Change in Annexin A3 Expression by Regulatory Factors of Hepatocyte Growth in Primary Cultured Rat Hepatocytes

Mizuho HARASHIMA, a Shingo NIIMI, a,b Hitomi KOYANAGI, a Masashi HYUGA, b Seiji NOMA, c Taiichiro SEKI, a Toyohiko ARIGA, a Toru KAWANISHI, b and Takao HAYAKAWA d

a Department of Nutrition and Physiology, Nihon University College of Bioresource Sciences; Kameino, Fujisawa 252–8510 Japan; b Division of Biological Chemistry and Biologicals, National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan; c The Japan Health Sciences Foundation; Kyodo Bld. 4F, 13–4 Nihombashi, Kodemmacho, Chuo-ku, Tokyo 103–0001, Japan; and d Pharmaceuticals and Medical Devices Agency; Shinkasumigaseki Bldg. 3–3–2 Kasumigaseki, Chiyoda-ku, Tokyo 100–0013, Japan.

We have recently reported that annexin (Anx) A3 expression is necessary for hepatocyte growth in cultured rat hepatocytes seeded at half the subconfluent density on collagen. In the present study, we investigated the effects of various regulatory factors of hepatocyte growth on AnxA3 expression. AnxA3 expression was significantly reduced in hepatocytes cultured under various growth inhibitory conditions such as presence of dexamethasone, culture at subconfluent cell density, and on EHS-Matrigel and lactose-carrying styrene polymer. On the other hand, hepatocyte growth factor and epidermal growth factor, stimulators of hepatocyte growth, significantly increased AnxA3 expression in hepatocytes cultured on EHS-Matrigel. These results show close correlation between known stimulatory or inhibitory actions of various factors to hepatocyte growth and increase or decrease in AnxA3 expression, and suggest the involvement of AnxA3 in their regulation of hepatocyte growth.

Key words annexin A3; hepatocyte growth; primary cultured rat hepatocyte

Annexin (Anx) A3, also called “lipocortin 3” or “placental anticoagulant protein 3” (PAP-III), is a member of the lipocortin/Anx family, which binds to phospholipids and membranes in a Ca2+-dependent manner. AnxA3 has been shown to have anticoagulant and anti-phospholipase A2 properties in vitro and to promote the Ca2+-dependent aggregation of isolated specific granules from human neutrophils. However, physiological functions have been completely unknown. AnxA3 has been detected in lung, spleen, placenta, and adrenal medulla, but not in liver and isolated hepatocytes.

We have recently reported that AnxA3 is expressed in cultured rat hepatocytes and that inhibition of AnxA3 expression by RNA interference results in a significant inhibition of hepatocyte growth. This evidence indicates that AnxA3 acts as a positive regulator on hepatocyte growth in cultured hepatocytes. In relation to our report, it is noteworthy that hepatocytes placed under culture conditions acquire a growth potential characterized by enhancement of hepatocyte growth dependent on several growth factors, whereas adult hepatocytes are normally quiescent in vivo. The correlation between AnxA3 expression and growth potential of hepatocytes described above suggests that AnxA3 is one of the factors necessary for hepatocytes placed under culture to acquire growth potential.

On the other hand, hepatocyte growth is regulated in cultured hepatocytes by various other factors including cell density, humoral factors such as dexamethasone (Dex), hepatocyte growth factor (HGF) and epidermal growth factor (EGF), and cellular substratum such as EHS-Matrigel and lactose-carrying styrene polymer (PVLA). In relation to our report, the question of whether regulation of hepatocyte growth by these factors could be mediated by concurrent change in AnxA3 expression seemed interesting; however, it remained to be elucidated whether these factors cause change in AnxA3 expression.

In the present study, we investigated the effects of various regulators of hepatocyte growth on the AnxA3 expression to examine the involvement of AnxA3 in their regulation of hepatocyte growth.

MATERIALS AND METHODS

Materials Recombinant human hepatocyte growth factor (HGF) was purchased from R&D Systems, Inc. (Minneapolis, MN, U.S.A.). Mouse epidermal growth factor (EGF) was purchased from Wako Pure Chemical, Ltd. (Osaka, Japan). Porcine dermal collagen type 1 (collagen) was purchased from Koken Co. (Tokyo, Japan). Lactose-carrying styrene polymer (PVLA) was purchased from Seikagaku Corp. (Tokyo, Japan). Dishes (10 cm) precoated with Matrigel were purchased from BD Biosciences (Bedford, MA, U.S.A.). Rabbit anti-human AnxA3 serum was a gift from Dr. F. Russo-Marie and Dr. C. Ragueness-Nicol. Rabbit anti-rat albumin IgG and rabbit anti-rat β-actin IgG were purchased from Cappel (Aurora, Ohio, U.S.A.) and Biologend, Inc. (San Diego, CA, U.S.A.), respectively.

