin (500 μl). After washing with sterile water, the cells were washed with 60% 2-propanol (500 μl) for 2–5 min and stained with Oil Red O (500 μl) for 5 min. The cells were rinsed with tap water and stained with Harris' haematoxylin (500 μl) for 1 min and rinsed with the water. Lipid vesicles were observed with microscope Biozero BZ-8000 (KEYENCE, Osaka, Japan).

Chondrogenic Differentiation—The cells (3×10^5) were washed with incomplete chondrogenic induction medium [Lonza (Cambrex) #PT-3003; chondrogenic basal medium containing dexamethasone, ascorbate, ITS (insulin-transferrin-sodium selenite) + supplement, sodium pyruvate, proline, penicillin/streptomycin, L-glutamine] and were resuspended in 0.5 ml of complete chondrogenic induction medium (CCIM; incomplete condrogenic induction medium supplemented with 10 ng/ml of TGF-β3) or MSCGM (as control) and cultured in 15 ml polypropylene culture tubes. The medium was replaced every 3–4 days and the cells were cultured for 24 days.

Safranin-O Stains for in vitro Chondrogenesis—The chondrogenic pellets were fixed in 10% neutral buffered formalin and paraffin embedded. The paraffin sections were stained with Weigert's iron hematoxylin (Wako 298-21741), 0.02% fast green FCF (MP biomedicals 195178) and 0.1% Safranin-O (Sigma HT 90432), followed by observation with microscope Biozero BZ-8000 (KEYENCE).

Total RNA Purification—The hMSCs were cultured on a 10 cm dish, lysed in 600 μl of Buffer RLT (RNeasy® Lysis Buffer) with β-mercaptoethanol and homogenized using a QIA shredder (QIAGEN, Düsseldorf, Germany). Total RNA was purified using RNeasy® mini spin columns according to manufacturer's protocol (QIAGEN). Total RNA was eluted with RNase-free water.

Microarray Analysis—Total RNA (100 ng or 1 μg) was reverse transcribed and amplified using a GeneChip® kit (Affymetrix, Santa Clara, California, USA) and the biotinylated cRNA was hybridized onto the GeneChip® Human Genome U133 Plus 2.0 Array (54,613 probe sets). The data was analysed using GeneChip Operating System software (versions 1.2–1.4), followed by statistical analysis. The data was also analysed using GeneSpring™ (version 7.3) (Agilent, Santa Clara, California, USA). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) (*17, 18*). They are accessible through GEO Series accession number GSE7637 for the data from 4F1560, and GSE7888 for the data obtained from all six batches. The statistical method for microarray data analysis has been also discussed elsewhere (*19*).

J. Biochem. **144,** 399–408 (2008)
doi:10.1093/jb/mvn082

Gene Expression Profiling of Human Mesenchymal Stem Cells for Identification of Novel Markers in Early- and Late-Stage Cell Culture

Shihori Tanabe*, Yoji Sato, Takayoshi Suzuki, Kazuhiro Suzuki, Taku Nagao and Teruhide Yamaguchi†

Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Tokyo 158-8501, Japan

Received January 9, 2008; accepted June 5, 2008; published online June 11, 2008

Human mesenchymal stem cells (hMSCs) are multipotent cells that differentiate into several cell types, and are expected to be a useful tool for cellular therapy. Although the hMSCs differentiate into osteogenic cells during early to middle stages, this differentiation capacity decreases during the late stages of cell culture. To test a hypothesis that there are biomarkers indicating the differentiation potential of hMSCs, we performed microarray analyses and profiled the gene expression in six batches of hMSCs (passages 4–28). At least four genes [necdin homolog (mouse) *(NDN)*, EPH receptor A5 *(EPHA5)*, nephroblastoma overexpressed gene *(NOV)* and runt-related transcription factor 2 *(RUNX2)*] were identified correlating with the passage numbers in all six batches. The results showed that the osteogenic differentiation capacity of hMSCs is down-regulated in the late stages of cell culture. It seemed that adipogenic differentiation capacity was also down-regulated in late stage of the culture. The cells in late stage are oligopotent and the genes identified in this study have the potential to act as quality-control markers of the osteogenic differentiation capacity of hMSCs.

Key words: cellular therapy, culture stage marker, differentiation, gene expression, stem cell.

Abbreviations: EPHA5, EPH receptor A5; hMSCs, human mesenchymal stem cells; NDN, necdin homolog (mouse); NOV, nephroblastoma overexpressed gene; PBS, phosphate buffered saline; RUNX2, runt-related transcription factor 2.

INTRODUCTION

'Cellular therapy' is a new concept in treating diseases with cells that have regeneration potential. Currently, it is at the clinical research stage; however, the use of cellular therapeutics in regular clinical settings will be implemented in near future. Cellular therapeutics involves the use of cells derived from human tissue, either cultured and/or modified, in regenerating and repairing damaged tissues and consequently improving the functions in the human body. Hence, tissue or embryonic stem cells that have the potential to differentiate into a variety of cell types are one of the prime candidate cells for cellular therapeutics. It is difficult to overview the entire discipline of cellular therapeutics since the cells themselves represent 'life'.

Stem cells, one of the candidates for cellular therapeutics, produce daughter cells identical to themselves that differentiate into other types of cells (*1*). The fate of the stem cells is determined by cellular signaling, although the underlying mechanism is still unknown. It is therefore important to investigate the gene expression patterns that influence the cellular signaling pathways and identify the representative biomarkers that can act as indicators of the differentiation potential of the stem cells. Recently, it has been reported that human somatic cells can be induced to pluripotent stem cells (*2*).

There have been several reports suggesting that cellular therapeutics is a promising treatment for several diseases. C-kit-expressing cells obtained from the bone marrow have been used in cardiac tissue repair in mice experiments (*3*). Previous studies have reported the use of autologous bone marrow cells transplantation for the post-infarction recovery of cardiac function (*4–9*). Cytotoxic T cells have also been used for cellular therapy to protect from infectious diseases in an immunodeficient condition following hematopoietic stem cell transplantation (*10*). Mesenchymal stem cells (MSCs) are also used for therapy expecting immunosuppressive effects (*11*, *12*). Previous studies on MSCs also indicate that these cells possess the ability for chondrogenic (*13*), osteogenic (*14*, *15*) and adipogenic differentiation, and possibly other differentiating capabilities (*16*). In a clinical setting, it is difficult to assess the overall profile of each batch of the cells. We hypothesized the existence of quality-control markers for the differentiation potential of human mesenchymal stem cells (hMSCs) and used gene expression profiling to identify these markers.

*To whom correspondence should be addressed. Tel: +81-3-3700-1141, Fax: +81-3-3700-9217, E-mail: stanabe@nihs.go.jp
†Present address: Division of Biological Chemistry and Biologicals, National Institute of Health Sciences

hepatectomy and its implications for initiation of liver regeneration. *Hepatology* **21**, 1695–1701

30. Mayanskii, D.N., Scherbakoff, V.I., Mayanskaya, N.N., and Panin, L.E. (1980) Lysosomal enzyme activity in hepatocytes and Kupffer cells from intact and partially hepatectomized rats. *Biochem. Exp. Biol.* **16**, 309–314
31. Olle, E.W., Ren, X., McClintock, S.D., Warner, R.L., Deogracias, M.P., Johnson, K.J., and Colletti, L.M. (2006) Matrix metalloproteinase-9 is an important factor in hepatic regeneration after partial hepatectomy in mice. *Hepatology* **44**, 540–549
32. Ben-Ze'ev, A., Robinson, G.S., Bucher, N.L., and Farmer, S.R. (1988) Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. *Proc. Natl Acad. Sci. USA* **85**, 2161–2165
33. Lindroos, P.M., Zarnegar, R., and Michalopoulos, G.K. (1991) Hepatocyte growth factor (hepatopoietin A) rapidly increases in plasma before DNA synthesis and liver regeneration stimulated by partial hepatectomy and carbon tetrachloride administration. *Hepatology* **13**, 743–750
34. Liu, F., Song, Y., and Liu, D. (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* **6**, 1258–1266

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.

