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Note

Localization of Alkaline Phosphatase and Cathepsin D during Cell Restoration after Colchicine Treatment in Primary Cultures of Fetal Rat Hepatocytes

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Localization of alkaline phosphatase (ALP) and cathepsin D (CAPD) in primary cultures of fetal rat hepatocytes was examined using double immunofluorescent staining in order to investigate the relationship between lysosome movement and the fate of ALP during cell restoration after microtubule disruption by colchicine. At 3 hr and 24 hr after colchicine treatment, numerous coarse dots containing ALP were observed throughout the cytoplasm, and some of these showed colocalization with CAPD. At 48 hr and 72 hr after colchicine treatment, although most of the dots containing ALP in the cytoplasm disappeared, dots containing CAPD remained. The present results suggest that the denatured ALP proteins remaining in the cytoplasm of hepatocytes during cell restoration after colchicine treatment are digested by lysosomes.

Key words: alkaline phosphatase, cathepsin D, colchicine, hepatocyte, primary culture

I. Introduction

Alkaline phosphatase (ALP) is predominantly localized in the bile canalicular membrane in adult rat hepatocytes and microtubules are involved in the transport of ALP to the bile canalicular membrane [1, 2, 4]. We previously reported that, in primary cultures of fetal rat hepatocytes, colchicine inhibits ALP transportation to the plasma membrane of bile canaliculus-like structures, and, consequently, numerous coarse dots containing ALP appear in the cytoplasm [6]. In addition, electron microscopy has revealed that autophagolysosome-like granules containing ALP are present in the cytoplasm of colchicine-treated rat hepatocytes [1, 4]. However, the relationship between lysosome movement and the fate of the ALP-containing dots in the cytoplasm remains to be fully elucidated. In the present study, the localization of ALP and the lysosome marker cathepsin D (CAPD) during cell restoration, after colchicine treatment in primary cultures of fetal rat hepatocytes, was examined in order to elucidate the relationship between lysosome movement and the fate of the ALP-containing dots in the cytoplasm.

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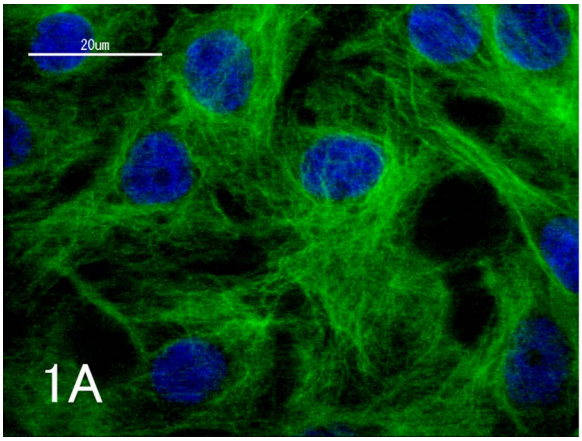
II. Materials and Methods

Hepatocytes were cultured as described previously [6]. Three days after the start of culture, 10^{-5} M colchicine was added to the medium and hepatocytes were incubated for 1 hr. Medium was then replaced with fresh normal medium and cells were further cultured for 3, 24, 48 or 72 hr. After the termination of culture, cells were fixed for 5 min at room temperature (RT) in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, followed by absolute methanol for 5 min at –20°C. Cells were washed with 0.01 M phosphate buffer, pH 7.2, containing 0.85% NaCl and 0.05% saponin (PBSS) at 4°C overnight and immersed for 5 min at RT in 0.1% Triton X-100 solution in PBSS. After washing with PBSS, cells were incubated for 1 hr at RT in 1:50 anti-rat β-tubulin monoclonal antibody (Chemicon International, Temecula, CA, USA) in PBSS. Cells were washed with PBSS and reacted for 30 min at RT with 1:150 fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG antibodies (Medical and Biological Laboratories, Nagoya, Japan) in PBSS.

After termination of culture, some cells were fixed in the same manner using Zamboni fixative solution in place of 4% paraformaldehyde. After washing with PBSS and Triton X-100 treatment, cells were incubated for 1 hr



