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Change in Localization of Alkaline Phosphatase and Mannosidase II by Colchicine Treatment of Primary Cultures of Fetal Rat Hepatocytes

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We examined the changes in localization of alkaline phosphatase (ALP) and mannosidase II (man II), a Golgi marker, after colchicine treatment of primary cultures of fetal rat hepatocytes, using double immunofluorescence staining and confocal laser microscopy. In hepatocytes cultured in basal medium, ALP was localized in the perinuclear cytoplasm, and man II was observed in the Golgi region of the cytoplasm. When hepatocytes were cultured in dexamethasone-supplemented medium, ALP was also localized in the plasma membrane surrounding the bile canaliculus-like structure that was formed between adjacent cells. In hepatocytes cultured in the same medium containing colchicine, the structure of microtubules in the cytoplasm was lost, man II exhibited granular distribution scattering throughout the cytoplasm, and ALP was localized in coarse granular sites of the cytoplasm. However, ALP was not colocalized at the same sites as man II. The present study indicated that colchicine inhibits the dexamethasone-promoted translocation of ALP to the plasma membrane surrounding the bile canaliculus-like structure in primary cultures of fetal rat hepatocytes by disassembling microtubules and discomposing the Golgi complex.

Key words: immunofluorescence, confocal laser microscopy, liver, microtubule, dexamethasone

I. Introduction

It is generally thought that glycoproteins in the plasma membrane are translocated via the Golgi complex to the plasma membrane after synthesis in the rough-surfaced endoplasmic reticulum [3, 19, 28, 29]. Alkaline phosphatase (ALP) in rat hepatocytes is mainly localized in the plasma membrane surrounding the bile canaliculus [1, 4, 14, 33, 34] and hydrolyzes the phospholipids in bile excreted by hepatocytes into the bile canalicular lumen [27]. Previous papers have reported that dexamethasone, in primary cultures of fetal rat hepatocytes, promotes formation of the bile canaliculus-like structure [6] and translocation of ALP from the limited perinuclear cytoplasm to the plasma membrane surrounding the bile canaliculus-like structure [10, 13]. Moreover, in the same culture system, it was demonstrated that dexamethasone represses DNA synthesis [6] and promotes the appearance of connexin 32 between adjacent hepatocytes [10, 13]. These observations indicate that hepatocyte differentiation promotes translocation of ALP from the cytoplasm to the plasma membrane. However, the mechanism of such ALP translocation in rat hepatocytes remains uncertain.

Previous studies using primary cultures of adult rat hepatocytes reported that colchicine, an anti-microtubule agent, disassembles microtubular fibers, and that ALP consequently exhibits coarse granular distribution patterns throughout the cytoplasm [9]. This suggests that microtubules participate in ALP translocation from the cytoplasm to the plasma membrane and that colchicine inhibits such ALP translocation by disassembling microtubules. Moreover, our previous studies using the rat hepatoma cell line McA-RH 7777 demonstrated that ALP and gamma-glutamyltranspeptidase (GGT) are localized at the same sites as Golgi markers (Golgi 58K protein and the substance that reacts to wheat germ agglutinin) [11] and that, using double immunofluorescence staining of ALP and the Golgi marker mannosidase II (man II), ALP is localized in the Golgi area of the cytoplasm in solitary McA-RH 7777 cells that are cultured at low concentrations and do not have cell to cell contacts, and that

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cell contact between McA-RH 7777 cells synchronously cultured at high concentrations promotes ALP translocation from the Golgi area to the plasma membrane between adjacent cells [12]. On the other hand, using man II as a Golgi marker, it was demonstrated that nocodazole, an anti-microtubule agent, alters the Golgi complex to form small Golgi stacks that are dispersed throughout the cytoplasm [32]. To investigate how microtubules participate in ALP translocation from the cytoplasm to the plasma membrane surrounding the bile canaliculus-like structure, the present study examined the changes in localization of ALP and man II after colchicine treatment of primary cultures of fetal rat hepatocytes, using double immunofluorescence staining and confocal laser microscopy.