This work is supported in part by the Grant-in-Aid for Cancer Research (15-2) from the Ministry of Health, Labor and Welfare. We wish to thank Mr Toshihiro Akiyama (Sankyo Labo Service Co.) and Dr Yukio Kodama (Division of Toxicology, National Institute of Health Sciences) for their technical assistance.

REFERENCES

1. Crumpton, M.J. and Dedman, J.R. (1990) Protein terminology tangle. *Nature* **345**, 212
2. Gerke, V. and Moss, S.E. (2002) Annexins: from structure to function. *Physiol. Rev.* **82**, 331–371
3. Moss, S.E. and Morgan, R.O. (2004) The annexins. *Genome Biol.* **5**, 219.211–219.218
4. Raynal, P. and Pollard, H.B. (1994) Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim. Biophys. Acta* **1197**, 63–93
5. Ernst, J.D., Hoye, E., Blackwood, R.A., and Jaye, D. (1990) Purification and characterization of an abundant cytosolic protein from human neutrophils that promotes Ca2(+)-dependent aggregation of isolated specific granules. *J. Clin. Invest.* **85**, 1065–1071
6. Tait, J.F., Sakata, M., McMullen, B.A., Miao, C.H., Funakoshi, T., Hendrickson, L.E., and Fujikawa, K. (1988) Placental anticoagulant proteins: isolation and comparative characterization four members of the lipocortin family. *Biochemistry* **27**, 6268–6276
7. Ernst, J.D. (1991) Annexin III translocates to the peripheagosomal region when neutrophils ingest opsonized yeast. *J. Immunol.* **146**, 3110–3114
8. Konishi, H., Namikawa, K., and Kiyama, H. (2006) Annexin III implicated in the microglial response to motor nerve injury. *Glia* **53**, 723–732
9. Le Cabec, V., Russo-Marie, F., and Maridonneau-Parini, I. (1992) Differential expression of two forms of annexin 3 in human neutrophils and monocytes and along their differentiation. *Biochem. Biophys. Res. Commun.* **189**, 1471–1476
10. Niimi, S., Harashima, M., Gamou, M., Hyuga, M., Seki, T., Ariga, T., Kawanishi, T., and Hayakawa, T. (2005) Expression of annexin A3 in primary cultured parenchymal rat hepatocytes and inhibition of DNA synthesis by suppression of annexin A3 expression using RNA interference. *Biol. Pharm. Bull.* **28**, 424–428
11. Park, J.E., Lee, D.H., Lee, J.A., Park, S.G., Kim, N.S., Park, B.C., and Cho, S. (2005) Annexin A3 is a potential angiogenic mediator. *Biochem. Biophys. Res. Commun.* **337**, 1283–1287
12. Niimi, S., Hyuga, M., Harashima, M., Seki, T., Ariga, T., Kawanishi, T., and Hayakawa, T. (2004) Isolated small rat hepatocytes express both annexin III and terminal differentiated hepatocyte markers, tyrosine aminotransferase and tryptophan oxygenase, at the mRNA level. *Biol. Pharm. Bull.* **27**, 1864–1866
13. Niimi, S., Oshizawa, T., Yamaguchi, T., Harashima, M., Seki, T., Ariga, T., Kawanishi, T., and Hayakawa, T. (2003) Specific expression of annexin III in rat-small-hepatocytes. *Biochem. Biophys. Res. Commun.* **300**, 770–774
14. Harashima, M., Niimi, S., Koyanagi, H., Hyuga, M., Noma, S., Seki, T., Ariga, T., Kawanishi, T., and Hayakawa, T. (2006) Change in annexin A3 expression by regulatory factors of hepatocyte growth in primary cultured rat hepatocytes. *Biol. Pharm. Bull.* **29**, 1339–1343
15. de Coupade, C., Gillet, R., Bennoun, M., Briand, P., Russo-Marie, F., and Solito, E. (2000) Annexin 1 expression and phosphorylation are upregulated during liver regeneration and transformation in antithrombin III SV40T large antigen transgenic mice. *Hepatology* **31**, 371–380
16. Masaki, T., Tokuda, M., Fujimura, T., Ohnishi, M., Tai, Y., Miyamoto, K., Itano, T., Matsui, H., Watanabe, S., Sogawa, K., Yamada, R., Konishi, M., and Nishioka, A. (1994) Involvement of annexin I and annexin II in hepatocyte proliferation: can annexins I and II be markers for proliferative hepatocytes? *Hepatology* **20**, 425–435
17. Higgins, G.M. and Anderson, R.M. (1931) Experimental pathology of the liver. I. Restration of the liver of the white rat following partial surgical removal. *Arch. Pathol.* **12**, 186–202
18. Tanaka, K., Sato, M., Tomita, Y., and Ichihara, A. (1978) Biochemical studies on liver functions in primary cultured hepatocytes of adult rats. I. Hormonal effects on cell viability and protein synthesis. *J. Biochem. (Tokyo)* **84**, 937–946
19. Shimaoka, S., Nakamura, T., and Ichihara, A. (1987) Stimulation of growth of primary cultured adult rat hepatocytes without growth factors by coculture with nonparenchymal liver cells. *Exp. Cell Res.* **172**, 228–242
20. Romisch, J., Schuler, E., Bastian, B., Burger, T., Dunkel, F.G., Schwinn, A., Hartmann, A.A., and Paques, E.P. (1992) Annexins I to VI: quantitative determination in different human cell types and in plasma after myocardial infarction. *Blood Coagul. Fibrinolysis* **3**, 11–17
21. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
22. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
23. Puissant, C. and Houdebine, L. M. (1990) An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Biotechniques* **8**, 148–149
24. Goyette, M., Petropoulos, C.J., Shank, P.R., and Fausto, N. (1983) Expression of a cellular oncogene during liver regeneration. *Science* **219**, 510–512
25. Goldshmidt, O., Yeikilis, R., Mawasi, N., Paizi, M., Gan, N., Ilan, N., Pappo, O., Vlodavsky, I., and Spira, G. (2004) Heparanase expression during normal liver development and following partial hepatectomy. *J. Pathol.* **203**, 594–602
26. Haruyama, T., Ajioka, I., Akaike, T., and Watanabe, Y. (2000) Regulation and significance of hepatocyte-derived matrix metalloproteinases in liver remodeling. *Biochem. Biophys. Res. Commun.* **272**, 681–686
27. Kim, T.H., Mars, W.M., Stolz, D.B., and Michalopoulos, G.K. (2000) Expression and activation of pro-MMP-2 and pro-MMP-9 during rat liver regeneration. *Hepatology* **31**, 75–82
28. Maianskaia, N.N., Shcherbakov, V.I., Panin, L.E., and Maianskii, D.N. (1978) Change in the state of the lysosomes in isolated Kupffer cells and hepatocytes in the process of liver reparative regeneration. *Tsitologiia* **20**, 1046–1051
29. Mars, W.M., Liu, M.L., Kitson, R.P., Goldfarb, R.H., Gabauer, M.K., and Michalopoulos, G.K. (1995) Immediate early detection of urokinase receptor after partial

