Effects of cytochalasin B on the formation of previllous ridges and the appearance of long microvillous-like processes in the organ culture system of chick embryonic intestine

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SUMMARY
Between 8 and 12 days of incubation the embryonic duodenum serially constructs with relative regularity the previllous ridges upon which the definitive villi later form. The effects of cytochalasin B (CB) on the formation of these previllous ridges of the duodena of developing chick embryos were studied, varying the concentrations and exposure time of CB in the organ culture system. The results were as follows: (1) CB inhibited the formation of new previllous ridges from the epithelial cell sheets of 8- to 11-day-old embryonic duodena at a cultured time of 24 h. (2) CB treatment blocked or delayed cytokinesis of the epithelial cells and the production of many long microvillous-like processes (long processes) from the surface of the epithelial cells. (3) These long processes elicited by CB contained actin filaments and their appearance was influenced by the developmental stages of embryos and local parts of epithelial cells. (4) With 11-day embryonic duodena, induction of long processes by CB was observed at various concentrations (1 μg/ml–16 μg/ml) and even after short exposure of 15 min. (5) Cytochalasin D (CD) and colchicine were used and long processes were induced by CD but not by colchicine itself. The appearance of long processes depended on the experimental concentration of CB, CD and colchicine.

In normal developments, such long processes appeared and disappeared within a confined area during the formation of previllous ridges (Noda, 1981). This study seemed to provide experimental support for the previous reported suggestion that the long processes might be one of the important factors in the formation of the previllous ridges of chick embryonic duodena.

INTRODUCTION
Since Hilton (1902), it has been well known that the villi of chick intestinal epithelium are derived from previllous ridges running along the length of the intestine. As a mechanism for the formation of previllous ridges Burgess (1975) suggested that actin contractions in groups of epithelial cells caused folding. These actin contractions are similar to the contraction by contractile microfilaments in epithelial cells in the amphibian neurulation. He showed that CB-treatment prevented the folding of the first previllous ridges and caused disruption of the

Key words: Cytochalasin B, previllous ridges, long microvillous-like processes.
bundles of microfilaments during the morphogenesis of chick embryonic duodena. Prior to his work, the effects of CB on morphogenesis of oviduct epithelium, salivary gland epithelium etc had been reported. In these cases too CB not only inhibited folding of epithelia, but also disrupted the structure of the microfilamentous bundles in those epithelial cells (Wrenn & Wessells, 1970; Wrenn, 1971; Cloney, 1972; Spooner & Wessells, 1972).

Previously, Noda (1979, 1981) observed that 9- to 10-day-old chick embryonic duodenal confined surface had long microvillous-like processes (long processes) containing core filaments. These long processes were observed on the slope near the foot or on the foot of previllous ridges while these ridges began to protrude and complete their development to high and slender form. Next these long processes disappeared in sequence from each portion scheduled for the formation of the next previllous ridges. They also formed a net by intertwining. From their features, Noda (1981) suggested that these long processes are one of the important factors in the morphogenesis of previllous ridges and that proliferation of epithelial cells having these long processes is inhibited in the troughs between the developing previllous ridges.

During an investigation of the effect of CB on these long processes, the author discovered that CB induced similar long processes and that long processes induced by CB seem to be related repressively to the morphogenesis of previllous ridges. Burgess & Grey reported in 1974 that CB elicited the elongation of microvilli. However, no mention was made of a relationship between formation of previllous ridges and elongation of microvilli, since the effect of CB on folding of previllous ridges was examined at a concentration of 1 µg/ml which rarely evoked elongation of microvilli.

But in this study concentrations of 2 µg/ml or even 1 µg/ml induced long processes and microfilamentous bundles did not seem to be always disrupted by CB. This paper discusses the effects of CB on the formation of previllous ridges and the appearance of long processes and their correlation.

CB has been used in the investigations of morphogenesis, cell movements and others ever since reports about its complicated effects (for a review, see Burnside & Manasek, 1972 and Holtzer & Sanger, 1972). In the present experiments using CB consideration was given to three parameters: embryonic age, CB concentration, and length of exposure to the agent. In addition, the effects on the epithelial cells of chick embryonic duodena were also described of both cytochalasin D (CD), which is more potent than CB, and colchicine, as a specific for tubulin.