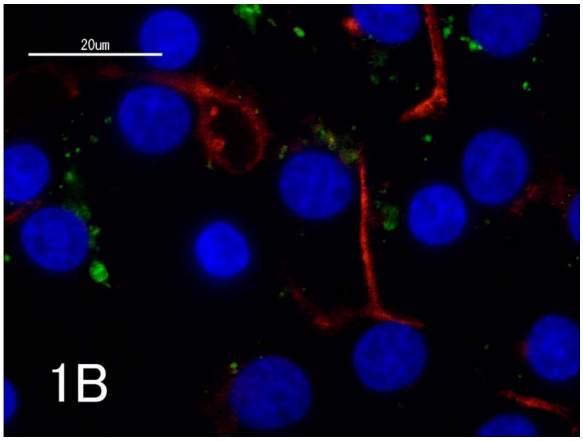
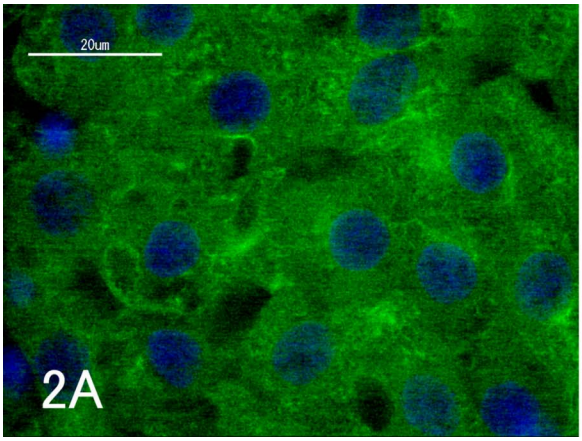


Fig. 1. Normal fetal rat hepatocytes in primary cultures. (**A**) Distribution of microtubules. Microtubules distribute radially from the nuclei to the peripheral cytoplasm. (**B**) Localization of ALP and CAPD. ALP is localized in the plasma membrane of bile canaliculus-like structures, while CAPD is localized in small dots in the cytoplasm. Bar=20 μm.



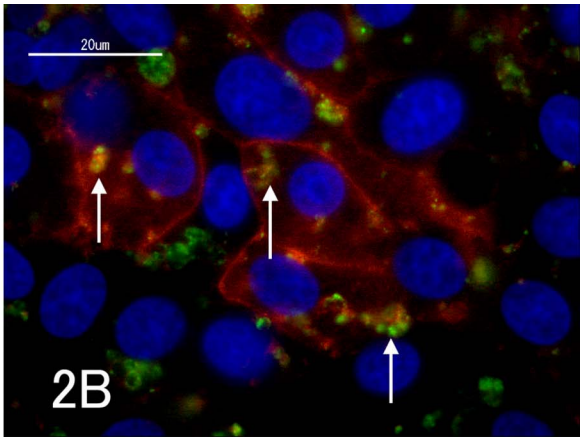
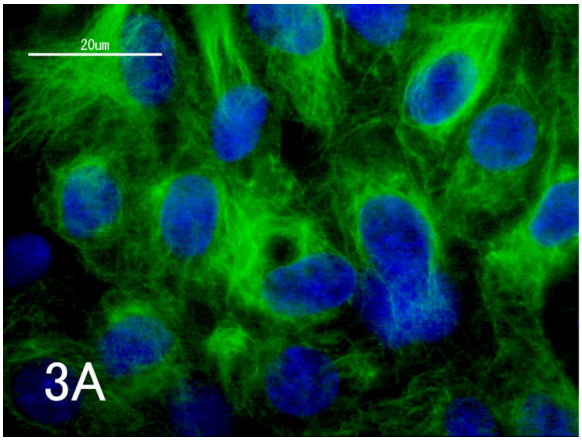


Fig. 2. Fetal rat hepatocytes at 3 hr after colchicine treatment in primary cultures. (**A**) Distribution of microtubules. Microtubule structure is disrupted, and numerous dots or fine nets showing specific fluorescence are observed throughout the cytoplasm. (**B**) Localization of ALP and CAPD. ALP is observed along cell borders between adjacent hepatocytes and in numerous coarse dots within the cytoplasm, and CAPD is colocalized in some coarse dots containing ALP in the cytoplasm (arrows). Bar=20 μm.



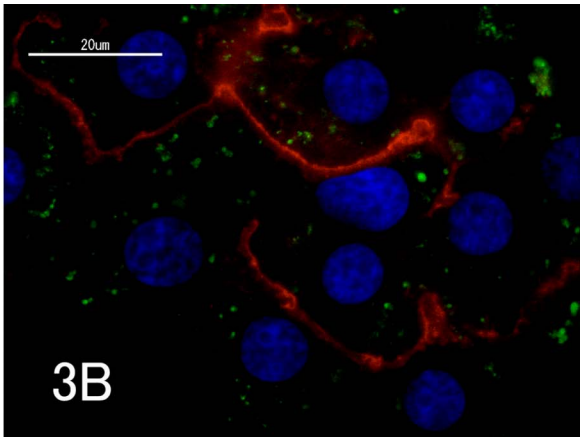


Fig. 3. Fetal rat hepatocytes at 72 hr after colchicine treatment in primary cultures. (**A**) Distribution of microtubules. Abundant microtubule structures are distributed from the nuclei to the peripheral cytoplasm. (**B**) Localization of ALP and CAPD. ALP is localized in the plasma membrane of bile canaliculus-like structures, but is scarcely seen in the cytoplasm, while CAPD is localized in dots of various sizes in the cytoplasm. Bar=20 μm.

at RT in mixed solution containing 1:50 anti-ALP rabbit serum [3] and 1:100 anti-CAPD goat antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBSS. Cells were then washed with PBSS and reacted for 30 min at RT with 1:50 rhodamine-labeled anti-rabbit IgG antibody (Medical and Biological Laboratories) and 1:150 FITC-labeled anti-goat IgG antibody (Medical and Biological Laboratories) in PBSS.

