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A New Function of Ito Cells in Liver Morphogenesis: Evidence Using a Novel Morphogenic Protein, Epimorphin, *in Vitro*

Miyoko Hirose, Sumio Watanabe, Hirosumi Oide, Tsuneo Kitamura, Akihisa Miyazaki, and Nobuhiro Sato[1]

Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo Bunkyo-ku, Tokyo 113, Japan

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A novel mesenchymal protein, epimorphin, has been reported to play a key role in morphogenesis of fetal organs. The morphological and functional features of epimorphin in adult liver were entirely unknown. This study was performed to determine the distribution and function of this protein in the adult mouse liver. Epimorphin was detected on sinusoidal Ito cells. It played an essential role in the formation of hepatocyte spheroids which maintained differentiated function in primary culture. Therefore, Ito cells might control the differentiation of parenchymal hepatocytes in the adult liver in the pathologic condition as well as in fetal organs.

BACKGROUND

In the histogenesis of embryonic tissues or organic restoration, the stream from cell proliferation, differentiation of epithelial cells, and subsequent acquisition of polarity as a tissue is essentially indispensable (1). In the process of morphogenesis of the organic restoration, the epithelial-mesenchymal interaction has been believed to play an important role. This interaction is generally organized by mesenchymal factors such as the extracellular matrix, cell surface-associated molecules, and soluble factors (2, 3, 4). Among them, the information of surface-associated molecules is lacking. Recently Hirai et al. reported that a novel 150 kd protein, epimorphin, is essential for epithelial morphogenesis including hair follicle, skin and lung epithelium (5, 6). In organ culture of embryonic tissues, a monoclonal antibody to epimorphin inhibited various processes of epithelial morphogenesis (5). However, the distribution and behavior of epimorphin in each tissue including liver during postnatal life is entirely unknown.

Koide et al. reported that, in the hepatocyte culture system, primary cultured adult rat hepatocytes formed spheroid shaped aggregates when they cultured with epidermal growth factor and insulin on the proteoglycan coated culture dish (7). These spheroid shaped hepatocyte aggregates maintain their differentiated function such as albumin secretion much longer than monolayer cultured hepatocytes (7). Therefore, these hepatocyte aggregates were thought to be differentiated functionally as well as morphologically (8).

In this study we assessed the cellular distribution of epimorphin and the role of this protein on the function and morphology of primary cultured hepatocytes.

MATERIALS AND METHODS

Cell Preparation

Adult mouse parenchymal and nonparenchymal liver cells were prepared from male Balb/c mice (8 weeks old) by the collagenase perfusion method (9) and isolated nonparenchymal cells were separated into Ito cells, Kupffer cells

[1] Corresponding author. Fax: 03-3813-8862.

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and endothelial cells by the metrizamide density gradient method (10, 11). Cells of each type were inoculated onto uncoated culture dishes (diameter 60 mm, Corning Glass Works, Corning, NY), and cultured in Dulbecco's medium (GIBCO Laboratories, Grand Island, NY) with 10% fetal bovine serum and antibiotics (streptomycin 100 μg/ml, penicillin 100 units/ml). These isolated cells were used for the identification of epimorphin. The purity of each cell was found to be more than 80% by immunohistochemical identification, phase contrast microscopy and electron microscopy and the viability was more than 90% according to the trypan blue exclusion test.

Primary cultured rat parenchymal hepatocytes were prepared from Wistar strain rats by collagenase digestion according to our previously reported methods (9) and cultured in L-15 medium supplemented with 10% fetal bovine serum. Parenchymal hepatocytes were inoculated onto uncoated culture dishes (diameter; 60 mm, Corning) or recombinant epimorphin-fragment coated dishes (6)(a generous gift from Dr. Y. Hirai) at a concentration of 10^6 cells per dish. These cultured cells were used for the experiment on spheroid formation.

Immunohistochemistry

In vivo. Frozen mouse liver was sectioned at 4 μm thickness on a cryostat. Glass-mounted sections were fixed and stained by the indirect immunofluorescence method using anti-mouse epimorphin antibody (MC-1)(provided by Dr. Hirai)(5) and anti-desmin antibody (Dako, Copenhagen, Denmark). The second antibodies used were fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG (Cappel, Durham, NC), rhodamine conjugated anti-rabbit IgG (Dako) or FITC conjugated anti-rabbit IgG (Cappel).