MATERIALS AND METHODS

Embryos

White Leghorn eggs were incubated at 37°C with 60–70 % relative humidity for 6–19 days. All stages were counted as days postincubation. The proximal ends of the duodenal loops were used for this study.
**Organ culture**

The excised duodenal fragments were quickly placed in warm Eagle's Minimal Essential Medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin and cut into fragments 1–2 mm in length. These fragments were slit open lengthwise with a sharp stainless-steel pincette. Tissues were cultured in Eagle's Essential Medium containing 20% foetal serum (MEM) and in 50 mm culture dishes (Heraeus Petriperm) in an atmosphere of 5% CO₂ in air. CB was dissolved in dimethylsulphoxide (DMSO) and added to culture media to achieve a final concentration of 2 μg/ml. For control cultures, a volume of DMSO equal to the experimental CB solution was added to the culture media. CB-treated experimental and control fragments from the 6–19th day chick embryo were cultured for about 1 h, 4–6 h or 24 h respectively except 6- and 7-day-old duodena. The effects of concentration or length of exposure of CB were studied next. Some duodenal fragments from 11-day-old embryos were cultured in CB (2 μg/ml medium) for 5, 10, 15, 30, 45, 90 min as well as 1, 4–6 and 24 h. In addition, to some experimental fragments from the same 11-day-old embryos, CB or CD was added to achieve a final concentration in the medium of 0.5, 1, 2, 4, 8, 16 (only CB) μg/ml and all fragments were cultured for 2 h. The effect of colchicine was studied in the presence of CB or without it. Colchicine was added to the medium (with or without 2 μg/ml CB) to achieve a final concentration of 0.5, 1, 2, 4, and 10 μg/ml and fragments were cultured for 2 h. In these cases, a volume of DMSO or MEM equal to the volume of CB and CD, or colchicine that was added to the experimental cultures, was added to the control dishes.

**Decoration with heavy meromyosin (HMM)**

HMM was kindly supplied by Dr Yutaka Shimada (University of Chiba). Some fragments of 11-day-old embryonic duodena were cultured in MEM containing CB (2 μg/ml) for 2 h and then rinsed in solution A: 60 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 10 mM TAME, 10 mM imidazole buffer at pH 7-3 (Mooseker & Tilney, 1975). They were then shaken in solution A containing HMM and saponin (4 mg HMM and 1 mg saponin/ml solution A) at room temperature for 60 min and immediately processed for transmisssional electron microscopic observation.

**Electron microscopy**

HMM-tested preparations were fixed in 1% glutaraldehyde containing 0.2% tannic acid, 0.1 M-phosphate buffer at pH 7.0 for 30 min and postfixed in 1% OsO₄ in 0.1 M-phosphate buffer at pH 6.0 for 25 min. The preparations were then washed two or three times with distilled water, stained en bloc with 1% uranyl acetate (aqueous) for 40 min. This method was based on Mooseker & Tilney (1975). Another set of preparations were fixed in 2.5% glutaraldehyde buffered in 0.1 M-phosphate buffer, pH 7.2 for 1–2 h at 4°C, and postfixed in 1% OsO₄. For scanning electron microscopic observations, the tissues were dehydrated in a graded series of ethanol, acetone and amylacetate, and then dried in a critical-point drier (HCP-1, Hitachi) using CO₂ as the transitional fluid. All samples were coated with gold in a vapour coater (Eiko 1B-3) and examined under a scanning electron microscope (JSM-35). For transmisssional electron microscopic observation, tissues were embedded in Epon-812 after dehydration and sectioned under a diamond knife on a Porter-Blum MT-2 ultramicrotome. Thin sections were stained in 2% uranyl acetate and lead citrate and examined with an electron microscope (JEM-100C).

**RESULTS**

**Scanning electron microscopic studies of control cultures**

In control tissues, the apical surface of epithelial cells in the 6th to 7th day specimen was studded with a few short and straight microvilli. Both the density
and length of these real microvilli increased gradually with the age of the chick embryo (Figs 1, 2) as in vivo.

The long microvillous-like processes (long processes) previously reported by Noda (1979, 1981) in normal development, were observed in control culture fragments of 8- to 12-day-old chick embryonic duodena. They were confined to the surface of cells that lie in troughs between previllous ridges throughout the course of formation of previllous ridges and entangled each other as a net. Those long processes seemed to be more frequent near the foot or on the parts of the previllous ridges which were starting zigzag folding during this period. In vitro such long processes were rarely observed before or after the period of formation of previllous ridges. From 8 to 12 days of incubation long processes were difficult to observe as the troughs were deeper and narrower. However the localization and structure of long processes in control cultures was similar to that described above for normal development (Figs 1, 2).

