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

Kohsuke Chida1 and Meiko Taguchi1

1Department of Anatomy, School of Allied Health Sciences, Kitasato University, Sagamihara, Kanagawa 228-8555, Japan

Received April 9, 2007; accepted December 25, 2007; published online February 22, 2008

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
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 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. 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...
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 canalculus-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 canalculus-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 canalculus-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 throughout the cytoplasm. In addition, ALP was present in numerous granules dispersed throughout the cytoplasm. 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 canalculus-like structure formed in cultured fetal rat hepatocytes, and consequently demonstrated that the bile canalculus-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.

Acknowledgments

We are grateful to Mr. Akira Takebe (Nikon Instech Co., Ltd.) for assistance with taking photographs during confocal laser microscopy.

References

1. Araki, N., Takashima, Y. and Makita, T. (1995) Redistribution and fate of colchicine-induced alkaline phosphatase in rat hepatocytes: possible formation of auto-phagosomes whose membrane is derived from excess plasma membrane. Histochem. Cell Biol. 104; 257–265.
2. Baribault, H. and Marceau, N. (1986) Dexamethasone and dimethylsulfoxide as distinct regulators of growth and differentiation of cultured suckling rat hepatocytes. J. Cell Physiol. 129; 77–84.
3. Bennett, G., Leblond, C. P. and Haddad, A. (1974) Migration of glycoprotein from the Golgi apparatus to the surface of various cell types as shown by radioautography after labelled fucose injection into rats. J. Cell Biol. 60; 258–284.
4. Chida, K. (1989) Changes in alkaline phosphatase and y-glutamyltranspeptidase during the process of rat hepatic cell proliferation. Acta Histochem. Cytochem. 22; 509–516.
5. Chida, K. (1991) Regulation on the expression of carcino-embryonic proteins in newborn rat hepatocytes by dexamethasone. An enzyme histochemical and immunohistochemical study. Okajimas Folia Anat. Jpn. 68; 235–242.
6. Chida, K. (1993) Regulation of the expression of gamma-glutamyltranspeptidase and alpha-fetoprotein in cultured fetal rat hepatocytes. Acta Histochem. Cytochem. 26; 45–50.
7. Chida, K. (1993) Immunohistochemical localization of alkaline phosphatase in rat liver. Acta Histochem. Cytochem. 26; 239–244.
8. Chida, K. (1993) Immunohistochemical detection of alkaline phosphatase in formalin-fixed and paraffin-embedded rat organs by means of avidin-biotin peroxidase complex methods. Okajimas Folia Anat. Jpn. 70; 203–208.
9. Chida, K. (1996) Effect of colchicine on intracellular transport of alkaline phosphatase in primary culture of adult rat hepatocytes-immunocytochemical study. Acta Histochem. Cytochem. 29; 45-
10. Chida, K. (1998) Localization of gamma-glutamyltranspeptidase and alkaline phosphatase in primary cultures of fetal rat hepatocytes. Acta Histochem. Cytochem. 31; 323–328.

11. Chida, K. (2000) Localization of gamma-glutamyltranspeptidase and alkaline phosphatase in rat hepatoma cell line, McA-RH 7777. J. Histochem. Cytochem. 33; 413–418.

12. Chida, K. and Taguchi, M. (2005) Localization of alkaline phosphatase and proteins related to intercellular junctions in primary cultures of fetal rat hepatocytes. Anat. Embryol. 210; 75–80.

13. Chida, K., Tamamushi, S. and Yamamoto, I. (1995) Enzyme cytochemical study of alkaline phosphatase in rat hepatocytes—change of location after colchicine administration or bile duct ligation. Okajimas Folia Anat. Jpn. 71; 371–380.

14. Chida, K. and Taguchi, M. (2005) Localization of alkaline phosphatase and proteins related to intercellular junctions in primary cultures of fetal rat hepatocytes. Anat. Embryol. 210; 75–80.

15. Cotariu, D., Barr-Nea, L., Papo, N. and Zaidman, J. L. (1988) Inhibition of alkaline phosphatase and proteins related to intercellular junctions in rat hepatoma cell line, McA-RH 7777. J. Histochem. Cytochem. 52; 979–983.

