

## Changes in Expression of Connexin 32, Bile Canaliculus-Like Structures, and Localization of Alkaline Phosphatase in Primary Cultures of Fetal Rat Hepatocytes

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We devised an experimental design in primary cultures of fetal rat hepatocytes for studying hepatocyte differentiation over a short period. In the present study, hepatocytes were first cultured for 3 days in dexamethasone-supplemented medium and then for an additional 3 days in dexamethasone- or epidermal growth factor-supplemented medium. In hepatocytes cultured continuously in dexamethasone-supplemented medium, the expression of connexin 32 increased and bile canaliculus-like structures and localization of alkaline phosphatase in the plasma membrane around bile canaliculus-like structures were maintained. Few cells incorporated bromodeoxyuridine. On the other hand, in most of the hepatocytes cultured in epidermal growth factor-supplemented medium, the expression of connexin 32 was minimally recognized, bile canaliculus-like structures were shortened or eliminated, and alkaline phosphatase was localized as numerous fine spots throughout the cytoplasm. More than 20% of all hepatocytes incorporated bromodeoxyuridine.

The present study suggests that in hepatocytes, there is a close relationship among connexin 32 expression, the maintenance of bile canaliculus-like structures, and the localization of alkaline phosphatase to the plasma membrane around the bile canaliculus-like structures, and this indicates that the present experimental model is useful for studying hepatocyte differentiation over a short period.

**Key words:** connexin 32, bile canaliculus, alkaline phosphatase, primary culture, hepatocyte

### I. Introduction

Gap junctions are involved in cell proliferation and differentiation [2, 3, 21, 23, 24]. Gap junctions in the hepatocytes of rat and human livers are composed of 90% connexin 32 (Cx32) and 5% connexin 26 [4, 15, 16, 20]. Connexin 32 expression in rat liver decreases after partial hepatectomy and during hepatocarcinogenesis [11, 12, 22]. On the other hand, glucocorticoids enhance Cx32 expression in primary cultured rat hepatocytes and MH<sub>1</sub>C<sub>1</sub> rat hepatoma cells [17]. Our previous study of primary cultured fetal rat hepatocytes demonstrated that dexamethasone

(DEX) induces the formation of bile canaliculus-like structures (BCLS), the localization of alkaline phosphatase (ALP) in the plasma membrane around BCLS, and the expression of Cx32 along the borders between adjacent cells [6, 8, 9]. However, we have not yet demonstrated whether Cx32 expression is related to BCLS and the localization of ALP in fetal rat hepatocytes. In the present study, we examined changes in Cx32 expression, BCLS, and localization of ALP using a new experimental model in primary cultures of fetal rat hepatocytes to elucidate the relationships among these parameters.

### II. Materials and Methods

#### *Primary culture of hepatocytes*

Pregnant Wistar rats were purchased from CLEA

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Japan Ltd. (Tokyo, Japan) and maintained in our laboratory animal facilities. The present study was approved by the Ethics Committee for Animal Experiments of Kitasato University. Livers were removed from fetal rats on gestational day 17 and hepatocytes were separated as previously described [10]. Collagen-coated coverslips (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) were placed inside 35-mm plastic culture dishes and hepatocytes were seeded at a concentration of  $8 \times 10^4$ – $1 \times 10^5$  cells/ml. After 24 hr of cell culture, the medium was replaced with fresh  $10^{-6}$  M DEX-supplemented Williams Medium E containing 20% fetal bovine serum and  $10^{-7}$  M insulin. Then, after 3 days of cell culture, the medium was replaced with DEX-supplemented or 20 ng/ml epidermal growth factor (EGF) (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan)-supplemented Williams Medium E containing 20% fetal bovine serum and insulin, and the cells were cultured for 3 more days.