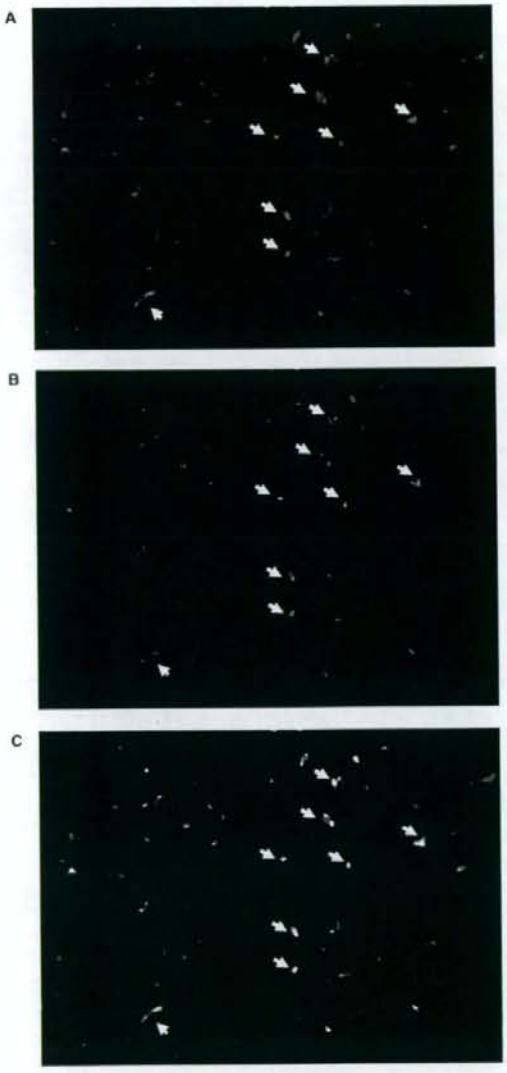


Fig. 9. **AnxA3 expression in hepatic sinusoidal endothelial cells in normal rat liver.** (A) AnxA3-positive cells; (B) SE-1-positive cells; (C) Merged image of AnxA3- and SE-1-positive cells. In (A–C), arrows show examples of positive immunoreactive cells.

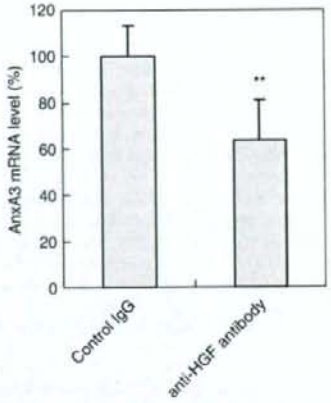


Fig. 10. **Effect of anti-HGF antibody on AnxA3 mRNA level in parenchymal hepatocytes following treatment with CCl_4.** Hepatocytes were isolated from liver in rats at 6 h following treatment with either anti-HGF IgG or control IgG, then CCl_4. AnxA3 levels were normalized to housekeeping gene, 28S rRNA. 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

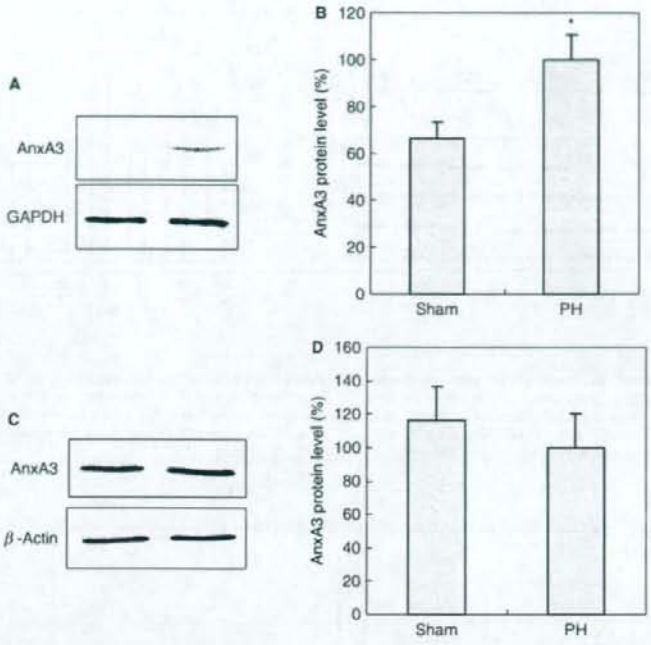


Fig. 7. **AnxA3 protein level in parenchymal hepatocytes and non-parenchymal liver cells after hepatectomy.** (A) Parenchymal hepatocytes and (C) non-parenchymal liver cells were isolated at 5 h after partial hepatectomy or sham operation. Data shown are representative of western blot analysis results 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.

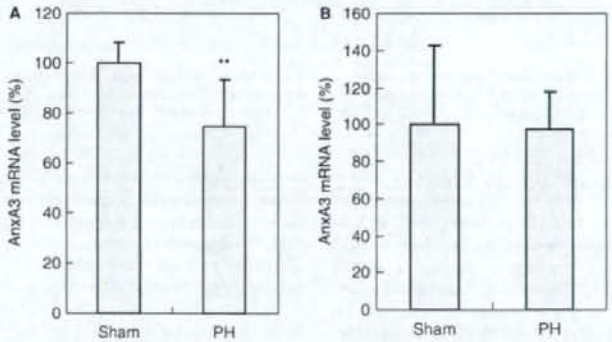


Fig. 8. **AnxA3 mRNA level in parenchymal hepatocytes and non-parenchymal liver cells after partial hepatectomy.** (A) Parenchymal hepatocytes and (B) non-parenchymal liver cells were isolated from liver in rats at 5 h after either partial hepatectomy or sham operation. AnxA3 mRNA levels were normalized 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.

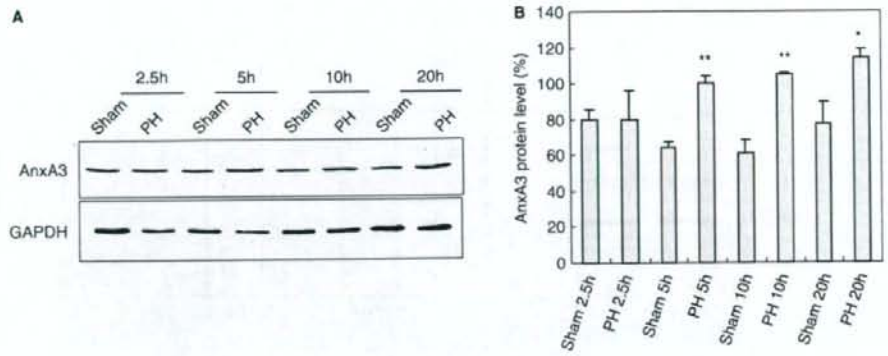


Fig. 5. **AnxA3 protein level in liver after partial hepatectomy.** (A) Data shown are representative of western blot analysis results. Approximately 35 and 1.5 μg of protein were used for detection of AnxA3 and GAPDH, respectively. (B) Results are presented relative to the values for liver in rats at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

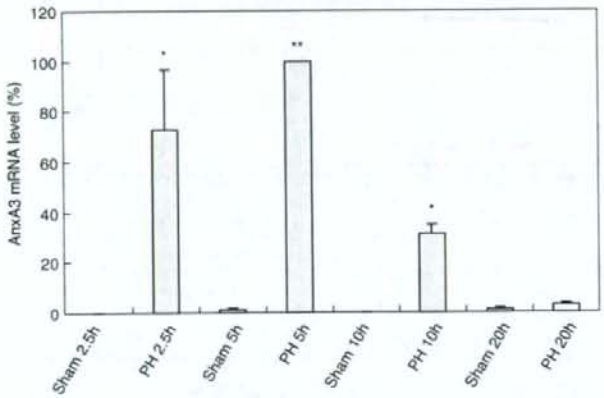


Fig. 6. **AnxA3 mRNA level in liver after partial hepatectomy.** Results are presented relative to the value produced by liver in rats at 5 h after partial hepatectomy. AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Data are expressed as mean ± SD ($n = 4$ at each time point) $^{*}P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

