VISUALIZATION OF VITAMIN D-DEPENDENT CALCIUM BINDING PROTEIN IN CHICK INTESTINAL TISSUE BY IMMUNO-SCANNING ELECTRON MICROSCOPY

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Vitamin D₃ is one of the essential factors in intestinal Ca transport. It is well established that vitamin D₃ is first metabolized to a hydroxylated product, 1,25-(OH)₂D₃, before it can act on the process of intestinal Ca transport. 1,25-(OH)₂D₃ is now regarded as a hormone which is secreted from the kidney and is accumulated specifically in the nuclei of the target tissues, such as the intestine, *via* a specific cytoplasmic receptor for 1,25-(OH)₂D₃. A part of its action mechanism can be elucidated by the stimulation of the synthesis of specific proteins (1).

With respect to the specific proteins induced by 1,25-(OH)₂D₃, CaBP is demonstrated in the intestinal mucosa (2). However, the role of CaBP in the intestinal Ca transport is still not clear. The definitive demonstration of intracellular localization of CaBP is necessary for understanding the role of CaBP. However, regarding the intracellular localization of CaBP, there are some discrepancies among investigators. TAYLOR and WASSERMAN demonstrated histochemically that immunofluorescence indicative of the presence of CaBP was found in the goblet cells and in association with the surface coat-microvillar region of all intestinal epithelial cells in normal and vitamin D-treated rachitic chicks (3). The other finding is that chick duodenal CaBP is located neither on microvillar membranes nor in goblet cells, and CaBP is located rather inside the intestinal epithelial cells (4).

The immuno-scanning electron microscopy technique has been developed for the visualization of antigen exposed to cell surface (5). If CaBP is located on brush borders as reported by TAYLOR and WASSERMAN, this method can be applied to the visualization of CaBP in the intestine.

Chick CaBP was purified from vitamin D₃ dosed chick duodenal mucosa by a slightly modified method of WASSERMAN *et al.* (6). The antibody for CaBP was raised

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Abbreviations: immuno-SEM; immuno-scanning electron microscopy, 1,25-(OH)₂D₃; 1,25-dihydroxyvitamin D₃, CaBP; calcium binding protein.

in rabbit (7). For the observation of CaBP by immuno-SEM, one-day-old chicks were raised on a vitamin D-deficient diet (8) for 5 weeks. Vitamin D-deficient chicks were dosed with either 2,000 IU of vitamin D₃ dissolved in 50 μ l of 95% ethanol or 50 μ l of 95% ethanol intravenously only. After 48 hours, the animals were sacrificed by decapitation and the duodena were used for estimation of CaBP content and immuno-SEM. CaBP content was estimated by radial immunodiffusion as described previously (7). On the other hand, for the immuno-SEM, the duodena were slit open and then fixed for 30 min at 0°C in 10% formalin in 0.2 M sodium phosphate buffer (pH 7.4) either immediately or after being well rinsed with ice-cold 0.2 M sodium phosphate buffer (pH 7.4). The fixed tissues were incubated with anti-CaBP rabbit γ -globulin for 1 hour at room temperature. This was followed by incubation with polystyrene latex (0.6 μ m, Dow Chem. Comp., Indianapolis, USA)-bound anti-rabbit goat γ -globulin (Miles Lab., UK) at 4°C overnight. Then tissues were fixed with 2.5% glutaraldehyde and prepared for scanning electron microscopy (5). In this method, polystyrene latex is used as a marker of CaBP.

CaBP content of duodenal mucosa was negligible in vitamin D-deficient chicks, while it was 180 ± 13.3 μ g/g tissue in vitamin D₃ dosed chicks. Obviously, CaBP was induced in the duodenal mucosa of vitamin D₃ dosed chicks.

Formalin-fixed duodenal tissues not washed by phosphate buffer and incubated with specific anti-CaBP rabbit γ -globulin and polystyrene latex bound goat γ -globulin, were viewed under a scanning electron microscope at low magnification. This revealed that the duodenal villi showed a large finger-like shape of uniform height. The apices of the goblet cells appeared as depressions within the coat of microvilli or were covered with their secretory substances. The difference between vitamin D-deficient and vitamin D₃ dosed chick was not observed in the gross views of the duodenal villi. However, it was faintly noticed that the duodenal villi of vitamin D₃ dosed chick were covered with polystyrene latex (Fig. 1a, b).

Further examination at higher magnification gave a view of polystyrene latex as round small particles. The polystyrene latex particles, indicative of the presence of CaBP, were observed on the secretory substances of goblet cells and the microvilli in the vitamin D₃ dosed chick duodenum, however, very few in the vitamin D-deficient chicks (Fig. 1c, d).

Fig. 1. Immuno-scanning electron micrograph of duodenal villi from vitamin D-deficient(a, c, e) and vitamin D₃-treated (b, d, f) chick. Either 50 μ l of 95% ethanol or 2,000 IU of vitamin D₃ in 50 μ l of 95% ethanol was dosed to vitamin D-deficient chicks 48 hours before sacrifice intravenously. Duodena were fixed in 10% formalin in phosphate buffer (pH 7.4) either immediately (a, b, c, and d) or after being well rinsed with ice-cold phosphate buffer (pH 7.4) (e and f), incubated with anti-CaBP rabbit γ -globulin followed by the incubation with polystyrene latex bound anti-rabbit goat γ -globulin. The tissues were fixed with glutaraldehyde and prepared for the scanning electron microscopy.
a and b: Observation of duodenal villi at low magnification. Black bar denotes 50 μ m.
c, d, e and f: Observation of duodenal villi at higher magnification. Black bar denotes 1 μ m. The apices of goblet cells (arrows) appeared as depression within the coat of microvilli (M) or were covered with their secretory substances(S). P, denotes polystyrene latex particles.

Fig. 1.

On the other hand, the polystyrene latex particles were not observed on the microvilli of vitamin D₃ dosed chick when the duodenal surface was well rinsed by phosphate buffer before fixation of tissues. In this case, the polystyrene latex particles were only observed on the secretory substances of goblet cells in the vitamin D₃ dosed chick duodenal villi (Fig. 1e, f). This suggests that CaBP is contained in the secretory substances of goblet cells and covers the intestinal surface, but it is not integrated into the microvillar membranes.

TAYLOR and WASSERMAN have demonstrated CaBP on the surface coat-microvillar region of all intestinal epithelial cells in normal and vitamin D₃-treated rachitic chick duodenum (3). In their study, tissues were frozen immediately after excising from chick. Then sections were cut using a cryostat microtome. The section was mounted on slide glass, fixed with 95% ethanol, and then treated with anti-CaBP rabbit γ -globulin. Namely, the duodenal surface was not rinsed by phosphate buffer before fixation. Therefore, it will be possible that CaBP demonstrated on the microvilli of vitamin D₃-treated chick duodenum by TAYLOR and WASSERMAN could be removed when the duodenal surface was well rinsed by phosphate buffer before freezing.

Immuno-SEM is not suitable for the observation of CaBP inside intestinal epithelial cell. Therefore, we can not comment on the presence of CaBP inside intestinal epithelial cells from these results, however, it is suggested that CaBP synthesized in goblet cells is secreted into the intestinal lumen and covers the intestinal villi.

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