With regard to the morphogenesis of previllous ridges, some differences were observed between normal intact tissues and controls cultured for 24 h. Previllous ridge formation in these control culture fragments was delayed to some extent compared with normal development. This delay was affected by the culture method and the consequent state of epithelial cell sheet (Burgess, 1975). In addition, newly formed previllous ridges in cultures of flat epithelial cell sheets from duodenal areas were almost irregular in contour (Fig. 3, Table 1), while ridges on in vivo duodenal luminal surfaces were regular (Figs 1, 6, Table 1). Whether these irregular ridges develop by the same mechanism as in vivo previllous ridges was not clear. However, in many cases long processes existed near the foot of those irregular ridges as in vivo (Fig. 4, Table 1).

Scanning electron microscopic studies of effects of CB on cultured duodena

Treatment with CB (2 μg/ml) for 24 h, resulted in an alteration of the epithelial lumen of the duodena. The lumina of 24 h-treated preparations were very small in

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Figs 1–5. Scanning electron micrographs of cultured duodenal lumens of chick embryos.

- Figs 1 and 2. The surfaces of the 10-day chick embryonic duodena cultured in control medium containing DMSO for 4 h. Fig. 2 is higher magnification of v in Fig. 1. Long processes (arrow) are observed on the valley (v) between previllous ridges (r). All epithelial cells exhibit short microvilli.

- Figs 3 and 4. The surfaces of the 9-day chick embryonic duodena cultured in control medium containing DMSO for 24 h. At the start of culture, the surface was flat and after 24 h irregular ridges (ir) were formed. Fig. 4 is one of the irregular ridges (ir) and long processes are observed on the foot (f).

- Fig. 5. The surface of low and wide previllous ridge of the 10-day chick embryonic duodena cultured in CB-added medium for 24 h. Long processes are induced on the surface as a whole and entangle each other.

Fig. 1, ×1350, Fig. 2, ×4400, Fig. 3, ×60, Fig. 4, ×1250, Fig. 5, ×4800.
CB and long processes

Figs 1–5.
size and delayed as compared to control cultures throughout this experiment. Cellular sensitivities to CB depended on the developmental degree (Table 2) and cellular localization (Table 1). The most dramatic effects (Table 1) were observed on the surface of cultured epithelial cell lumen of 8- to 12-day-old duodenal fragments especially the 9- to 11-day-old fragments as shown in Table 2. In this case, induction of long processes was accompanied by inhibition or delay of formation or development of previllous ridges and these phenomena showed clear localization. Concerning the induction of long processes (Table 2), the effect of

Table 1. Alteration of experimental (cultured) epithelial cell sheet caused by the differences of starting status (I and II) of specimen and cultured time in duodena of 8–11 day old chick embryos

<table>
<thead>
<tr>
<th>group</th>
<th>cultured time</th>
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<td>II</td>
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<td>localization of production of long processes by CB is observed.</td>
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<tr>
<td>control</td>
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<tr>
<td>CB</td>
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= epithelial cell sheet

= serosa

= real or cytochalasin B (CB)-induced long processes
Table 2. Effects of cytochalasin B (CB) on the appearance of long processes in 6- to 19-day-old chick embryonic duodena

<table>
<thead>
<tr>
<th>Time of culture in CB (h)</th>
<th>Age of embryonic duodena (days)</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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0 = long processes by CB was absent  
+ = long processes by CB was slight  
++ = long processes by CB was moderate  
+++ = long processes by CB was marked