Cell Isolation and Monolayer Cultures Parenchymal hepatocytes were isolated from adult male Wistar rats weighing 180–200 g, by in situ perfusion of the liver with collagenase. All animal care and procedure protocols were approved by the institutional animal care committee. The cells were then suspended at a density of 2.5×105 cells/ml or 5.0×105 cells/ml in Williams medium E (WE) containing 5% fetal bovine serum, 1 μM insulin and 1 μg/ml aprotinin, and cultured at a density of 0.5×105 cells/cm2 or 1.0×105 cells/cm2 in a 10-cm dish precoated with 0.03% collagen. Alternatively, the cells were then suspended at a density of 2.5×105 cells/ml in WE containing 1 μM insulin and 1 μg/ml aprotinin and cultured at a density of 0.5×105 cells/cm2 in a 10-
cm dish precoated with Matrigel or a 10-cm dish precoated with 100 μg/ml PVLA. The cells were cultured in a humidified chamber at 37 °C in 5% CO₂ and 30% O₂ in air. After 2.5 h of culture, the medium was replaced with a serum- and hormone-free medium containing aprotinin (1 μg/ml), and then various humoral factors to be tested were added and the cells were further cultured for 1 d. After 1 d culture, the medium was replaced as described above, and then the humoral factors were again added and the cells were further cultured for 1 d.

**Western Blot Analysis** Cell lysates were prepared from the cells 2 d after the start of culture by modification of a method previously described.25) The cells were washed twice with 5 ml of phosphate-buffered saline and then once with 5 ml of buffer A (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, and 10 mM EDTA). The cells were then harvested after the addition of 20 μl of buffer A. The cells were suspended, shaken for 15 min at room temperature, and sonicated four times for 15 s each time while in an ice bath after the addition of 1/5 [v/v] of 5× buffer A containing 2.5% Triton X-100 and 1/100 [v/v] of a protease inhibitor cocktail (SIGMA). After centrifugation at 10000×g, an equal amount of cytosolic protein in each experiment was subjected to SDS-PAGE on a 10% gel and electroblotted to a PVDF membrane (GVHP; Millipore). After blocking the membrane with 5% skimmed milk, a Western blot analysis was performed using rabbit anti-human AnxA3 antibody serum at a dilution of 1:21000, rabbit anti-rat albumin IgG at a dilution of 1:80000, or rabbit anti-rat β-actin IgG at a dilution of 1:500. Detection was performed using an ECL detection system (Amersham Bioscience). We used albumin or β-actin as a housekeeping protein based on the results of the preliminary studies. The intensity of each band was measured over a proportional range in the experiments. A computer assisted-analyzer was used to quantitatively analyze the intensity, and the intensity of each AnxA3 band was normalized to the intensity of the housekeeping protein.

**Total RNA Extraction and Real-Time Quantitative PCR** Total RNA was extracted from the cells 1 d after the start of culture using Trizol reagent (Invitrogen) in accordance with the manufacturer’s protocol. An equal amount of RNA (approximately 1 μg) in each experiment was reverse-transcribed using a THERMOSCRIPT™ RT-PCR System (Invitrogen) and oligo(dT)₁₆ at a final volume of 40 μl in accordance with the manufacturer’s protocol, and then diluted two-fold with ultrapure water. Subsequently, 2 μl of cDNA was used as a template for real-time PCR analysis in a Light-Cycler system (Rosche), in accordance with the manufacturer’s instructions. For AnxA3 and albumin, the PCR program consisted of 40 cycles of 10 s at 94°C, 10 s at 60°C, and 12 s at 72°C. For 18S rRNA, the PCR program consisted of 40 cycles of 10 s at 94°C, 10 s at 60°C, and 20 s at 72°C. The sequences of AnxA3-specific primers were 5′-CAATTGACGAGATCCTGT-3′ and 5′-TGGAGTGGTTCG-3′,12) those of the albumin-specific primers were 5′-AAGGCACCACCCGATTACTCCG-3′ and 5′-TGCGAACAGTTTCGCT-3′, and those of 18S rRNA-specific primers were 5′-CCAGCAGCAAGTGGCTTTCAGCAAC-3′ and 5′-GCCATTCAAGACCGTATTGTGCT-3′. The 18S rRNA PCR product specificity was confirmed by DNA sequencing using an ABI Prism 377 Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analyses. We used albumin or 18S rRNA as a housekeeping gene based on the results of the preliminary studies. And the AnxA3 levels were normalized to the levels of the housekeeping gene.

**RESULTS**

**Effects of Dex and HGF on AnxA3 Expression by Hepatocytes Cultured on Collagen** We investigated the effect of Dex and HGF on AnxA3 expression by hepatocytes cultured on collagen. Dex (100 nm) suppressed the increase of AnxA3 protein level during culture by approximately 80% (Fig. 1). On the other hand, HGF (20 ng/ml) had no effect on the AnxA3 protein level (Fig. 1). Dex (100 nm) suppressed the increase of AnxA3 mRNA level by approximately 80% (Fig. 2).