In vitro. To identify epimorphin-producing cells in the liver, cultured mouse parenchymal cells and nonparenchymal cells were fixed on a dish with cold pure ethanol for 30s and incubated with anti-mouse epimorphin antibody (MC-1) overnight at 4°C. All cells were rinsed with PBS and stained with FITC-conjugated anti-rat IgG (Cappel). In order to confirm Ito cells, cultured cells were double-stained with anti-epimorphin antibody and anti-desmin polyclonal antibody by the immunofluorescence method described in our previous report (12).

Hepatocyte Spheroid Formation

Isolated rat hepatocytes were inoculated onto uncoated or epimorphin-coated plastic culture dishes at a concentration of 10^6 cells per dish and cultured in L-15 medium without addition of growth factors. The cultures were kept at 37°C in a moist atmosphere for 7 days. The process of spheroid formation was monitored under phase contrast microscope equipped with a time-lapse video disc recorder (Sony, Tokyo, Japan).

Albumin Measurement

During the process of spheroid formation, the concentration of rat albumin was determined. The supernatant of culture medium from uncoated or epimorphin-coated culture dishes was collected on day 7. The albumin content of the supernatant were measured by enzyme immune assay with polystyrene beads according to Kominami et al (13). Anti-rat albumin antibody and peroxidase-conjugated anti-rabbit IgG were purchased from Cappel. The reaction was determined with 2 2′-azino-di-[3-ethylbenzthiazoline] sulfate (ABTS) reagent (Boehringer, Mannheim, Germany).

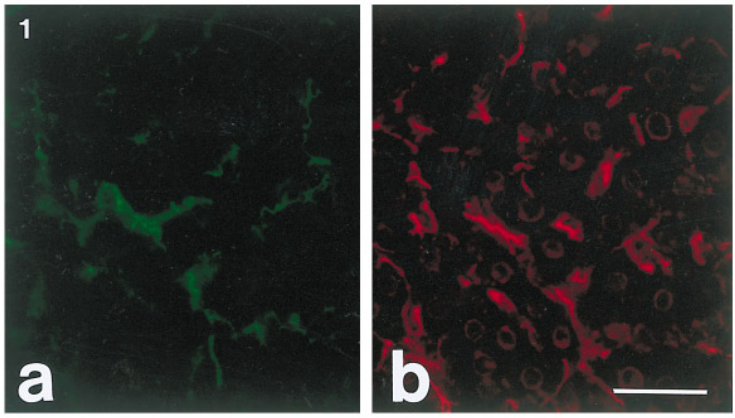
RESULTS

Detection of Epimorphin in the Liver and Cultured Cells

Immunohistochemical analysis clearly showed that epimorphin was present on the sinusoidal Ito cells which were also positive for desmin, a marker protein of Ito cells, in normal adult mouse liver (Figure 1). In the cultured cells, epimorphin was not detected on parenchymal hepatocytes, Kupffer cells or endothelial cells, it was detected only on desmin-positive cultured Ito cells (Figure 2).

Hepatocyte Spheroid Formation

Primary cultured hepatocytes showed cell spreading during the culture and formed a cell sheet 7 days after inoculation in uncoated plastic culture dishes (Figure 3). In epimorphin-coated dishes, inoculated cells initially attached to the dish surface and formed monolayer cell islands within 24 h. These cell islands were then detached and aggregated and gradually formed small hepatocyte spheroids. These spheroids gradually increased in size and some became floating spheroids (diameter about 150 μm) 7 days after inoculation (Figure 3). In transmission electron micrographs of hepatocyte spheroids 7 days after inoculation, bile canaliculi-like structures were easily seen between adjacent hepatocytes and these structures were sealed with