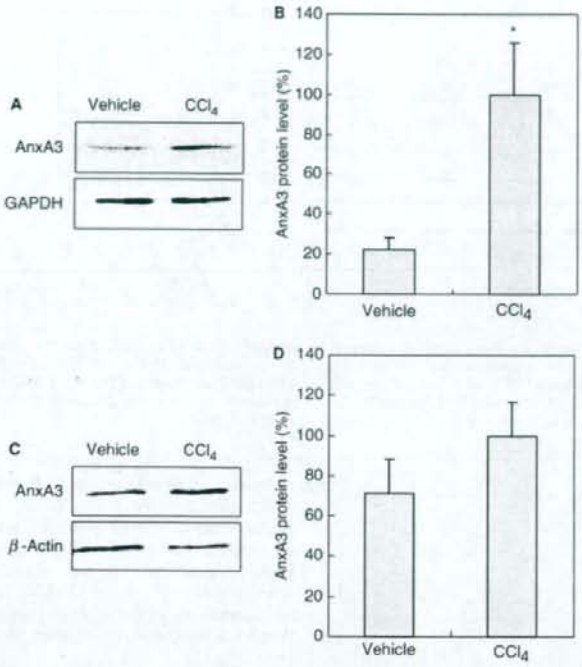


Fig. 3. **AnxA3 protein level in parenchymal hepatocytes and non-parenchymal cells isolated from liver in rats following treatment with CCl_4.** (A) Parenchymal hepatocytes and (C) non-parenchymal cells were isolated from liver in rats at 6 h after either CCl_4 or olive oil treatment. Data shown are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

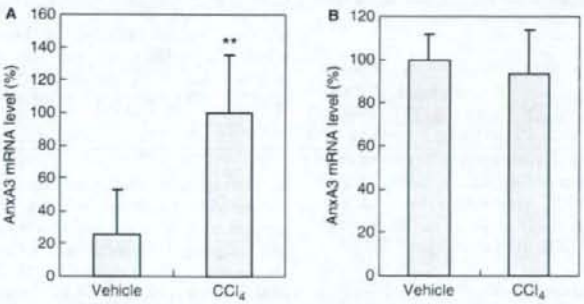
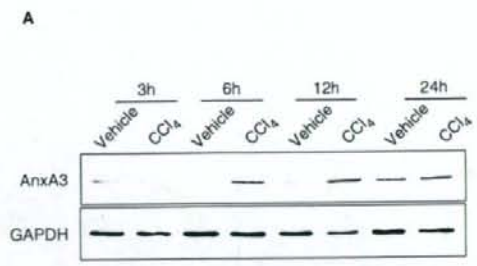


Fig. 4. **AnxA3 mRNA level in parenchymal hepatocytes and non-parenchymal cells isolated from livers in rats following treatment with CCl_4.** (A) Parenchymal hepatocytes and (B) non-parenchymal liver cells were isolated from liver in rats at 6 h after either CCl_4 or olive oil treatment. AnxA3 levels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



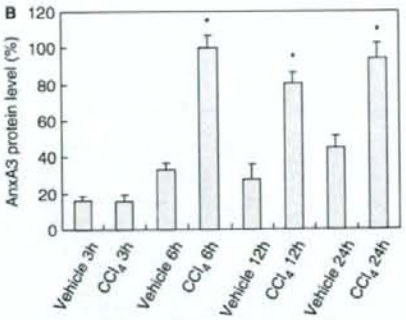


Fig. 1. **AnxA3 protein level in liver following treatment with CCl_4.** (A) Data shown are representative of western blot analysis results. Approximately 35 and 1.5 μg of protein were used for detection of AnxA3 and GAPDH, respectively. (B) Results are presented relative to the value produced by liver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

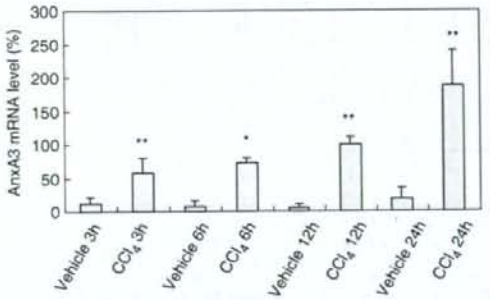


Fig. 2. **AnxA3 mRNA level in liver following treatment with CCl_4.** Results are presented relative to the value produced by liver in rats at 6 h after CCl_4 administration ($n = 4$ at each time point). AnxA3 mRNA levels were normalized to housekeeping gene, 28S rRNA. Data are expressed as the mean ± 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.

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).

J. Biochem. **143,** 537–545 (2008)
doi:10.1093/jb/mvm248

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

Mizuho Harashima[1], Kayo Harada[1], Yoshimasa Ito[1], Masashi Hyuga[2], Taiichiro Seki[1], Toyohiko Ariga[1], Teruhide Yamaguchi[2] and Shingo Niimi[2,*]

[1]Department of Nutrition and Physiology, Nihon University College of Bioresource Sciences, Kameino Fujisawa 252-8510; and [2]Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received November 14, 2007; accepted December 18, 2007; published online January 7, 2008

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

*To whom correspondence should be addressed. Tel: +81-3-3700-9347, Fax: +81-3-3700-9084, E-mail: niimi@nihs.go.jp

に含まれるため，ヘパリンの精製の指標として有用であると考えられる．今後，国内ヘパリンナトリウム中へのDSの含有量の実態を正確に把握した上で，規制が必要か否か検討していく必要がある．

謝　辞

有益なご助言をいただきました，国立医薬品食品衛生研究所医薬安全科学部鹿庭なほ子室長，独立行政法人医薬品医療機器総合機構早川堯夫顧問及び小嶋茂雄顧問，並びにヘパリンナトリウムをご供与いただきました日本バルク薬品㈱に深く御礼申し上げます．本研究の遂行にご協力いただきました厚生労働省医薬食品局審査管理課鷲田淳専門官，鈴木克之主査，独立行政法人医薬品医療機器総合機構品質管理部基準課上野清美課長，森田收氏，仁後知子氏に感謝いたします．また，有益なご討論をいただいた日本薬局方原案審議委員会生物薬品委員会，FDA，米国薬局方，欧州薬局方，及びヘパリンナトリウム製剤製造会社の関係各位にお礼申し上げます．

本研究の一部は厚生労働省の支援によって行われたものである．

文　献

1) http://www.fda.gov/cder/drug/infopage/heparin/default.htm.
2) Sugahara, K.: *Trends in Glycoscience and Glycotechnology*, **12**, 321-349 (2000).
3) Guerrini, M., Beccati, D., Shriver, Z., *et al.*: *Nat Biotechnol*, **26**(6), 669-675 (2008).
4) Kishimoto, T. K., Viswanathan, K., Ganguly, T., *et al.*: *N Engl J Med*, **358**(23), 2457-2467 (2008).
5) Maruyama, T., Toida, T., Imanari, T., Yu, G., Linhardt, R. J.: *Carbohydr Res*, **306**(1-2), 35-43 (1998).
6) http://www.usp.org/hottopics/heparin.html.
7) http://www.edqm.eu/medias/fichiers/Heparin_sodium_monograph_Revised.pdf.
8) Montgomery, R. I., Lidholt, K., Flay, N. W., Liang, J., Vertel, B., Lindahl, U., Esko, J. D.: *Proc Natl Acad Sci U S A*, **89**(23), 11327-11331 (1992).