![Fig. 1. Effects of Dex and HGF on AnxA3 Protein Level by Hepatocytes Cultured on Collagen](image1)

(A) The data shown are representative of the Western blot analysis results. Approximately 7.5 μg of protein was used for the detection of AnxA3 and β-actin. (B) The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10⁵ cells/cm² on collagen in the absence of humoral factors, shown as None. The data are expressed as the mean±S.D. of 3 experiments. *p<0.01, compared with the value of None.

![Fig. 2. Effects of Dex on AnxA3 mRNA Level by Hepatocytes Cultured on Collagen](image2)

The AnxA3 levels were normalized to the levels of a housekeeping gene, albumin. The data are expressed as the mean±S.D. of 3 experiments. The results are shown relative to the value produced by hepatocytes cultured at the cell density of 0.5×10⁵ cells/cm² on collagen in the absence of humoral factors, shown as None. **p<0.05, compared with the value of None.
Effect of Cell Density on AnxA3 Expression by Hepatocytes Cultured on Collagen

We investigated the effect of cell density on AnxA3 expression by hepatocytes cultured on collagen. AnxA3 protein and mRNA levels produced by the hepatocytes seeded at subconfluent cell density (1×10^5 cells/cm^2) were approximately 70% and 50% lower than those by the hepatocytes seeded at half of subconfluent cell density (0.5×10^5 cells/cm^2), respectively (Figs. 3, 4).

AnxA3 Expression by Hepatocytes Cultured on EHS-Matrigel and PVLA

We investigated AnxA3 expression by hepatocytes cultured on EHS-Matrigel and PVLA, and compared the expression with that by hepatocytes cultured on collagen. AnxA3 protein was not detected in hepatocytes cultured on EHS-Matrigel (Fig. 5). And AnxA3 protein level produced by hepatocytes cultured on PVLA was approximately 70% lower than that by hepatocytes cultured on collagen (Fig. 5). mRNA levels produced by hepatocytes cultured on EHS-Matrigel and PVLA were approximately 80% and 40% lower than that by hepatocytes cultured on collagen, respectively (Fig. 6).

Effect of HGF and EGF on AnxA3 Expression by Hepatocytes Cultured on EHS-Matrigel

We investigated the effect of HGF and EGF on AnxA3 expression by hepatocytes cultured on EHS-Matrigel. HGF and EGF significantly increased AnxA3 protein level, from an initially undetectable level (Fig. 7). This result suggests that the failure of increase of AnxA3 protein level by HGF, as shown in Fig. 1, is due to maximal stimulation of AnxA3 expression in hepatocytes cultured on collagen. In relation to these findings, it is seen that the stimulation of AnxA3 expression in hepatocytes cultured on collagen is not due to the stimulation of HGF synthesis, because hepatocytes do not synthesize HGF.27) HGF and EGF also increased AnxA3 mRNA levels by approximately 3.2-fold and 2.5-fold (Fig. 8), respectively.
DISCUSSION

In the present study, we showed that AnxA3 expression is changed by various factors of hepatocyte growth. These factors can be classified as stimulators and inhibitors of hepatocyte growth. EGF and HGF are typical stimulators of hepatocyte growth in cultured hepatocytes. Other factors belong to the inhibitors group, as follows: Dex suppresses hepatocyte growth in cultured hepatocytes. Other factors could be involved in their regulation of hepatocyte growth.

The most marked observation in the present study seems to be that AnxA3 protein is not detected in hepatocytes cultured on EHS-Matrigel (Fig. 5). Interestingly, hepatocytes cultured on EHS-Matrigel show small round-shaped morphology compared with those cultured on the collagen, resembling those in vivo, whereas hepatocytes cultured on the collagen were uniformly spread flat. This evidence suggests that AnxA3 expression is dramatically reduced in cultured hepatocytes that show round-shaped morphology. This possibility may be supported by the finding that inverse correlation between DNA synthesis and roundness of hepatocytes is observed in cultured hepatocytes showing various morphologies from round shape to flat shape by coating dishes with different concentrations of PVLA.

There are many reports showing that various factors regulate expression of other Anxs. For example, Dex and other glucocorticoids stimulate AnxA1 expression in vitro and in vivo, in contrast to the present finding. This inconsistency suggests that the mode of regulation by glucocorticoids differs among species of Anxs, tissues, and cells. HGF and EGF stimulate expression of AnxA1, AnxA2, AnxA5, and AnxA6 in primary cultured rat hepatocytes. Interleukin-6 and 12-O-tetradecanoylphorbol β-acetate stimulate AnxA1 expression in A549 cells and cultured astrocytes, respectively.

In conclusion, the present study shows a close correlation between the known actions of various factors to hepatocyte growth and change in AnxA3 expression, and suggests that the changes in AnxA3 expression associated with these factors could be involved in their regulation of hepatocyte growth.

Acknowledgement This work was supported in part by the Gant-in-Aid for Cancer Research (15-2) from the Ministry of Health, Labor and Welfare.

REFERENCES