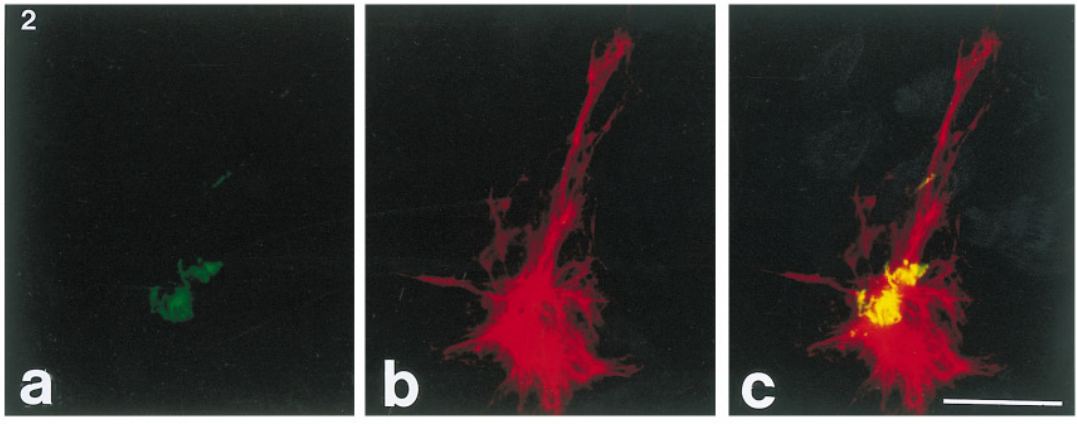


FIG. 1. Double staining fluorescence microscopy for epimorphin and desmin in the rat liver. Epimorphin was detected on sinusoidal Ito cells (a) which contained desmin, a marker for Ito cells (b). Bar, 100 μm.

FIG. 2. Double staining fluorescent microscopy for epimorphin and desmin in cultured Ito cells 4 days after the inoculation. (a) Epimorphin, (b) desmin, (c) superimposition of (a) and (b). Both epimorphin and desmin were detected on the identical cell. Bar, 50 μm.

tight junctions (Figure 4). The production of albumin during 7 days was 20.6±2.4 μg/day in uncoated culture dishes and 41.7±4.3 μg/day in epimorphin-coated dishes ($p<0.01$, Student' t test $n=5$).

DISCUSSION

Epimorphin has been first described by Hirai et al. as a mesenchymal cell surface-associated protein that induces branching morphogenesis in embryonic skin and lung by direct cell contact and the matured protein is thought to be anchored in the membrane via glycosylphosphatidylinositol linkage (5, 6).

In the present study, we detected epimorphin on hepatic sinusoidal Ito cells in adult mouse liver by the immunohistochemical double-staining technique with desmin, a marker for Ito cells. This finding was also confirmed in primary cultured Ito cells by identical procedures. Therefore, this protein was produced and localized on sinusoidal Ito cells in the adult liver. The function of epimorphin was also investigated and it was found that this protein induced the formation of hepatocyte spheroids without addition of growth factors and extracellular matrix. These findings are interesting because hepatocyte spheroids have been reported to be