exposure times of 1–6 h was almost the same as that of 24 h. When the duodenal luminal surface was flat and previlous ridges were not yet formed at the start of culture, the surface of duodenal epithelial cells did not show new previlous ridges and was covered by long processes as a whole instead (Fig. 5, Table 1). On the other hand, when the luminal surface had already formed some previlous ridges at the start of culture, the surface of duodenal epithelial cells was maintained without subsequent formation and development of previlous ridges, established ridges were stable in the presence of CB, and long processes were induced locally as shown next. If the previlous ridges on the luminal surface were low and wide at the start of culture, these ridges became completely covered with long processes. If the previlous ridges were higher and narrower at the start of culture, only the under slope and the foot of these ridges were occupied by cells which possessed long processes, while the tip of the previlous ridges or the upper slope did not possess long processes but only short microvilli. Those epithelial cells with no long processes were larger in size and their surfaces showed hexagonal or similar forms (Figs 7, 8 and 9). In the cases of the complete previlous ridges, long processes did not appear on the tip or on the slope in many cases (as shown in Table 1). The limit to the height of previlous ridges which would result in the appearance of long processes was not determined quantitatively. But the qualitative feature at the cellular level that decided the appearance of long processes was distinguished as shown later. The effect of CB on stages other than the 8–12th day period was influenced by the length of exposure as shown in Table 2. However even if long processes were induced by treatment of 4–6 h or 24 h, the number of processes was less than in 8–12th day embryos. In these cases long processes were formed on the parts which seemed capable of cell proliferation.  
Morphological differences between the long processes of control samples and of CB-treated samples were not recognized using the SEM. It was not possible to distinguish whether long processes on the valley between previlous ridges were real long processes or ones induced by CB. The diameter of long processes and normal-sized microvilli was about 0.1 μm, but their lengths were difficult to
determine because they branched in some cases and entangled each other as shown in Figs 5 and 9. Despite the fact that long processes seemed to cover all the surface as a net, all cells did not exhibit them. On 9- to 11-day-old embryonic duodena the appearance of long processes began at exposure time of 5–15 min and was very evident after 30 min treatment with CB concentration of 2 μg/ml as shown in Figs 10 and 11. An exposure of 1–4 h of CB seemed to evoke maximum elongation of long processes. In these experiments, long processes induced by CB appeared much more frequently on the frame of fragments than on other portions (this may be related to cellular pressure) and spreading of the epithelial frame parts was not observed in contrast to control (Table 1).

**Transmissional electron microscopic studies**

Ultrastructural observation revealed that long processes of both control and CB-treated duodena contained similar core filaments to long processes in control duodena (Figs 12, 13). At high magnification, CB-induced long processes seemed to have two characteristic structures; (1) bridges connecting actin filaments to each other and (2) other electron-dense patches on the inside surface of the membrane (Fig. 14) (Mooseker & Tilney, 1975; Matsudaira & Burgess, 1982). In this experiment with HMM, statistical analysis of directions of their arrowed structures was impossible but HMM-core filament complexes were recognized in the long processes induced by CB (Fig. 16). From these results, long processes seemed to have a similar actin fine structure to normal microvilli.

On the other hand, in some CB-treated fragments longer bundles of actin-like filaments protruded from mainly normal microvilli deep into the cytoplasm (Fig. 15). This phenomenon seemed to relate to the amount of membrane available for long processes. It resembled the structure reported by Burgess & Grey (1974). In addition, CB seemed to block or delay cytokinesis without blocking nuclear division in the case of 24 h culture (Fig. 17). As a result, CB-treated fragments seemed to be smaller and delayed in their development as compared to control cultures. In the epithelial cells of CB-treated fragments, microvilli containing core

Figs 6–9. Scanning electron micrographs of duodenal lumens of the 10-day chick embryo cultured for 4 h. At the start of culture, some previllous ridges have already formed on the luminal surface.

Fig. 6. The surface of the fragment cultured in control-medium containing DMSO. Long processes are scarcely observed on the valley (v) between higher previllous ridge (hr) and low and wide previllous ridge (lr).

Fig. 7. The surface of the fragment cultured in CB-added medium. Long processes are observed on the surface of the valley (v) and low and wide previllous ridge (lr) as a whole. Long processes are not induced on the top region of higher previllous ridge (hr).

Figs 8 and 9. Higher magnification of the surfaces of another parts of fragment of Fig. 7. Fig. 8 shows the induction of long processes on the slope (s) of higher previllous ridge and Fig. 9 shows a remarkable induction as in Fig. 7. The surfaces of cells exhibit hexagonal or similar forms (arrows). Long processes are not observed there.

Figs 6 and 7, ×1500, Fig. 8, ×2450, Fig. 9, ×2300.
CB and long processes

Figs 6–9.
filaments protruded into the lumen of vacuole (Fig. 18). This phenomenon may be related to the cellular status at the start time of culture. The cells may have just started their cytokinesis at the beginning of culture CB. In the cytoplasm of CB-treated cells masses of finely granular material were observed as shown in the report by Burgess (1975). Not all bundles of the microfilaments were disrupted by CB. Bundles of microfilaments are sometimes present running parallel to the apical plasmamembrane in the apical region of CB-treated epithelial cytoplasm, especially in cells lining the valley between previllous ridges (Fig. 19). Increasingly some vesicles were recognized in the cytoplasm after CB-treatment (Figs 13 and 22).

