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STIMULATION OF FOLLISTATIN PRODUCTION BY EPIDERMAL GROWTH FACTOR IN CULTURED RAT HEPATOCYTES

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Production of follistatin in cultured rat hepatocytes was studied by measuring follistatin release with a protein-binding assay using [^{125}I]activin A. Follistatin was detected in conditioned medium of cultured hepatocytes. Ligand blotting using [^{125}I]activin revealed that follistatin released into the medium consisted of two different forms with molecular weight of approximately 40 K-Da. Epidermal growth factor (EGF) elicited dose-dependent increases in DNA synthesis and follistatin release. Dose-response relationship for EGF-induced follistatin release correlated well with that for EGF-induced DNA synthesis. In EGF-stimulated cells, a marked increase in DNA synthesis occurred after 48 hrs. Similarly, follistatin release was markedly augmented after 48 hrs. Amount of cell-bound follistatin was not changed by the treatment with EGF. These results indicate that cultured hepatocytes synthesize and release follistatin. The activin-follistatin system operates in cultured rat hepatocytes and may modulate DNA synthesis by altering the action of activin A.

Follistatin was isolated from ovarian follicular fluid as a stimulator of follicle-stimulating hormone (FSH) secretion in cultured pituitary cells (1,2). It was structurally different from either inhibins or activins and was considered to be a novel regulator of FSH secretion originated from ovarian cells. Subsequent studies revealed that follistatin gene is a single copy gene (3,4) and two different mRNAs are generated by alternative splicing (5-6). Two precursor proteins containing two asparagine-linked glycosylation sites are translated and, as a result, multiple forms of mature follistatin exist in tissues (7-8). Despite that follistatin was originally thought to be an independent inhibitor of FSH secretion, Nakamura et al. (9) brought

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a new insight in this field by showing that follistatin binds to activin in a stoichiometric manner and blocks the effect of activin. They suggested that follistatin modulates various cell functions as an autocrine or paracrine factor by modulating the action of activins. Another intriguing aspect of follistatin is that it associates with heparan sulfate chains of proteoglycans (10). Taken together, follistatin binds to extracellular matrix and may modulate the action of activins. Given that activins affect various cellular functions including cellular growth and differentiation, follistatin may modulate cellular functions by altering the effect of activins.

We have recently reported that activin A acts as an autocrine negative regulator of DNA synthesis in cultured hepatocytes (11). Since expression of immunoreactive follistatin in liver is reported (12), it is possible that follistatin is synthesized in parenchymal liver cells and affects growth of hepatocytes by modulating the action of an autocrine factor, activin A. In the present study, we assessed the production of follistatin in cultured rat parenchymal liver cells by measuring concentrations of follistatin with a protein-binding assay using radioactive activin (13).

Materials and Methods

Materials

Recombinant human activin A and human follistatin were provided by Dr.Y.Eto of Central Laboratory, Ajinomoto Inc. (Kawasaki, Japan). Epidermal growth factor (EGF) was purchased from Collaborative Research (Lexington, MA). [^{3}H]Thymidine and Na[^{125}I] was obtained from Du-Pont New England Nuclear (Boston, MA). Sulfated-cellulofine (sulfated cellulose gel) was from Seikagaku Kogyo (Tokyo, Japan). All other chemicals were of analytical reagent grade.

Culture of Rat Hepatocytes

Parenchymal liver cells were prepared from male Wistar rats (170-200 g) by the method of Berry and Friend (14). Cells were plated in a 24-well collagen-coated dish at a density of 5 x 10^4/well in Williams' E medium containing 5 % fetal calf serum, 10 nM insulin, 1 nM dexamethasone, streptomycin and penicillin. After 3 hrs, attached cells were washed and were then incubated for indicated time in serum-free medium containig 0.1 nM insulin, 0.1 % bovine serum albumin (BSA) and EGF (11).