Table 4　キャピラリー電気泳動を用いるDS限度試験共同検定結果

DS % (w/w)	ピーク面積値		
	機関A	機関B	機関C
1.0	24707	75734	54647
2.0	48633	153927	85043
4.0	78022	425538	142533

考　察

1. OSCS

FDAから公開されているキャピラリー電気泳動を用いる分析法では，ヘパリンナトリウムとOSCSの分離は不完全であった．分析能パラメータの評価により10%における添加回収率（真度）は41%（RSD=2.18%）であり，本試験法のOSCSに対する特異性は低かった．また，共同検定で得られた本試験法の検出限界は1.5%程度であることから，本試験法は，1.5%程度の限度試験であり，OSCSの含量が1.5%以下であることを保証する試験法であると解釈された．OSCSは，有害事象の原因物質であると考えられていること，また，製造工程由来物質や目的物質関連物質として混入する可能性がないことから，ヘパリンナトリウム中に検出されるべきではない．したがって，本試験法における規格は，「ヘパリンナトリウム中にOSCSに由来するピークが検出されないこと」が適当であると考えられる．OSCSについては，厚生労働省医薬食品局審査管理課長通知薬食審査発第0701001号（平成20年7月1日）において，「ヘパリンナトリウムに関する日本薬局方の一部改正に伴う取り扱いについて」として，NMR法による限度試験（0.5%）が規定された．しかしながら，キャピラリー電気泳動法を用いる試験法は1.0%未満のOSCSの混入を検出することができず，OSCSを対象としたヘパリンナトリウムの日本薬局方の純度試験法として，現状では採用できないと判定される．しかし，キャピラリー電気泳動はヘパリンナトリウム中に混入するOSCSを検出できる限られた分析法の一つであり，分析条件の検討によりヘパリンナトリウムとOSCSの分離が達成されれば，OSCSの限度試験として利用することは可能であると考えられる．

2. DS

近畿大学で実施した分析能パラメータ評価では，DSとヘパリンの識別が可能であること，また，1.0～10.0%（w/w）の範囲で直線性があることが確認された．再現性については併行精度が2.15%，室内再現精度が2.48%であり，定量性と特異性を有することが明らかにされた．また，共同検定の結果からも，キャピラリー電気泳動法によるDS分析を日本薬局方の試験法として，ヘパリンナトリウム中のDSの混入が1.0%以下であることを保障する限度試験として設定することは可能であると判断される．

国内3機関による共同検定の結果，キャピラリー電気泳動装置によりDSとヘパリンナトリウムのピークを分離できること（同程度の特異性），1.0%以上のDSを検出できること（同程度の検出限界）が確認された．今回，共同検定に参加した3機関はキャピラリーカラムへの試料導入法としていずれも加圧法を使用したが，試料導入法は加圧法の他，吸引法や落差法なども利用でき，他メーカーの装置を用いた場合でも同程度の検出限界を得るためには，分析に使用する試料量を規定することが重要である．例えば，「試料はヘパリンナトリウムのピーク強度がフルスケールの10%となるように注入する」あるいは「試料はヘパリンナトリウムのピーク最大吸光度が0.010～0.015となるように注入する」などとし，今回と同様のバリデーションスタディを実施しなければならない．その結果，同程度の特異性と検出限界が確認できれば，DSを対象とするヘパリン純度試験法として有用である．

なお，DSの規制の必要性については，DSはヘパリンとは異なる物質であるので，純度試験として適切に規制するべきとする意見と，これまでに毒性等の報告がなく，純度試験等により規制する必要はないとする意見があり，国際的にも見解が分かれている．しかし，DSはヘパリンを調製する際の原料

10.0%になるように試料溶液を調製し，キャピラリー電気泳動装置を用いて測定したとき，1.0%のDSを確認することができた．したがって，検量線のデータ（下記参照）から検出限界は1.0%と判定された（Fig. 5）．

2.3　直線性，範囲

1.0～10%（w/w）のDSを添加したヘパリンナトリウム溶液を用いて，キャピラリー電気泳動装置により測定した．DSのピーク面積は，1.0～10%の範囲で直線性が確認され，その相関係数は0.9991であった（Fig. 6）．

2.4　真度並びに精度

DSを1.0%（w/w）含むヘパリンナトリウム溶液を用いて，添加回収率（真度）を求めたところ，添加回収率は82%（RSD=1.78%）であった．また，同溶液を用いて，1試験日内に6回測定を行った．1試験日内でのDSピーク面積の再現性（併行精度，n=6）は，相対標準偏差（RSD）として2.15%であった（Table 3）．一方，異なる試験日におけるDSピーク面積の再現性（室内再現精度，n=6）は相対標準偏差（RSD）として2.48%であった（Table 3）．

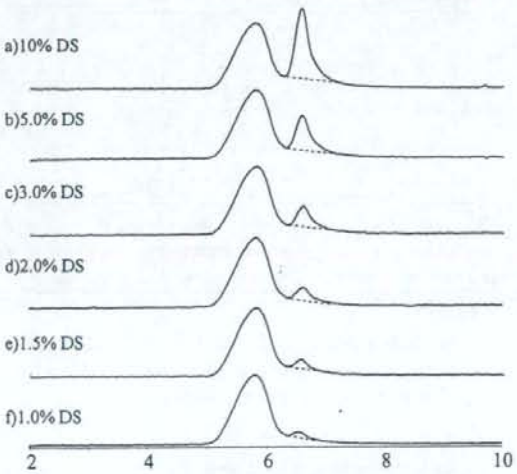


Fig. 5　DSを含むヘパリンナトリウムのエレクトロフェログラム

a)～f)：10 mg/mLのヘパリンナトリウム溶液に1.0～10.0%（w/w）のDSを添加した試験溶液．

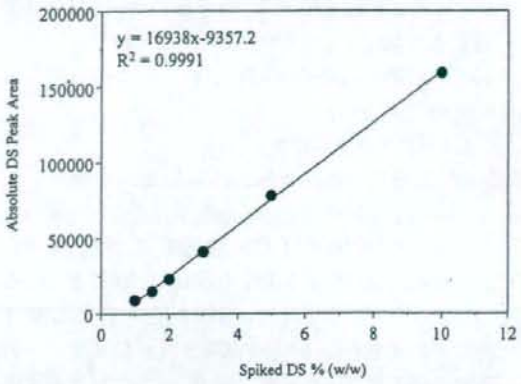


Fig. 6　キャピラリー電気泳動法によるDSの直線性

10 mg/mLのヘパリンナトリウム溶液に1.0～10.0%（w/w）のDSを添加したときのDSピークの絶対面積値をプロットした．

Table 3　キャピラリー電気泳動によるDS分析の分析能パラメータ

分析能パラメータ	結果
真度[a]	82 % (SD=1.78 %)
併行精度[a]	2.15 %
室内再現精度[a]	2.48 %
特異性[b]	Fig.4 参照
検出限界	1.0 % (w/w)
定量限界	1.0 % (w/w)
直線性	Y= 16938X - 9357.2 (R^2 = 0.9991) Fig.6 参照
範囲	1.0 - 10.0 % (w/w)

[a] 1.0 % DSを用いた(n=6).

[b] 5.0% DSを用いた(n=6).