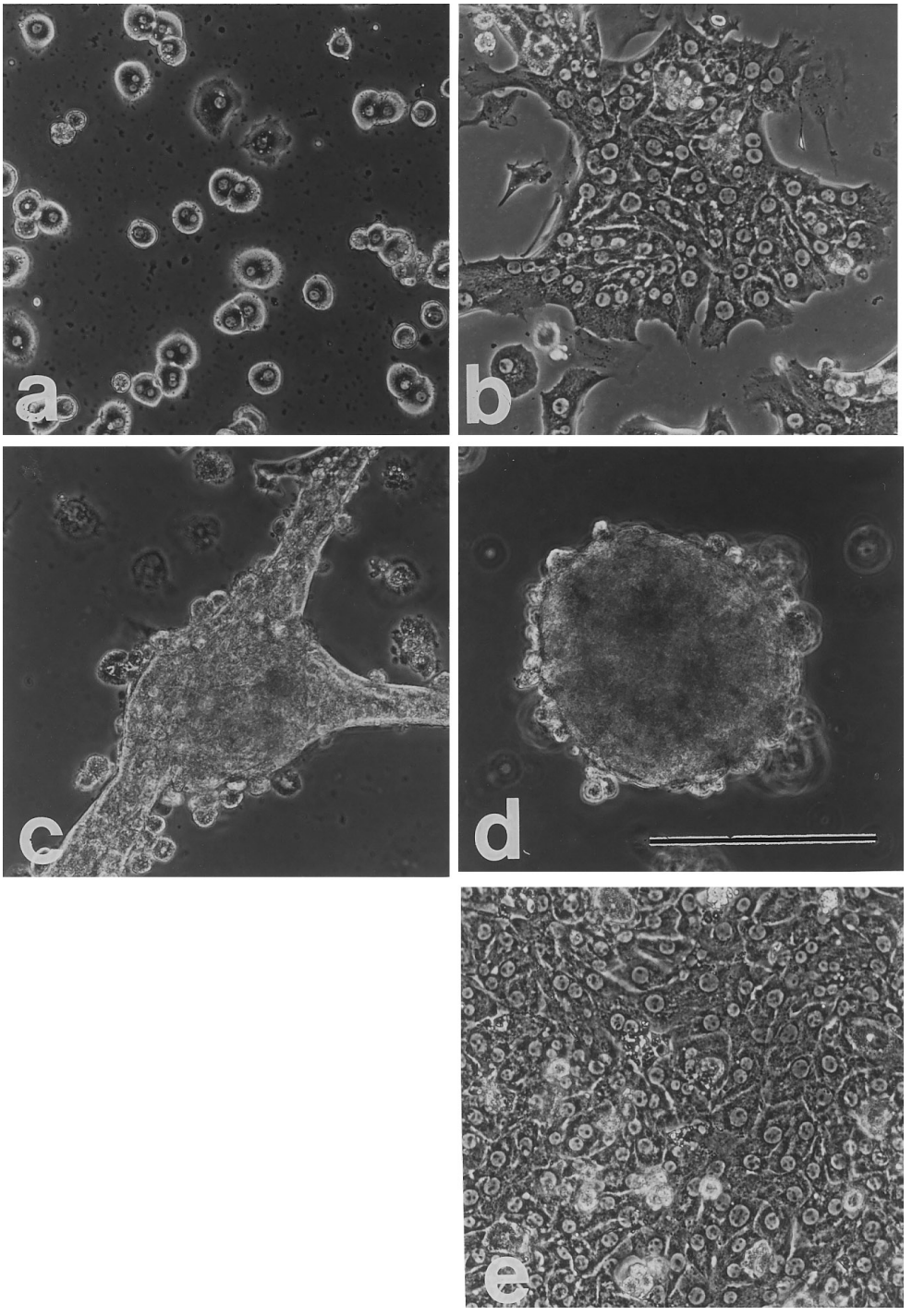


FIG. 3. Phase contrast microscopy of primary cultured hepatocytes. On epimorphin-coated dishes, parenchymal hepatocytes formed spheroids without adding growth factors. (a) 2 h after the inoculation, (b) 24 h, (c) 4 days, (d) 7 days. On uncoated dishes, primary cultured hepatocytes formed monolayer cell sheets 7 days after the inoculation (e). Bar, 250 μm.

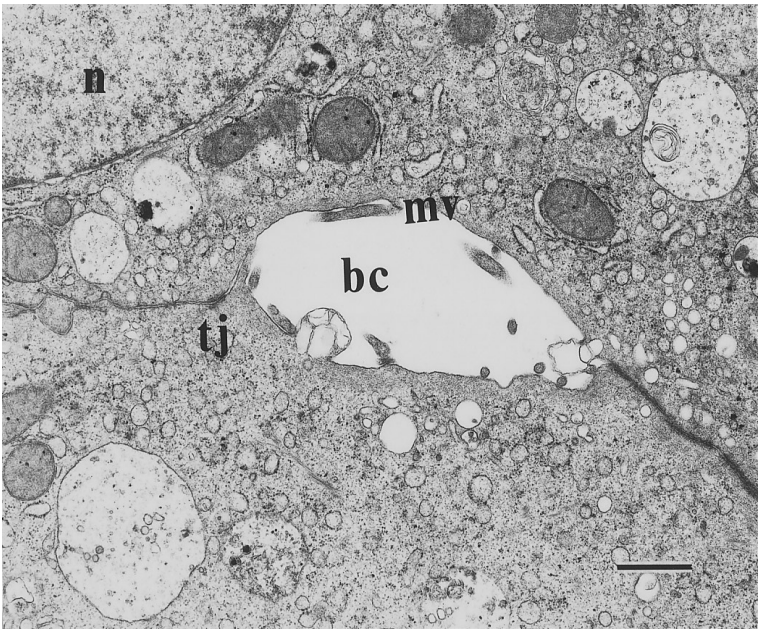


FIG. 4. Transmission electron microscopy. In the spheroids, bile canaliculus which was sealed with tight junctions was detected. bc: bile canaliculus, tj: tight junction, mv: microvilli, n: nucleus. Bar, 1 μm.

formed by adding epidermal growth factor and insulin to the medium in proteoglycan-coated dishes (7) or Primaria dishes (14). In this study we induced hepatocyte spheroids by coating culture dishes with just the insoluble active site of epimorphin without adding growth factors. In addition, these spheroids contained bile canaliculi-like structures and maintained albumin production for a long period. Therefore, epimorphin might induce morphogenesis of cultured parenchymal hepatocytes. Ito cells have been reported to have various functions in vivo. Among them, the major functions of this cell are its contribution to vitamin A metabolism (15), fibrogenesis after liver injury (16), and regulation of sinusoidal blood flow (11, 17). The present results added a new function of Ito cells in the pathogenesis and regeneration of liver diseases. Ito cells might play an important role in morphogenesis during liver regeneration in the pathogenic conditions.

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