II. Materials and Methods

Dissociation of liver and primary culture of hepatocytes

We purchased pregnant female rats from CLEA Japan and kept them 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 at gestation day 17 under aseptic conditions. We dissociated the livers by modifying the method of Cotariu *et al.* [15]. Livers were cut into small pieces, which were then incubated with stirring for 10 min at 37°C in 0.05% collagenase (Type II: Worthington Biochemical Co., Freehold, NJ, USA) dissolved in Hanks' balanced salt solution containing 0.5% serum albumin. After removing the enzyme solution by centrifugation, liver fragments were again incubated with stirring for 20 min at 37°C in fresh enzyme solution containing 73.5 mg/dl calcium. Collagenase-digested liver fragments were gently dissociated using pipettes. After filtration through gauze, the filtrate was centrifuged. Pellets were resuspended in culture medium and were washed twice with culture medium by centrifugation. We used Williams Medium E containing 20% fetal bovine serum, 10^{-7}M insulin and 20 ng/ml mouse epidermal growth factor (Takara Shuzo, Kyoto, Japan) as basal culture medium. Separated hepatocytes were seeded on collagen-coated coverslips (Nippon Becton Dickinson, Tokyo, Japan) at a concentration of 5×10^4 cells/ml and were incubated in basal culture medium. After 24 hr, medium was replaced with fresh basal medium or basal medium containing 10^{-6}M dexamethasone, and cells were cultured for 3 days in a CO_2 incubator. For some cells, the dexamethasone-supplemented medium was replaced with the same medium containing 10^{-5}M colchicine at 4 hr before the termination of culture, and cells were thereafter incubated in colchicine-containing medium.

Detection of microtubules

After washing three times with 0.01 M phosphate-buffered saline solution (PBS, pH 7.2) containing 0.85% NaCl, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 5 min at room temperature followed by absolute methanol for 5 min at –20°C. After fixation, cells were washed with PBS containing 0.05% saponin (PBSS) and were then incubated for 1 hr at room temperature in anti-rat β-tubulin monoclonal antibody (Zymed Laboratories Inc., South San Francisco, CA) solution diluted 1:50. After washing with PBSS, cells were incubated for 30 min at room temperature in fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibodies (Medical and Biological Laboratories; Nagoya, Japan). Cells were washed with PBSS and then mounted in Fluoro-Guard Antifade Reagent (Bio-Rad, Hemel Hempstead, UK) after nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Localization of the antigen in cells was examined by confocal laser microscopy (C1si, Nikon, Kawasaki, Japan).

Double staining for ALP and man II

After washing with PBS, cells were fixed in Zamboni solution (2% paraformaldehyde, 15% saturated picric acid in 0.1 M phosphate buffer, pH 7.4) for 10 min at room temperature. After fixation, cells were washed with PBS containing 0.05% saponin (PBSS) and were then placed in PBSS overnight at 4°C. Cells were immersed in 0.1% Triton X-100 solution dissolved in PBSS for 5 min at room temperature before immunoreaction. For double staining, cells were incubated for 1 hr at room temperature in mixed solution containing anti-rat ALP rabbit serum [7, 8] diluted 1:50, and anti-man II monoclonal antibody (Covance; Research Products, Richmond, CA) diluted 1:10,000. As a control for staining, normal rabbit serum and mouse IgG were used in place of antiserum and monoclonal antibodies. After washing with PBSS, cells were incubated for 30 min at room temperature in mixed solution containing rhodamine-labeled anti-rabbit IgG antibodies (Medical and Biological Laboratories) and FITC-labeled anti-mouse IgG antibodies. Cells were washed with PBSS and then mounted in Fluoro-Guard Antifade Reagent after nuclei were stained with DAPI. Localization of antigens in cells was examined by confocal laser microscopy.

III. Results and Discussion

In hepatocytes cultured in basal or dexamethasone-supplemented medium, numerous microtubular fibers were observed in the cytoplasm around the nucleus (Fig. 1A, 1B). On the other hand, in hepatocytes incubated in colchicine-containing medium, the fibrous microtubule structure disappeared and numerous coarse granules with weak fluorescence were scattered throughout the cytoplasm (Fig. 1C). On double staining with ALP and man II, in hepatocytes cultured in basal medium, ALP was localized in the limited perinuclear cytoplasm and man II was localized in the Golgi region of the cytoplasm (Fig. 2A). In some cells, ALP was colocalized at the same sites as man II (Fig. 2A). In hepatocytes cultured in dexamethasone-supplemented medium, ALP was localized in the limited cytoplasm around the nucleus and the plasma membrane surrounding the bile canaliculus-like structure, whereas man II was distributed