2.4　キャピラリー電気泳動法における特異性及び検出限界に関する共同検定

3機関において，1.0，2.0及び4.0%（w/w）のDSを添加したヘパリンナトリウム試料溶液をキャピラリー電気泳動装置を用いて測定し，DSのピーク面積を求めた．DSに由来するピークは，6.5～7.8分の範囲に観察された．各機関で得られたDSのピーク面積をTable 4に示す．全機関で1.0%以上のDSを確認することができた．

ナトリウム溶液を用いて，キャピラリー電気泳動装置により測定した．OSCSのピーク面積は，1.5～10%の範囲で直線性が確認され，その相関係数は0.9758であった．

1.4 真度並びに精度

OSCSを10.0%（w/w）添加したヘパリンナトリウム溶液を用いて，添加回収率（真度）を求めたところ，添加回収率は41%（RSD=2.18%）であった．また，OSCSを5.0%（w/w）になるように添加したヘパリンナトリウム溶液を用いて，1試験日内に6回測定を行った．1試験日内のOSCSピーク面積の再現性（併行精度，n=6）は，相対標準偏差（RSD）として1.36%であった（Table 1）．一方，異なる6試験日におけるOSCSピーク面積の再現性（室内再現精度）は相対標準偏差（RSD）として2.17%であった（Table 1）．

1.5 キャピラリー電気泳動法における特異性及び検出限界に関する共同検定

3機関において，OSCSを2.0，3.0及び4.0%（w/w）になるようにヘパリンナトリウム溶液に添加し，キャピラリー電気泳動装置を用いて測定した．

Table 1　キャピラリー電気泳動によるOSCS分析の分析能パラメータ

分析能パラメータ	結果
真度[a]	41 % (RSD= 2.18 %)
併行精度[b]	1.36 %
室内再現精度[b]	2.17 %
特異性	Fig.2 参照
検出限界	1.5 % (w/w)
定量限界	1.5 % (w/w)
直線性	$Y = 36663X - 367.1$ ($R^2 = 0.9758$)
範囲	1.5 - 10.0 % (w/w)

[a] 10.0 % OSCSを用いた(n=6).
[b] 5.0% OSCSを用いた(n=6).

OSCSに由来するピークは，いずれの機関でも5.4～6.3分に観察された．各機関で得られたOSCSのピーク面積をTable 2に示す．全機関で2.0%以上のOSCSを確認することができたが，ヘパリンナトリウムのピークと分離が十分でないため，2.0%以下のOSCSを検出することは難しく，本分析法の検出限界は2.0%程度と判断された．

2. DSの分析

2.1 特異性

ヘパリンナトリウムに5.0%（w/w）のDSを添加して測定したところ，DSに由来するピークは，6.5分をピーク中心とし6.2～7.0分に観察され，ヘパリンナトリウムのピークとDSのピークを完全に分離することができた（Fig. 4）．

2.2 検出限界

ヘパリンナトリウムに対するDSの濃度が1.0～

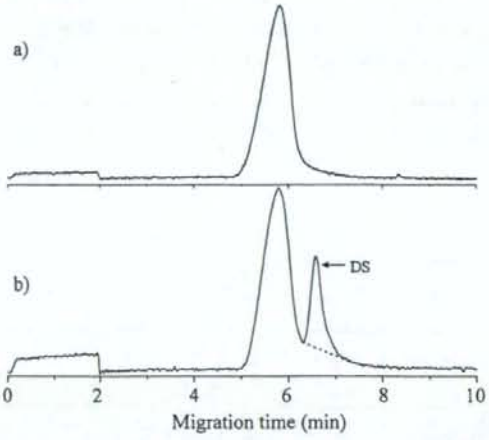


Fig. 4　キャピラリー電気泳動法によるDS検出の特異性
a) 10 mg/mL のヘパリンナトリウム溶液，b) 5.0%（w/w）のDSを10 mg/mLのヘパリンナトリウム溶液に添加した溶液．

Table 2　キャピラリー電気泳動を用いるOSCS限度試験共同検定結果

OSCS % (w/w)	ピーク面積値		
	機関A	機関B	機関C
2.0	6902	36665	7457
3.0	9290	78759	17481
4.0	15336	124710	29949

ム溶液（20 mg/mL）0.50 mL を添加し，次いで精製水 0.5，0.45，0.425，及び 0.40 mL を加えて混和し，ヘパリンナトリウム中に OSCS がそれぞれ 0，2.0，3.0，及び 4.0%（w/w）含む共同検定用試験溶液とした．共同検定用試験溶液はキャピラリー電気泳動装置を用いて測定し，OSCS のピーク面積を求めた．

4.2　DS

4 mg の DS を 1.0 mL の精製水に溶解し，DS 溶液（4.0 mg/mL）を調製した．この液 0，0.025，0.050，及び 0.10 mL にヘパリンナトリウム溶液（20 mg/mL）0.50 mL を添加し，次いで精製水 0.5，0.475，0.45，及び 0.40 mL を加えて混和し，ヘパリンナトリウム中に DS がそれぞれ 0，1.0，2.0，及び 4.0%（w/w）含む共同検定用試験溶液とした．共同検定用試験溶液はキャピラリー電気泳動装置を用いて測定し，DS のピーク面積を求めた．

結　　果

1．OSCS の分析

1.1　特異性

ヘパリンナトリウム 20 mg を 1.0 mL の精製水に溶解し，この液 0.5 mL に精製水 0.5 mL を加え 10 mg/mL の濃度とし，FDA から公開されている分析条件に従って測定した．その結果，ヘパリンナトリウムに由来するピークは 5.8 分をピーク頂点とし，5.0～6.5 分に泳動された（Fig. 2a）．次に，ヘパリンナトリウム溶液（20 mg/mL）0.5 mL と OSCS 溶液（4.0 mg/mL）0.125 mL と精製水 0.375 mL を添加したものを試験溶液（5.0% OSCS）として測定した．測定の結果，OSCS に由来するピークは，5.5 分をピーク中心として泳動されたが，ヘパリンナトリウムのピークと完全に分離しなかった（Fig. 2b）．

1.2　検出限界

OSCS を 0.5～5.0%（w/w）になるようにヘパリンナトリウムに添加した溶液を測定し，本分析法の検出限界を確認した．Fig. 3 に示すように，OSCS 含量が 1.5% では OSCS を確認することができたが，OSCS が 1.0% では確認することができず，本試験法の検出限界は 1.5% 程度と判定された．

1.3　直線性，範囲

0.5～10%（w/w）の OSCS を添加したヘパリン

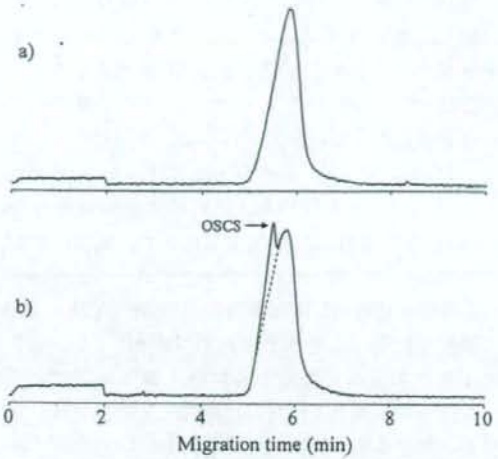


Fig. 2　キャピラリー電気泳動法による OSCS 検出の特異性
a）10 mg/mL のヘパリンナトリウム溶液，b）5.0%（w/w）の OSCS を 10 mg/mL のヘパリンナトリウム溶液に添加した溶液．