Measurement of DNA Synthesis

DNA synthesis was determined by measuring [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable materials. [^{3}H]Thymidine (0.5 μCi/ml) was included from 48 to 72 hrs unless otherwise specified. [^{3}H]Thymidine incorporation was measured as described by McNiel et al. (15).

Iodination of Activin A

Activin A was iodinated by the loctoperoxidase method (11). Briefly, ativin A (9.4 μg) in 5 μl of 70 mM sodium acetate buffer was added to 25 μl of 0.4 M sodium acetate buffer (pH 5.0). The solution, 500 μCi of Na[^{125}I] in 10 μl of 0.2 M phosphate

buffer (pH 7.5), 5 µl of lactoperoxidase solution (20 µg/ml 0.1 M sodium acetate buffer, PH 5.0) and 5 µl of H_2O_2 solution were added and incubated for 5 min at room temperature. To dilute and terminate the reaction, 50 µl of NaN_3 solution (10 µg/ml in distilled water) containing 0.1 % Triton X-100 was added. Then the mixture was chromatographed on Sephadex G-10 column (1 x 6 cm) equilibrated with phosphate buffered saline containing 1 % bovine serum albumin (BSA-PBS). The column was eluted with BSA-PBS and the labeled activin A was collected. Specific activity of [^{125}I]activin A ranged from 10000 to 20000 cpm/ng.

Measurement of Follistatin and Ligand Blotting

Follistatin in the conditioned medium was measured by the method of Saito et al. (13) using recombinant human follistatin as standard. Briefly, 200 µl aliquots of samples or standard solution of follistatin were added to glass tubes and 50 µl sulfated-cellulofine gel was added to each tube. After shaking at room temperature for 24 hrs, the supernatant was discarded and the gels were washed three times with 1 ml of assay buffer containing 50 mM Tris/HCl (pH 7.3), 0.3 M NaCl, 2 mM EDTA and 1 % BSA. Thereafter, [^{125}I]activin A solution was added to each tube with or without unlabeled activin A and the solution was incubated for 24 hrs at 4° C. After the incubation, the gels were washed with the washing buffer containing 150 mM NaCl and 0.02 % Tween 20 and the radioactivity was counted in a gamma counter. To measure cell-bound follistatin, cells washed twice with ice-cold PBS were incubated with 20 mM Hepes buffer (pH 7.4) containing 2 M NaCl at 4° C for 5 min. Then the solution was collected and was diluted with the assay buffer and concentration of follistatin was measured. Statistical significance was analysed by Student's t-test. Ligand blotting was carried out as described by Nakamura et al. (9) using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Results

We quantitated follistatin by a protein binding assay using radioactive activin A (13). Figure 1 depicts a typical standard curve of the binding assay for follistatin. This assay has a detection limit of 0.84 ng/ml (0.21 ng/tube), defined as the recombinant human follistatin concentration which corresponds to the mean ± 2 S.D. of the response in the absence of follistain. Employing this assay, we measured the activity of follistatin in culture medium of rat parenchymal liver cells. Samples obtained by serial dilution of conditioned medium of hepatocytes were parallel with the recombinant human follistatin. The inter- and intra-asay coefficients of variation for folliistatin concentration of 6.25 ng/ml were 6.67 and 7.29 % respectively. When hepatocytes were incubated for 72 hrs in William's medium containing 0.1 nM insulin and 1 nM dexamethasone, a considerable activity of follistatin was released into culture medium. To elucidate molecular weight of follistatin released from hepatocytes, we performed ligand blotting of released follistatin using [^{125}I]activin A. As shown in Figure 2, two broad bands with molecular weights of approximately 38 and 42 K-Da were visualized by autoradiogram with [^{125}I]activin A, which were disappeared by the addition of unlabeled excess activin A. As demonstrated in