On the control transmissional electron micrographs the author tried to compare epithelial cells producing no long processes in the presence of CB with epithelial cells induced to produce long processes by CB. The following morphological differences were recognized between the two sets of cells as shown in Figs 20 and 21. In the case of epithelial cells producing no long processes by CB, their form and size were larger and wider than the cells producing long processes by CB and their electron density was low. The direction of the organelles and cellular interdigitations beneath luminal surfaces was parallel with the luminal surface in many cases (Fig. 20). These structures did not change after CB-treatment and seemed to be stable to CB in this experimental condition. In contrast, in the case of epithelial cells producing long processes by CB their size and form were irregular and the electron density of the cytoplasm was high in comparison. The direction of their organelles of supranuclear region of the cells such as mitochondria and endoplasmic reticulum and cellular interdigitation seemed to be parallel with long axis of the cells in some cases, but was not regular. After CB-treatment, these structures scarcely changed except in the appearance of many vesicles in the cytoplasm and many long processes on luminal surface of epithelial cells (Fig. 22).

CD had a similar effect to CB, but CD was more potent than CB on the induction of long processes and CD of lower concentration than CB caused damage to epithelial cells as shown next. CB concentration of 0.5 µg/ml scarcely protruded long processes. Doses from 1 to 16 µg/ml produced remarkably long

Figs 10 and 11. Scanning electron micrographs of the surfaces of low and wide previllous ridges of the 11-day chick embryo. Fragments incubated in CB-added medium for 5 min (Fig. 10) or 30 min (Fig. 11). CB-treatment for 30 min induced remarkably long processes compared with 5 min culture. ×3050.

Fig. 12. Transmissional electron micrograph of long processes containing core filaments in the valley between previllous ridges of the 10-day chick embryo during normal development. Microfilamentous bundles (arrow) were observed in the apical region of cytoplasm. A substance with a very high electron density (arrowhead) was sometimes observed. This seems to be contracted materials of many microfilaments but it is not clear. l, lumen. ×21 000.

Fig. 13. Transmissional electron micrograph of long processes containing core filaments of the 9-day chick embryo cultured in CB-added medium for 24 h. Apical cytoplasm contains some vesicles (arrow) and mitochondria (mt). l, lumen. ×21 000.
Figs 14–18.
CB and long processes

But at a concentration of 16 μg/ml, long processes were still observed but decreased in number and bulges lacking both microvilli and long processes began to appear on the top region of previllous ridges (Fig. 23). In the case of a CD concentration of 0.5 μg/ml, more long processes appeared than with the same CB concentration, but were comparatively shorter. Dose of 1 μg/ml of CD evoked a large number of long processes (Fig. 24). At concentrations from 2 to 4 μg/ml the induction of long processes decreased by degree and in the case of 4 μg/ml bulges began to appear. At a CD concentration of 8 μg/ml, the epithelial cells and long processes were not very healthy, with a resulting drop in the number of cells.

In the presence of CB (2 μg/ml) and colchicine, the effect of CB on the induction of long processes was removed by increasing the concentration of colchicine. When the concentration of colchicine was 0.5 or 1 μg/ml, long processes were observed in the same status as with CB alone (Fig. 25). But, doses from 2 to 10 μg/ml greatly decreased the production of long processes and increased the production of bulges (Fig. 26). In the case of colchicine alone, any concentration from 0.5 μg/ml to 10 μg/ml failed to induce long processes. In these fragments, the area between two previllous ridges was lower and wider as compared with control fragments, and development of previllous ridges seemed to be repressed. In this experiment colchicine seems to have an influence on the tubulin in the cytoplasm directly, but as a result to have an inhibitory effect on the production of long processes by CB or development of previllous ridges. More detailed experiments will be needed to clarify these mechanisms.

DISCUSSION

The author reported previously that long processes in normal development would be one of the important factors for the formulation of previllous ridges.

Figs 14–18. Transmissional electron micrographs of duodenal lumens of 9-day chick embryo cultured in CB-added medium for 24 h (Figs 14, 15, 17, 18) and the 11th day chick embryo cultured in CB-added medium for 4 h (Fig. 16).