#### Immunofluorescence

At the end of the cell culture period, monolayers of hepatocytes were washed three times with phosphate-buffered saline (PBS) and fixed with Zamboni solution for 10 min at room temperature. Cells were then washed with PBS containing 0.05% saponin (PBSS) overnight in a refrigerator and immersed in 0.1% Triton X-100 solution dissolved in PBSS for 5 min at room temperature. After washing with PBSS, cells were incubated with a 1:100 dilution of anti-Cx32 monoclonal antibody (Invitrogen Co., Camarillo, CA) for 1 hr at room temperature. Some cells were incubated in a 1:50 dilution of anti-rat ALP rabbit serum [7] for 1 hr at room temperature. As a control, normal mouse IgG and rabbit serum were used in place of monoclonal antibodies and rabbit antiserum. After washing with PBSS, cells were incubated with a 1:150 dilution of fluorescein isothiocyanate-labeled anti-mouse IgG antibodies (Medical and Biological Laboratories, Nagoya, Japan) or a 1:50 dilution of rhodamine-labeled anti-rabbit IgG antibodies (Medical and Biological Laboratories) for 30 min at room temperature. After the reaction, cells were washed with PBS and nuclei were stained with 4',6-diamino-2-

phenyl-indole (DAPI). Samples were examined under a fluorescent microscope (ECLIPSE E-600; Nikon, Tokyo, Japan) and photographed using a digital camera (DS-L2; Nikon) equipped with a fluorescein figure analysis system (Lumina Vision; Mitani Co., Tokyo, Japan).

#### Detection of cell proliferation

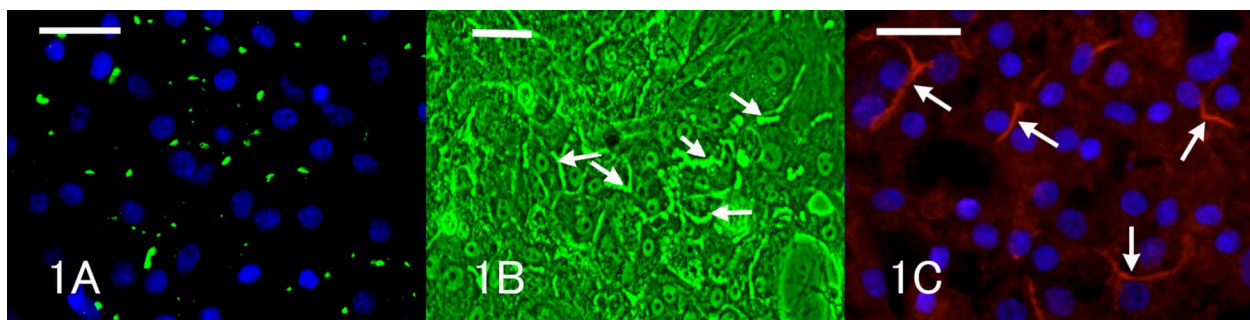
We used the 5-bromo-2'-deoxy-uridine (BrdU) labeling and detection kit I (Roche Diagnostics Japan, Tokyo, Japan) to detect cell proliferation.

#### Quantitative analysis

Connexin 32 in hepatocytes cultured in DEX- or EGF-supplemented medium was quantitatively measured on a computer using ImageJ image analysis software [19]. Thirty sites in each Cx32-stained sample were photographed and spots of Cx32 in photographs were quantified with ImageJ software. The measurement was expressed as pixels per cell. Quantities of bile canaliculus-like structures and localization of ALP in the plasma membranes surrounding BCLS were counted as numbers per 10 cells in photographs using a phase-contrast or fluorescent microscope. The index of BrdU labeling was calculated as the percentage of hepatocytes labeled by BrdU. Significant differences ( $p < 0.05$ ) between data in hepatocytes under two culture conditions were determined by the Student's *t*-test. The same experiments were repeated on six independent occasions.