After immunostaining, all cell nuclei were stained with 4',6-diamidino-2-phenyl-indole. Samples were examined under a fluorescence microscope (ECLIPSE E-600; Nikon, Tokyo, Japan) and photographed using a digital camera (DS-L2; Nikon) equipped with a fluorescein figure analysis system (LuminaVision; Mitani Co., Tokyo, Japan).

The present study was approved by the Ethics Committee for Animal Experiments of Kitasato University.

III. Results and Discussion

In normal hepatocytes, microtubules were observed to distribute radially from the cytoplasm around the nuclei to the peripheral cytoplasm (Fig. 1A), and ALP was localized in the plasma membrane along bile canaliculus-like cell borders (Fig. 1B). We previously demonstrated that the long stretches of plasma membrane exhibiting ALP localization between cell borders of normal hepatocytes are those surrounding the bile canaliculus-like structure, as the bile canaliculus marker occludin is colocalized in these long stretches of plasma membrane [5]. On the other hand, CAPD was observed in small scattered dots in the cytoplasm (Fig. 1B). Three hours after colchicine treatment, the microtubule structures in hepatocytes were destroyed, and numerous dots or fine nets showing specific fluorescence were observed throughout the cytoplasm (Fig. 2A).

Microtubular structures are known to reappear by wash-out with normal medium after treatment with anti-microtubular agents. It has been reported that, in Madin-Darby canine kidney (MDCK) cells treated with colcemid for 4 hr and cultured in normal medium for 2 hr, microtubule networks were reconstructed and reverted to normal shape [8]. On the other hand, we found that, in McA-RH 7777 cells incubated for 8 hr in basal medium after 4-hr colchicine treatment, microtubular structures did not reappear in the cytoplasm, as observed in the present study [9]. The difference in these results may be the result of differences in anti-microtubular agent or cell type.

On double staining for ALP and CAPD, at 3 hr after colchicine treatment, ALP was observed along cell borders between adjacent hepatocytes and in numerous coarse dots within the cytoplasm, and CAPD was colocalized in some of these coarse dots (Fig. 2B). These dots, in which ALP and CARD were colocalized, appear to be secondary lysosomes showing fusion of autophagosomes containing ALP and granular primary lysosomes containing CAPD and other proteinases. ALP in autophagosomes may be digested by these lysosomal enzymes. At 24 hr after colchicine treatment, microtubule structures showed a random distribution in the cytoplasm, but coarse dots showing positive reactions for ALP and autophagolysosome-like dots containing ALP and CAPD remained in the cytoplasm (data not shown). This indicates that transportation of ALP to the plasma membrane is not fully restored at 24 hr after colchicine treatment.

At 48 hr and 72 hr after colchicine treatment, abundant microtubule structures were distributed from the nuclei to the peripheral cytoplasm (Fig. 3A). ALP was localized in the plasma membrane of bile canaliculus-like structures but was scarcely seen in the cytoplasm, while CAPD was localized in dots of various sizes in the cytoplasm (Fig. 3B). In the same culture system, we observed small granules containing early endosomal antigen 1 (EEA1) along the plasma membrane showing positive reactions for ALP at 3 hr and 24 hr after colchicine treatment, but these granules were located in the peripheral cytoplasm in normal hepatocytes or hepatocytes at 48 hr and 72 hr after colchicine treatment (unpublished data). This suggests that ALP may be transported to the plasma membrane of bile canaliculus-like structures from the cytoplasm via endosomes during cell restoration after colchicine treatment. Accordingly, denatured ALP enzyme proteins in the cytoplasm may be taken up by autophagosomes and digested by lysosomes.

It has been reported that the apical membrane protein B10 in rat hepatocytes is localized in numerous vesicles in the cytoplasm when hepatocytes are cultured in the presence of colchicine for 24 hr, and that it is mainly localized in vacuoles resembling lysosomal structures when hepatocytes are cultured in the presence of nocodazole for 48 hr [7]. After microtubule disruption, bile canalicular membrane proteins such as ALP and B10 may be taken up by autophagosomes and endosomes and digested by lysosomes or transported to the plasma membrane via endosomes during cell restoration.

IV. References

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