Fig. 14. High magnification of long processes. In addition to core filaments, bridges connecting actin filaments to each other within the bundles (arrows) and electron-dense patches (arrowheads) on the inside surface of the membrane can be seen. ×71,000.

Fig. 15. Longer bundles of filaments (arrow) that protruded deep into the cytoplasm can be seen. Long processes (arrowhead) of a neighbouring cell can be seen. ×11,000.

Fig. 16. HMM-treated long processes and short ‘real’ microvilli of the surface of low and wide previllous ridge cultured in CB-added medium. Core filaments within both long processes and short ‘real’ microvilli form HMM-filament complexes (arrow). l, lumen. ×29,000.

Fig. 17. Transmissional electron micrograph showing block or delay of cytokinesis without blocking nuclear division in duodenal epithelial cells. l shows the lumen side. ×4200.

Fig. 18. Vacuole (arrows) that protruded microvillus-like processes in the vacuolar lumen (v) can be seen in the duodenal epithelial cells. n shows nuclei of the neighbouring cells. ×5700. Inset: Core filaments within the microvillus-like processes are observed. ×11,350.
Figs 19–26.
because of their morphological and topological characteristics. In the present experiments, similar long processes could be induced by CB. This remarkable effect of CB was limited to between 8 and 12 days of incubation. This period coincided with the existing period of formation of long processes during normal development. Long processes induced by CB resembled normal long processes morphologically and functionally. Thus these experimental results using CB suggest that my above mentioned speculation about the function of long processes in normal development may be correct.

The function of these long processes should be discussed in relation to other mechanisms for previllous ridge formation such as the contraction of microfilamentous bundles (Burgess (1975)). Both factors seem to be compatible. The present author observed these bundles in the apical region of the cytoplasm and supports the supposition by Burgess that contraction of these bundles causes folding and the establishment of previllous ridges. At the time of folding or after folding, long processes appeared in the region of the folding part. It seems that

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Fig. 19. Transmissional electron micrograph of duodenal epithelial cells of the valley of the 10-day embryo cultured in CB-added medium for 24 h. Microfilamentous bundles (arrow) and the substance with a very high electron density (arrowhead) (as in Fig. 12) were observed in the apical region of cytoplasm. ×17000.

Fig. 20. Transmissional electron micrograph of duodenal epithelial cells of the top region of previllous ridge of the 10-day chick embryo in control medium for 2 h. Many mitochondria (mt) and cellular interdigitations (arrow) beneath their luminal surface ran parallel with the luminal surface, so many mitochondria can be seen as cross sections. ×5550.

Fig. 21. Transmissional electron micrograph of duodenal epithelial cells of low and wide previllous ridge of the 10-day chick embryo cultured in control medium for 2 h. Compared with Fig. 20, cellular size and form were irregular and smaller (slender) and the electron density of the cytoplasm was high. Directions of cellular organelles and interdigitiation (arrow) were irregular. ×5550.

Fig. 22. Transmissional electron micrograph of duodenal epithelial cells of low and wide previllous ridge of the 10-day chick embryo cultured in CB-added medium for 2 h. There were no prominent changes compared with Fig. 21 except the appearance of long processes and increase of vesicles (arrow). ×9200.

Fig. 23. Scanning electron micrograph of top region of previllous ridge of the 11-day embryonic duodena cultured in CB-added medium (16 μg/ml) for 2 h. Bulges (arrow) having no real microvilli began to appear on the top of previllous ridge at first. ×950.

Fig. 24. Scanning electron micrograph of epithelial surface of the 11-day embryonic duodena cultured in CD-added medium (1 μg/ml) for 2 h. Long processes are very apparent on the slope (s) of higher previllous ridge, valley (v) and low and wide previllous ridge (br) except the cells of the top region (t). ×1550.

Fig. 25. Scanning electron micrograph of duodenal epithelial cells of low and wide previllous ridge of the 11-day chick embryo cultured in the medium containing both colchicine (0.5 μg/ml) and CB (2 μg/ml). Long processes are induced as a whole in this case. ×2900.

Fig. 26. Scanning electron micrograph of duodenal epithelial cells of low and wide previllous ridge of the 11-day chick embryo cultured in the medium containing both colchicine (10 μg/ml) and CB (2 μg/ml). Bulges such as Fig. 23 and a few long processes are observed. ×3300.
epithelial cells having long processes are inhibited or delayed their cell divisions and comprise the base of previllous ridges, while previllous ridges continue developing after folding. These long processes disappeared in sequence from each portion scheduled for the formation of the next previllous ridges. Folding and development of previllous ridge repeat along the length of the intestine. These suppositions seemed to be supported as follows: (1) Overton & Shoup (1964) reported that the first increase of cell division in intestinal mucosa was observed in 9- to 11-day-old chick embryo. This agrees with the period for the induction of long processes by CB. Burgess (1975) reported that the first folding happened before the first peak of cell division and cell division is not necessary for folding.