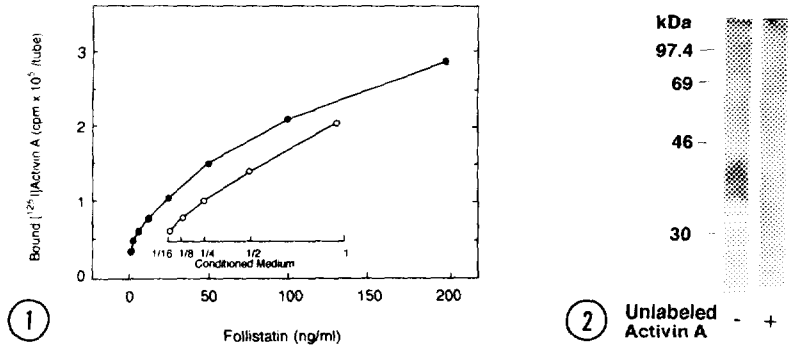


Figure 1. Standard Curve of the Binding Assay for Follistatin.

Various amounts of recombinant human follistatin (●) and serially diluted conditioned medium of cultured hepatocytes (O) were applied for the protein binding assay for follistatin. Conditioned medium was obtained by culturing hepatocytes in William's E medium containing 0.1 nM insulin and 1 nM EGF for 72 hrs in a 100-mm dish.

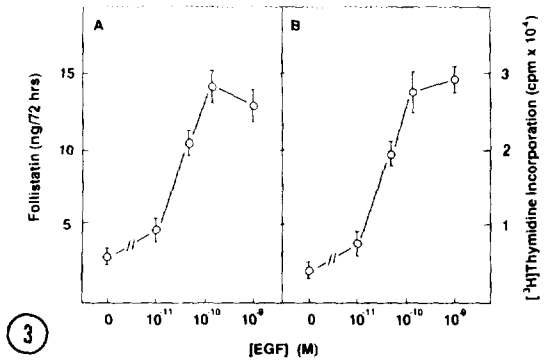
Figure 2 Ligand Blotting of Follistatin Released From Hepatocytes.

Hepatocytes were cultured for 72 hrs with 1 nM EGF and ligand blotting was performed using the conditioned medium. After PAGE, samples were transferred to the membrane and [^{125}I]activin A was added to visualize follistatin in the presence and absence of excess unlabeled activin A.

Figure 3A, EGF augmented release of follistatin into medium. The effect of EGF was dose-dependent, being maximal at 10^{-10} M. At this concentration, EGF elicited more than four-fold increase in the release of follistatin. The dose-response relationship for EGF-mediated follistatin release correlated with that for EGF-induced DNA synthesis (Figure 3B). Thus, EGF elicited the maximal stimulation of DNA synthesis at a concentration of 10^{-10} M.

Figure 4A depicts time course of release of follistatin in EGF-treated cells. The medium was changed every 12 hrs and follistatin released into medium was determined. Follistatin was released constantly up until 48 hrs of incubation with 1 nM EGF. Then, the rate of follistatin release was increased in the next 12 hrs approximately three-fold. Figure 4B shows time course of [^{3}H]thymidine incorporation in EGF-stimulated hepatocytes. [^{3}H]Thymidine incorporation was detected later than 36 hrs and the peak of incorporation was obtained between 48 and 60 hrs.

It is reported that follistatin binds to heparan sulfate chain of proteoglycan (10). It is quite likely that at least some of follistatin produced in hepatocytes binds to



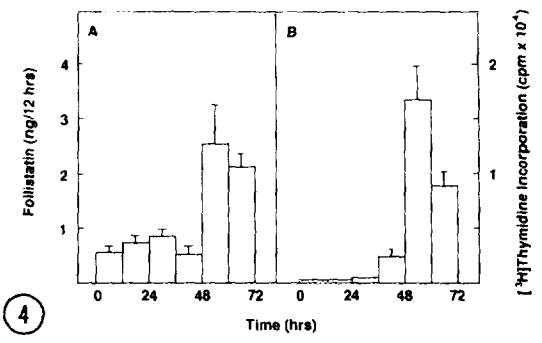


Figure 3. Dose-response Relationships For EGF-induced Follistatin Release and DNA Synthesis.