16. Durand-Schneider, A. M., Bouanga, J. C., Feldmann, G. and Maurice, M. (1991) Microtubule disruption interferes with the structural and functional integrity of the apical pole in primary cultures of rat hepatocytes. Eur. J. Cell Biol. 56; 260–268.

17. Farquhar, M. G. and Palade, G. E. (1981) The Golgi apparatus—(1954–1981)—from artifact to center stage. J. Cell Biol. 91; 77s–103s.

18. Eilers, U., Klumperman, J. and Hauri, H. P. (1989) Nocodazole, a microtubule-active drug, interferes with apical protein delivery in cultured intestinal epithelial cells (Caco-2). J. Cell Biol. 108; 13–22.

19. Ezzell, R. M., Toner, M., Hendricks, K., Dunn, J. C., Tompkins, R. G. and Yarmush, M. L. (1993) Effect of collagen gel configuration on the cytoskeleton in cultured rat hepatocytes. Exp. Cell Res. 208; 442–452.

20. Farquhar, M. G. and Palade, G. E. (1981) The Golgi apparatus (complex)—(1954–1981)—from artifact to center stage. J. Cell Biol. 91; 77s–103s.

21. Hasegawa, H., Watanabe, K., Nakamura, T. and Nagura, H. (1987) Immunocytochemical localization of alkaline phosphatase in absorptive cells of rat small intestine after colchicine treatment. Cell Tissue Res. 250; 521–529.

22. Knop, E., Bader, A., Böker, K., Pichlmayr, R. and Sewing, K.-F. (1995) Ultrastructural and functional differentiation of hepatocytes under long-term culture conditions. Anat. Rec. 242; 337–349.

23. Kreis, T. E. (1990) Role of microtubules in the organization of the Golgi apparatus. Cell Motil. Cytoskel. 15; 67–70.

24. Musat, A. I., Sattler, C. A., Sattler, G. L. and Pitot, H. C. (1993) Reestablishment of cell polarity of rat hepatocytes in primary culture. Hepatology 18; 198–205.

25. Oda, K. and Ikehara, Y. (1981) Inhibitory effect of colchicine on the cytoskeleton in cultured rat hepatocytes. J. Biochem. 100; 433–438.

26. Oda, K. and Ikehara, Y. (1981) Inhibitory effect of colchicine on the cytoskeleton in cultured rat hepatocytes. J. Biochem. 100; 433–438.

27. Pekarthy, J. M., Short, J., Lansing, A. I. and Lieberman, I. (1972) Function and control of liver alkaline phosphatase. J. Biol. Chem. 247; 1767–1774.

28. Polishchuk, R., Pintina, A. D. and Lippincott-Schwartz, J. (2004) Delivery of raft-associated, GPI-anchored proteins to the apical surface of polarized MDCK cells by a transcytotic pathway. Nat. Cell Biol. 6; 297–307.

29. Sabatini, D. D., Kreibich, G, Morimoto, T. and Adesnik, M. (1982) Mechanisms for the incorporation of proteins in membranes and organelles. J. Cell Biol. 92; 1–22.

30. Suzuki, T., Matsuzaki, T., Hagiwara, H., Aoki, T., Tajika-Takahashi, Y. and Takata, K. (2006) Apical localization of sodium-dependent glucose transporter SGLT1 is maintained by cholestrol and microtubules. Acta Histochem. Cytochem. 39; 155–161.

31. Thyberg, J. and Moskalowski, S. (1985) Microtubules and the organization of the Golgi complex. Exp. Cell Res. 159; 1–16.

32. Thyberg, J. and Moskalowski, S. (1992) Reorganization of the Golgi complex in association with mitosis: redistribution of mannosidase II to the endoplasmic reticulum and effects of brefeldin A. J. Submicrosc. Cytol. Pathol. 24; 495–508.

33. Wolf-Peeters, C. D., Vilain, J. and Desmet, V. (1972) Electron microscopy and histochemistry of canalicular differentiation in fetal and neonatal rat liver. Tissue Cell 4; 379–388.

34. Yamamoto, K. and Ogawa, K. (1983) Effects of NaOH–PIPER buffer used in aldehyde fixative on alkaline phosphatase activity in rat hepatocytes. Histochemistry 77; 339–351.

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.