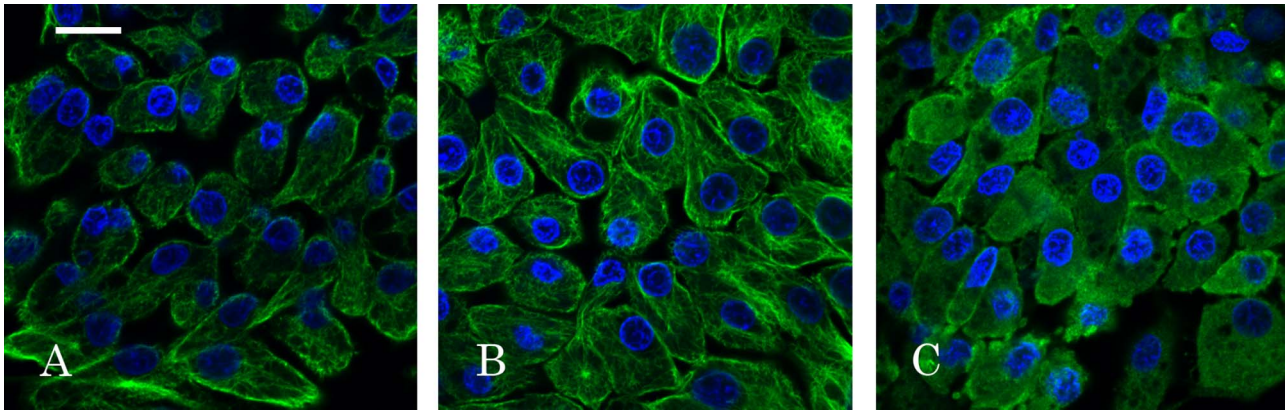

Fig. 1. Distribution of microtubules in primary cultures of fetal rat hepatocytes. **A:** Hepatocytes cultured in basal medium. **B:** Hepatocytes cultured in dexamethasone-supplemented medium. **C:** Hepatocytes cultured in dexamethasone-supplemented medium and further incubated for 4 hr in the same medium containing colchicine. In hepatocytes cultured in basal or dexamethasone-supplemented medium, abundant microtubular fibers are observed from the cytoplasm around the nucleus to the cell surface. However, microtubular fibers disappear in hepatocytes treated with colchicine. Bar=20 μm.

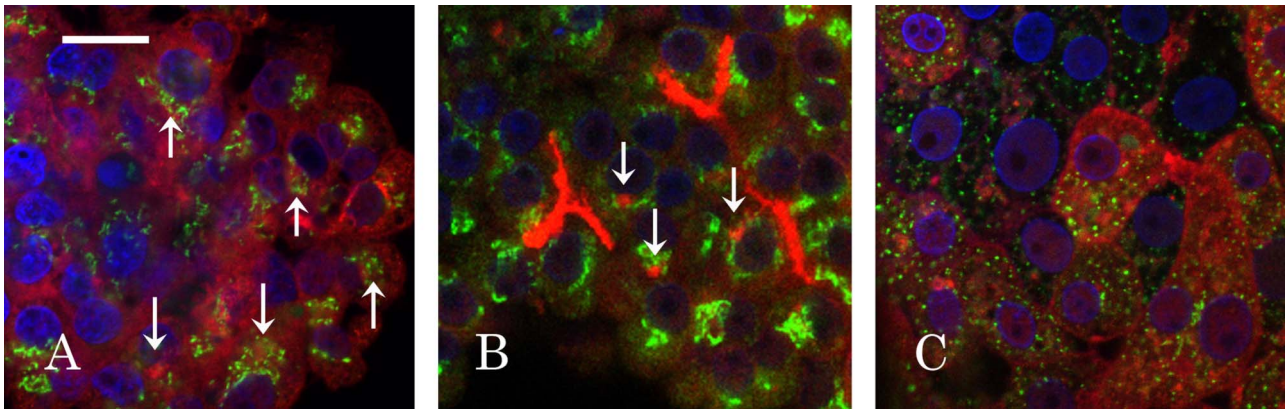

Fig. 2. Localization of alkaline phosphatase and mannosidase II in primary cultures of fetal rat hepatocytes. **A:** Hepatocytes cultured in basal medium. ALP is localized in the limited perinuclear cytoplasm. Man II is localized in the Golgi region of the cytoplasm. In some cells, both are colocalized at the same sites (arrows). **B:** Hepatocytes cultured in dexamethasone-supplemented medium. ALP is localized in the limited perinuclear cytoplasm and in the plasma membrane around the bile canaliculus-like structure. Man II is localized in the Golgi region of the cytoplasm. Colocalization of ALP and man II is observed at the same sites of a few cells (arrows). **C:** Hepatocytes cultured in dexamethasone-supplemented medium and further incubated for 4 hr in the same medium containing colchicine. ALP is located in the coarse granular sites in the cytoplasm, while man II is observed in the granular sites scattered throughout the cytoplasm. ALP and man II are not colocalized at the same sites. Bar=20 μm.

in the Golgi region of the cytoplasm around the nucleus (Fig. 2B). Colocalization of ALP and man II was observed in the cytoplasm of a few cells (Fig. 2B). When hepatocytes were incubated in colchicine-containing medium, ALP and man II were dispersed throughout the cytoplasm as numerous coarse and fine granules, respectively, but were not colocalized at the same sites (Fig. 2C).