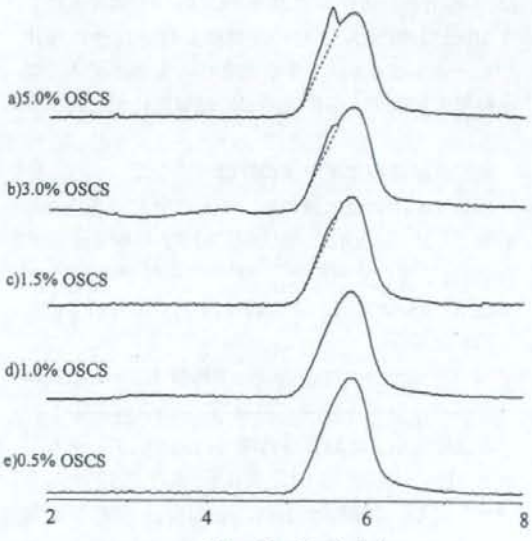


Fig. 3　OSCS を含むヘパリンナトリウムのエレクトロフェログラム
a）～e）：10 mg/mL のヘパリンナトリウム溶液に 0.5～5.0%（w/w）の OSCS を添加した試験溶液．

2. 分析条件

ヘパリンナトリウム，OSCS及びDSは精製水に溶解し，ポアサイズ0.45 μmの酢酸セルロース製メンブランフィルターでろ過し試料溶液とした．この液についてFDAのWebサイトにて公開されている分析条件に従い分析を行った．キャピラリーカラムは内径50 μm，全長66 cmのフューズドシリカキャピラリーを使用し，試料導入末端側から56 cmの位置を紫外部吸収検出窓とした．電気泳動用緩衝液は，リン酸二水素一ナトリウム一水和物1.0 gを蒸留水195 mLに溶解し，リン酸でpHを3.5に調整した後，蒸留水を加えて200 mLとし，ポアサイズ0.45 μmの酢酸セルロース製メンブランフィルターでろ過して用いた．印加電圧の極性は，試料導入側を陰極，廃液側を陽極とし，ヘパリンの泳動時間が6±1分となるように調整した．分析温度は25℃とし，検出は200 nmの紫外部吸収検出により行った．また，試料注入はヘパリンナトリウムの最大ピーク強度が0.010～0.014 AUとなるように加圧法により注入した．キャピラリーカラムは0.1 M水酸化ナトリウムで10分間，続いて蒸留水により10分間洗浄し，3回の空試験を行った後に使用した．キャピラリーは分析ごとに，蒸留水で4分間，泳動用緩衝液で4分間洗浄後，試験に使用した．

3. 分析能パラメータの評価

近畿大学薬学部において，キャピラリー電気泳動装置としてBeckman P/ACE MDQ Glycoprotein Systemを用いて実施した．ピーク面積値は，Beckman 32 Karat Gold Softwareを用いて算出した．

3.1 OSCS

ヘパリンナトリウム20 mgを精製水1 mLに溶解してヘパリンナトリウム溶液（20 mg/mL）とした．この液0.5 mLにOSCS溶液（4 mg/mL）をそれぞれ0.0125，0.025，0.037，0.075，及び0.125 mLを添加し，次いで精製水0.487，0.475，0.462，0.425，及び0.375 mLを加えて混和し，ヘパリンナトリウムに対してOSCSをそれぞれ0.5，1.0，1.5，3.0，及び5.0%（w/w）含む溶液とした．これらの溶液を分析能パラメータ評価用試験溶液とし，キャピラリー電気泳動装置を用いて測定した．OSCSのピーク面積は最小ピーク幅設定値を2秒とし，OSCSのピーク開始点とピーク終了点を結ぶ傾斜線をベースラインとして検出されるピークの積算値から求めた．真度は10% OSCSを含むヘパリンナトリウム溶液，併行精度並びに室内再現精度は5.0% OSCSを含むヘパリンナトリウム溶液を試験溶液として6回分析を行いOSCSのピーク面積値より算出した．また，検出限界についてはOSCSを0.5～5.0%含むヘパリンナトリウム試験溶液をそれぞれ6回分析し算出した．

3.2 DS

ヘパリンナトリウム20 mgを精製水1 mLに溶解してヘパリンナトリウム溶液（20 mg/mL）とした．この液0.5 mLにDS溶液（4 mg/mL）をそれぞれ0.025，0.037，0.050，0.075，0.125，及び0.25 mLを添加し，次いで精製水0.475，0.462，0.450，0.425，0.375，0.25 mLを加えて混和し，DSをそれぞれ1.0，1.5，2.0，3.0，5.0，及び10.0%(w/w)含む溶液とした．これらの溶液を分析能パラメータ評価用試験溶液とし，キャピラリー電気泳動装置を用いて測定した．DSのピーク面積は最小ピーク幅設定値を2秒とし，DSのピーク開始点とピーク終了点を結ぶ傾斜線をベースラインとして検出されるピークの積算値から求めた．真度，併行精度並びに室内再現精度は1.0% DSを含むヘパリンナトリウム溶液を試験溶液として6回分析を行いDSのピーク面積値より算出した．また，検出限界についてはDSを1.0～10.0%含むヘパリンナトリウム試験溶液をそれぞれ6回分析し算出した．

4. キャピラリー電気泳動法における特異性及び検出限界に関する共同検定

近畿大学薬学部，㈱大塚製薬工場，及び扶桑薬品工業㈱が参加した．ここでは便宜上試験室A～Cと記す（順不同）．キャピラリー電気泳動装置として，機関Aは Beckman P/ACE MDQ Glycoprotein System及び Beckman 32 Karat Gold software，機関Bは Beckman P/ACE 5510及び Waters Empower，機関Cは Beckman P/ACE MDQ Molecular Characterization System及びP/ACEシステム MDQワークステーション Ver 2.2を使用した．

4.1 OSCS

1 mgのOSCSを0.25 mLの精製水に溶解し，OSCS溶液（4.0 mg/mL）を調製した．この液0，0.050，0.075，及び0.10 mLとヘパリンナトリウ

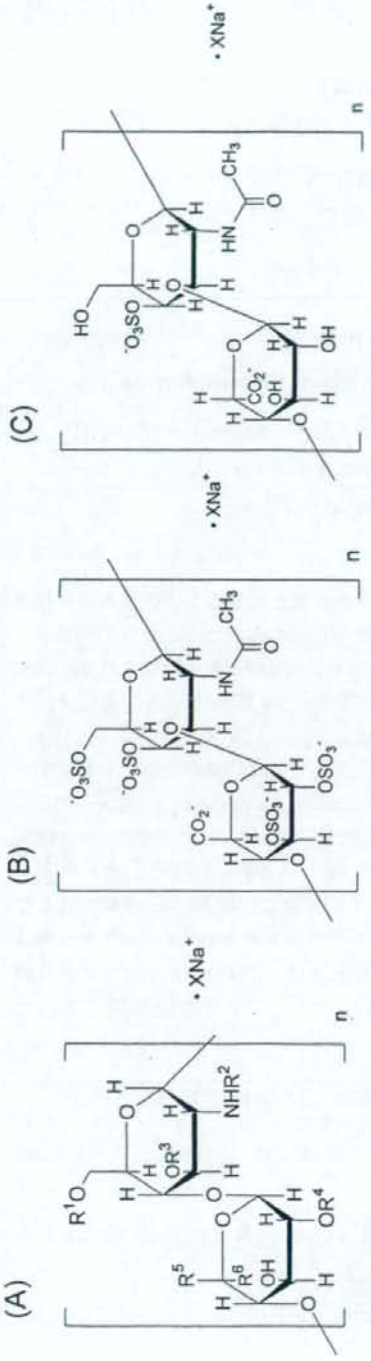


Fig. 1　ヘパリンナトリウム（A），OSCS（B）及びDS（C）の構造

(Fig. 1B)[3~5]．その後，有害事象を引き起こしたヘパリンナトリウム中に，OSCSに加えて，デルマタン硫酸（DS：別名：コンドロイチン硫酸B）(Fig. 1C) が多く含まれていることが明かにされた．