Hepatocytes were incubated for 72 hrs with various concentrations of EGF. Follistatin released into medium (A) and DNA synthesis (B) were measured. Values are the mean ± S.E. for six (A) and four (B) experiments.

Figure 4. Time Course of EGF-induced Follistatin Release and DNA Synthesis.

Hepatocytes were incubated for up to 72 hrs with 1 nM EGF. For measurement of follistatin release (A), the medium was changed every 12 hrs and follistatin released during the period was measured. For measuredment of DNA synthesis (B), [^{3}H]thymidine (0.5 μCi/ml) was added at 0,12,24,36,48 and 60 hrs and [^{3}H]thymidine incorporation during the subsequent 12 hrs was measured. Values are the mean ± S.E. for six (A) and four (B) experiments.

extracellular matrix. We therefore measured amount of cell-bound follistatin in EGF-treated hepatocytes. Cell-bound follistatin was extracted by incubating with high concentration of NaCl. As shown in Table 1, amount of cell-bound follistatin was not changed significantly by treatment with EGF.

Discussion

In the present study, we have studied the production of follistatin in cultured hepatocytes. The results clearly indicate that cultured parenchymal liver cells synthesize and release considerable amount of follistatin. Of interest is the fact that release of follistatin is augmented by the treatment with EGF. Time course of follistatin output implicates that release of follistatin is markedly enhanced when hepatocyte DNA synthesis occurs. Given that amount of cell-bound follistatin is not changed significantly during the course of 72 hrs of culture, it is conceivable that release of follistatin is due largely to de novo production in hepatocytes. In addition, dose-response relationship for EGF-induced follistatin release correlates well with

Table 1. Effect of EGF on Cell-bound Follistatin

Addition	Follistatin (ng/2x10^5 cells)	
	0	60 hrs
none	72.5±2.8	72.6±9.8
EGF	78.5±17.5	88.3±3.2

Hepatocytes were incubated for indicated time with William's medium containing 0.1 nM insulin and 0.1 % BSA in the presence and absence of 1 nM EGF. Cell-bound follistatin was measured as described in Methods. Values are the mean ± S.E. for four experiments.

that for EGF-induced DNA synthesis. Production of follistatin may be linked by some mechanism to the initiation of DNA synthesis. With regard to the cell-cycle dependent production of follistatin, it is possible that production of follistatin might be enhanced by activin A, which is synthesized and released in middle to late G_1 phase of the cell-cycle in cultured hepatocytes (11). In pituitary gonadotrophs, production of follistatin is shown to be stimulated by activin B, an autocrine factor of gonadotrophs. (16). Activin B enhances FSH secretion by an autocrine mechanism in one hand and simultaneously reduces its own action by enhancing production of follistatin (16). Activin-follistatin system may be under a similar complex control in cultured hepatocytes. Activin A, an autocrine negative regulator of hapatocyte DNA synthesis, is expressed in the late G_1 phase, which is followed by an elevation of release of follistatin. Follistatin released from hepatocytes may modulate the action of activin A. However, since exogenous addition of excess follistatin augments DNA synthesis induced by EGF or hepatocyte growth factor in cultured hepatocytes (11), endogenous follistatin is not sufficient enough to block the action of endogenous activin A on DNA synthesis in vitro. We have recently observed that an intraportal administration of excess follistatin after partial hepatectomy accelerates liver regeneration (submitted for publication). Follistatin released from hepatocytes in vivo is also insufficient to block the action of endogenous activin A at least in the initial round of DNA synthesis. At present, the role of follistatin expressed in hepatocytes is not totally clear. Despite that follistatin is unable to block the action of endogenous activin A in the initial round of DNA synthesis, it is possible that

follistatin modifies the action of activin A at later time points. Further study is needed to elucidate the significance of follistatin in the regulation of growth of hepatocytes.

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