It has been reported that, in monolayer culture systems, adult rat hepatocytes cultured on a single collagen gel rapidly dedifferentiate, but cells sandwiched between two layers of collagen gel maintain cellular morphology and function for long periods [18, 21]. However, in the present culture system of fetal rat hepatocytes, dexamethasone induced GGT translocation, the formation of bile canaliculus-like structures and the appearance of connexin 32 at cell borders [6, 10, 13]. The above results coincided with those reported in primary rat hepatocytes overlaid with a basement membrane-like matrix extracted from Engelbreth-Holm-Swarm mouse tumor [25]. It is known that dexamethasone promotes the differentiation of suckling rat hepatocytes [2, 5, 23], although it exerts other effects, such as an increase in GGT activity and an accumulation of glycogen [5]. We therefore believe that dexamethasone and the present culture system are useful for investigating the mechanisms of ALP translocation in rat hepatocytes.

It was previously reported that, in fetal rat hepatocytes

cultured primarily in basal medium without dexamethasone, ALP and GGT are localized in the limited perinuclear cytoplasm [10]. Another study reported that, in McA-RH 7777 cells cultured at low concentration and without contact between adjacent cells, both enzymes were localized in the limited cytoplasm around the nucleus [11]. We recently demonstrated using double staining for ALP and man II that the limited cytoplasm around the nucleus in McA-RH 7777 cells is the Golgi region [12].

The present study also demonstrated that ALP is localized in the perinuclear cytoplasm containing the Golgi region in fetal rat hepatocytes cultured primarily in basal medium without dexamethasone, while it is localized in the perinuclear cytoplasm and plasma membrane surrounding the bile canaliculus-like structure in hepatocytes cultured in dexamethasone-supplemented medium. In a previous paper, ALP and proteins (ZO-1 and occludin) related to tight junctions were colocalized in the long stretches of plasma membrane between cell borders of hepatocytes cultured in dexamethasone-supplemented medium, but a protein (E-cadherin) related to adherence junctions was not colocalized in such stretches of plasma membrane [13]. This indicates that the above long stretches of plasma membrane are those surrounding the bile canaliculus-like structure. Thus, in primarily cultured fetal rat hepatocytes, dexamethasone promotes translocation of ALP from the perinuclear cytoplasm to the plasma membrane surrounding the bile canaliculus-like structure.

On the other hand, it was reported that anti-microtubular agents, such as colchicine, nocodazole and colcemid, interfere with the intracellular transport of plasma membrane proteins located in the apical domain of epithelial cells [16, 17, 20, 30]. This suggests that microtubules participate in the intracellular transport of ALP, a protein localized in the bile canalicular membranes of rat hepatocytes. Microtubules are also known to play a role in maintaining Golgi complex composition in the cytoplasm [22, 31]. This role is evident from the fact that microtubule disruption by colchicine or nocodazole alters the composition and intracellular distribution of the Golgi complex [20, 32].

The present study confirmed that, in fetal rat hepatocytes incubated in colchicine-containing medium, the fibrous structure of microtubules disappears and man II is present in numerous granules dispersed throughout the cytoplasm. In addition, ALP was present in the coarse granules scattered throughout the cytoplasm after colchicine treatment. These results indicate that microtubule disruption by colchicine decomposed the Golgi complex and inhibited ALP translocation from the limited perinuclear cytoplasm to the plasma membrane.

Moreover, we examined whether inhibition of ALP translocation by colchicine treatment disrupts the bile canaliculus-like structure formed in cultured fetal rat hepatocytes, and consequently demonstrated that the bile canaliculus-like structure does not change, even if ALP translocation is inhibited (unpublished). Durand-Schneider *et al.* reported that, in rat hepatocytes cultured as primary monolayers, colchicine does not inhibit the structural reconstitution of bile canaliculi, but impairs the concentration of protein B10 of the apical plasma membrane domain on the bile canalicular membrane [16]. These results suggest that the translocation of ALP to the bile canalicular membrane is not related to formation of bile canaliculus.

There are other papers regarding the inhibitory effects of colchicine on ALP translocation in adult rat hepatocytes *in vivo* and *in vitro* [24, 26]. These papers reported that at the early stages of treatment, colchicine causes accumulation of ALP in the Golgi fraction and delays transport of ALP from the cytoplasm to the plasma membrane. The results of the present study are seemingly consistent with these reports. However, ALP was not colocalized with man II in the granular sites that appeared in the cytoplasm after colchicine treatment. This indicates that ALP is not localized in the structurally altered Golgi stacks after colchicine treatment. Instead, ALP was located in the coarse granules following colchicine treatment. We are currently planning investigations into the origins of these coarse granules.

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