FDAは急性炎症反応の原因物質としてOSCSを特定したことを公表するとほぼ同時に[1]，^{1}H-核磁気共鳴スペクトル測定法（NMR）とキャピラリー電気泳動法を用いたOSCS検出法をインターネット上に公開した[1]．^{1}H-NMRは，ヘパリンのGlcNAcの*N*-アセチル基とOSCSのGalNAcの*N*-アセチル基の化学シフトの違いを利用する方法であり，キャピラリー電気泳動法は，ヘパリンとOSCSが分子量及び硫酸基数の違いで分離できることを利用する方法である．各国は，FDAが公開した分析法を用いてヘパリンナトリウムの分析を行うとともに，OSCSの存在が確認されたヘパリンナトリウムの回収を行う等の対応をとった．一方で，世界的にヘパリン関連医薬品の供給不足への懸念が広がり，ヘパリンナトリウム製剤の安定供給のために，ヘパリンナトリウム原料中のOSCS及びDSの分析法の整備が緊急課題となっている．我が国でも，この事態に迅速に対応するために，日本薬局方ヘパリンナトリウム各条にOSCS及びDS試験の追加を検討するに至った[6,7]．

本研究では，我が国におけるヘパリンナトリウムの品質·安全性確保を目的として，FDAの方法を参考に，キャピラリー電気泳動によるOSCS及びDS分析法を確立するとともに，日本薬局方各条ヘパリンナトリウム純度試験としての適用可能性を検証した．

実験方法

1．試　料

ヘパリンナトリウムは日本薬局方ヘパリンナトリウム標準品を使用した．共同検定に参加した製薬企業2社は，各社のヘパリンナトリウムを使用した．OSCSは日本バルク薬品㈱から供与されたOSCSを含むヘパリンナトリウムから，弱塩基性陰イオン交換HPLCにより精製して用いた[8]．DS（ブタ皮膚由来）は生化学工業㈱から購入した．その他の試薬は特級あるいはHPLCグレードを使用した．

——資　　料——

医薬品研究
Pharm. Regul. Sci.
39(11)713～720(2008)

ヘパリン純度試験に関する研究（第3報）
キャピラリー電気泳動法によるヘパリンナトリウム不純物の分析

掛樋　一晃[*1]，椿　　直孝[*1]，木下　充弘[*1]，橋井　則貴[*2]，川崎　ナナ[*2]，
寺尾　敏光[*3]，河合　健蔵[*4]，余田　　光[*4]，山口　照英[*2]

（受付：平成20年9月8日，受理：平成20年10月24日）

Studies on the Heparin Purity Test (Part 3)
Analysis of Contaminants in Heparin Sodium by Capillary Electrophoresis

Kazuaki KAKEHI[*1], Naotaka KAKOI[*1], Mitsuhiro KINOSHITA[*1], Noritaka HASHII[*2],
Nana KAWASAKI[*2], Toshimitsu TERAO[*3], Kenzo KAWAI[*4],
Hikaru YODEN[*4] and Teruhide YAMAGUCHI[*2]

緒　　言

ヘパリンナトリウムは，ウロン酸（L-イズロン酸又はD-グルクロン酸）とグルコサミン（GlcN）の2糖を構成単位とする硫酸化グリコサミノグリカンのナトリウム塩で，構成2糖単位に2～3個硫酸基を持つ構造からなる（Fig. 1A）．ヘパリンナトリウムは，血液透析その他の体外循環装置使用時の血液凝固の防止剤として世界中で汎用されており，日本薬局方にも収載されている．また，様々な低分子量ヘパリン製剤の原料としても使用されている．

2007年12月以降米国において，特定のヘパリンナトリウム製剤（以下「ヘパリン製剤」という）投与後に低血圧や急性過敏症反応などの重篤な副作用症例の発生の増加が認められたことから，2008年1月以降，当該ヘパリン製剤が自主回収された．米国食品医薬品庁（FDA）は2008年3月に急性炎症反応の原因物質として，ヘパリン製剤原料のヘパリンナトリウムに混入していた過硫酸化コンドロイチン硫酸（over-sulfated chondroitin sulfate; OSCS）を特定した[1]．天然に存在するコンドロイチン硫酸は，グルクロン酸と*N*-アセチルガラクトサミン（GalNAc）の2糖単位に硫酸基が1～3個結合したグリコサミノグリカンであるが[2]，ヘパリン製剤に混入していたOSCSは，2糖単位中のすべての水酸基が硫酸化されたコンドロイチン硫酸であった

*1 近畿大学薬学部　大阪府東大阪市小若江3-4-1（〒577-8502）
School of Pharmacy, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577-8502, Japan
*2 国立医薬品食品衛生研究所生物薬品部　東京都世田谷区上用賀1-18-1（〒158-8501）
Division on Biological Chemistry & Bioloicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
*3 ㈱大塚製薬工場　徳島県鳴門市撫養町立岩字芥原115（〒772-8601）
Otsuka Pharmaceutical Factory, Inc., 115 Kuguhara, Tateiwa, Muya-cho, Naruto, Tokushima 772-8601, Japan
*4 扶桑薬品工業㈱研究開発センター　大阪市城東区森之宮2-3-30（〒536-8523）
Research and Development Center, Fuso Pharmaceutical Industries, 2-3-30 Morinomiya, Joto-ku, Osaka 536-8523, Japan
Corresponding author: Kazuaki Kakehi, School of Pharmacy, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577-8502, Japan
E-mail: k_kakehi@phar.kindai.ac.jp

くお礼申し上げます．また，本研究遂行にご協力いただきました厚生労働省医薬食品局審査管理課鷲田淳専門官，鈴木克之主査，監視指導・麻薬対策課風間秀元 GMP 指導官，独立行政法人医薬品医療機器総合機構品質管理部基準課上野清美課長，森田收氏，仁後知子氏にお礼申し上げます．

本研究の一部は，厚生労働省による支援，並びに平成 20 年度日本公定書協会の「日本薬局方標準品に関する研究（研究者戸井田敏彦）」により実施したものである．

文　献

1) http://www.fda.gov/cder/drug/infopage/heparin/default.htm.
2) Guerrini, M., Beccati, D., Shriver, Z., *et al.*: *Nat Biotechnol*, **26**(6), 669-675 (2008).
3) Kishimoto, T. K., Viswanathan, K., Ganguly, T., *et al.*: *N Engl J Med*, **358**(23), 2457-2467 (2008).
4) 「日本薬局方」平成 18 年厚生労働省告示第 285 号，平成 19 年厚生労働省告示第 316 号，平成 20 年厚生労働省告示第 285 号．
5) 「日本薬局方外医薬品規格 2002」平成 14 年医薬発第 0920001 号，厚生労働省医薬局長通知，平成 20 年 7 月 31 日薬食発第 0731015 号．
6) Maruyama, T., Toida, T., Imanari, T., Yu, G., Linhardt, R. J.: *Carbohydr Res*, **306**(1-2), 35-43 (1998).
7) Montgomery, R. I., Lidholt, K., Flay, N. W., Liang, J., Vertel, B., Lindahl, U., Esko, J. D.: *Proc Natl Acad Sci U S A*, **89**(23), 11327-11331 (1992).
8) Suzuki, A., Toyoda, H., Toida, T., Imanari, T.: *Glycobiology*, **11**(1), 57-64 (2001).