(2) Tsukita & Ishikawa (1984) reported that G-actin was polymerized into bidirectional filaments in the presence of single-layered erythrocyte membranes and CB. It is known that intracellular microfilaments such as terminal web, apical microfilamentous bundles or contractile circle do not show constant distribution and arrangement or form, but these change with time and according to their functions (Lazarides & Weber, 1974 and Taylor & Wang, 1980). In this study, it seemed that actin materials such as G-actin scheduled for any intracellular microfilaments was used as a component of the core filaments of long processes. It is unlikely that terminal web serves as an origin for the core filaments since they begin to appear in the approx. 12-day-old embryonic intestine (Burgess & Grey, 1974). In the experiment using CB apical microfilamentous bundles did not always disrupt, but formation or development of previllous ridges was inhibited and many long processes were induced by CB. Therefore formation of core filaments of long processes and the inhibition of formation of previllous ridges by CB cannot be explained by the disruption of microfilamentous bundles alone. The failure of CB to abolish microfilamentous bundles in all cells was reported by Burgess (1975).

The reason why these two results are inconsistent with the reports of the effect of CB on otherwise developing epithelia (Spooner & Wessells, 1972) are unclear. But it may depend on differences between animal species and experimental conditions, especially the effect of changes in concentration. However, there is the possibility that actin materials scheduled for microfilamentous bundles have been used as a component of the core filaments.

The epithelial cells which fail to produce any long processes when treated with CB seemed from their morphological features to be structurally stabilized and differentiated cells and to have no possibility of cell division (Eguchi, 1982). On the other hand the epithelial cells which produce long processes when treated with CB can undergo cell division and CB treatment, seem to influence their proliferation too. As seen in Figs 11 and 12 short treatment of CB (15–30 min) results in protrusion of long processes and great shortening of the period of the contractile circle. For these reasons, it is suggested that actin materials scheduled for the contractile circle rather than the existing contractile circle itself seem to be used as a main component of the core filament of the long processes. There is the possibility that this also occurs during normal development.
From the 13th till 19th day long processes were not induced by CB in spite of an increase in cell numbers. The following is suggested as a reason. In the case of the 13-day embryo, almost all previllous ridges have finished their development and these previllous ridges seem to be occupied by structurally stabilized cells. During the period from 13 or 14 days to 19 days, the constituents of the cellular milieu increase in concentration and complexity, and this seems to have an inhibitory effect on the induction of long processes by CB. For example Kalliecharan & Hall (1974) reported a peak in the corticosteroids assay in the plasma between 15 and 19 days. Alkaline phosphatase and maltase activities also appeared and began to increase in that period (Noda, 1979). In the same period the terminal web appeared and remained visible. This idea seems to be supported by the fact that short treatment did not induce long processes possibly because the cellular milieu was not changed yet, and that prolonged treatment induced long processes presumably because the cellular milieu had undergone a change as dedifferentiation occurred. Before the 8th day, actin materials or their regulating substances in the cytoplasm are apparently immature.

It is well known that cytoskeleton materials such as actin or tubulin etc contribute to early morphogenesis, cell division and maintenance of cell and tissue shape etc. However, the detailed mechanism is not clear. These experiments suggest that the production of long processes may be one of the causes of the formation of previllous ridges and that cellular differentiation and sensitivities to CB depend on the intracellular status of actin materials. However, it is not clear what kind of factor controls the appearance of long processes. It may be cellular pressure by contraction of microfilamentous bundles or the existence of substances such as CB, CD or colchicine in this study or the influence of mesenchyme (Burgess, 1975). Furthermore the possibility must be entertained that the existence of actin-binding proteins is intimately related to the existing state of the actin substances.

Concerning future work, CB, CD or colchicine seem to be very useful and interesting tools to clarify the mechanisms of morphogenesis of the previllous ridges in the chick embryonic duodena under appropriate conditions. The use of immunological techniques is necessary to observe in detail the existing status of cytoskeletons and their regulating proteins.

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