### III. Results and Discussion

In hepatocytes cultured in DEX-supplemented medium, numerous coarse spots of Cx32 were observed along the cell borders between adjacent hepatocytes (Fig. 1A). On the other hand, in hepatocytes cultured in EGF-supplemented medium, a few spots of Cx32 were present at the same sites as in the above hepatocytes. Quantitative analysis by Image J software revealed a statistically significant difference between hepatocytes cultured in DEX-supplemented medium and hepatocytes cultured in



**Fig. 1.** Distribution of connexin 32, bile canaliculus-like structures, and localization of alkaline phosphatase in fetal rat hepatocytes cultured in dexamethasone-supplemented medium. (A) Connexin 32. Numerous coarse spots of connexin 32 are observed along cell borders between adjacent hepatocytes. (B) Bile canaliculus-like structures. Bile canaliculus-like structures are observed along the borders between adjacent cells (arrows). (C) Alkaline phosphatase. Alkaline phosphatase is localized in the plasma membrane surrounding bile canaliculus-like structures (arrows). Bar=50  $\mu$ m.

**Table 1.** Quantitative comparison of connexin 32 (Cx32) expression, bile canaliculus-like structures (BCLS), localization of alkaline phosphatase (ALP) in bile canaliculus-like structures, and the index of cell proliferation (BrdU) between hepatocytes cultured in dexamethasone-supplemented medium and those in epidermal growth factor-supplemented medium

	Cx32 (pixels/cell) <sup>1)</sup>	BCLS (no./10 cells) <sup>2)</sup>	ALP in BCLS (no./10 cells) <sup>3)</sup>	BrdU (%) <sup>4)</sup>
DEX	418.6±19.3	6.25±0.74	2.67±0.14	4.99±1.66
EGF	49.7±12.2	1.50±0.25	0.58±0.18	21.8±1.02

Four parameters show simultaneous quantitative changes in hepatocytes cultured in dexamethasone-supplemented medium and in epidermal growth factor-supplemented medium. Data=mean±standard error.

1)  $p < 0.0001$ , 2)  $p < 0.001$ , 3)  $p < 0.001$ , 4)  $p < 0.0001$ .

EGF-supplemented medium (Table 1). Bile canaliculus-like structures in hepatocytes cultured in DEX-supplemented medium were maintained and lengthened (Fig. 1B), whereas BCLS in hepatocytes cultured in EGF-supplemented medium were shortened or had disappeared. Alkaline phosphatase was localized in the plasma membrane surrounding BCLS in hepatocytes cultured in DEX-supplemented medium (Fig. 1C), whereas ALP in hepatocytes cultured in EGF-supplemented medium was observed as numerous fine spots throughout the cytoplasm. The index of cell proliferation was approximately 5% in hepatocytes cultured in DEX-supplemented medium, whereas it was more than 20% in hepatocytes cultured in EGF-supplemented medium (Table 1).

Dexamethasone promotes hepatocyte differentiation [1, 5, 14]. On the other hand, EGF induces hepatocyte proliferation [18]. In the present study, after hepatocyte differentiation was first induced by DEX, hepatocyte differentiation was continuously maintained by DEX or hepatocyte proliferation was induced by EGF. Hepatocytes continuously cultured in DEX-supplemented medium maintained Cx32 expression, BCLS, and the localization of ALP in the plasma membrane surrounding BCLS. On the other hand, hepatocytes cultured in EGF-supplemented medium were characterized by decreased Cx32 expression, shortened or no BCLS, a lack of localized ALP in BCLS sites, and increased hepatocyte proliferation. These results indicate that Cx32 expression, BCLS, and localization of ALP simultaneously change during hepatocyte differentiation and proliferation.

On the relationship between gap junctions and tight junctions, it had been reported that Cx32 expression induces the formation of tight junctions in rat hepatocytes [13]. Tight junctions separate the plasma membrane of hepatocytes into sinusoidal-lateral and bile canalicular membranes and form bile canaliculi between adjacent hepatocytes. Consequently, Cx32 expression is considered to induce the formation of bile canaliculi and to maintain the bile canaliculus structure. Moreover, it is thought that the formation of tight junctions and polarization of the plasma membrane in hepatocytes result in localizing ALP into bile canalicular membranes. The present study suggests that Cx32 expression in hepatocytes is closely related to the maintenance of BCLS and localization of ALP to the plasma membrane around BCLS, and this indicates that the present experimen-

tal model is useful for studying hepatocyte differentiation over a short period.

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