Inverse Effects of Colchicine on the Induction of Long
Microvillous-like Processes by Cytochalasin B in Cultured
Chick Embryonic Duodena

Setsuko NODA

Department of Anatomy, School of Medicine,
Tokai University
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Previously, the author reported that long microvillous-like processes (long processes) containing core actin filaments seemed to be related to the formation of previllous ridges in the chick embryonic duodenum and that similar long processes were induced by cytochalasin B (CB) treatment (8,9,10). The appearance of long processes by CB (polymerization of core actin filaments) seemed to be related to the ability of cell proliferation (10).

In this study, the effect of colchicine on the induction of long processes by CB was examined in 11-day-old chick embryonic duodena in the culture system. The results were as follows: (1) The induction of long processes by CB was inversely influenced by the concentration of colchicine; (2) the appearance of long processes by CB was differently influenced by local parts of the epithelial cells in the presence of colchicine; and (3) alteration of the arrangement of intracellular actin elicited by CB and colchicine was confirmed by immunohistochemistry using actin antiserum.

It was also suggested that sudden intracellular alterations elicited by higher concentrations of colchicine may act as a trigger of cell division differing from normal conditions.

(Key Words: Chick embryonic duodenum, cell division, long microvillus-like processes, cytochalasin B, colchicine.)

INTRODUCTION

Since the report of Hilton (4) it has been well known that the villi of the intestinal epithelium in the chicken are derived from previllous ridges. Noda (9) reported previously that 9- to 11-day-old chick embryonic duodenal confined surface had long processes containing core actin filaments and suggested that these long processes are one of the important factors in the morphogenesis of previllous ridges in addition to the microfilamentous bundles by suggested Burgess (1).

Recently, Noda observed that similar long processes were induced on the surface of the epithelial cells by CB and that of this time formation of new previllous ridges was inhibited. This effect of CB was limited to between 8 and 12 days of incubation, and the epithelial cells producing long processes in the presence of CB seemed to be structurally unstabilized and undifferentiated cells and to have the possibility of cell division. In addition, this effect of CB was inhibited by doses of from 2 to 10 μg/ml of colchicine concentration in the culture system (10).

CB has been used in the investigation of morphogenesis, cell movements and others items ever since reports about its complicated effects appeared (2,5). Interestingly, colchicine is reported to have inverse effects not only as an inhibitor of karyokinesis, but also as promoter of cell proliferation (3,11).

These facts suggested some interaction between CB (actin) and colchicine (tubulin) in the induction of long processes. During a subsequent investigation of the interaction between CB and colchicine, the author found mainly by
scanning electron microscopy that the induction of long processes by CB was influenced differently according to the concentrations of colchicine and local parts of the epithelial cells. This paper describes the inverse effects of colchicine on the induction of long processes by CB and discusses the interaction between the production of long processes and cell division.

MATERIALS AND METHODS

Embryos and organ culture:
White leghorn eggs were incubated at 37.5°C and 60-70% relative humidity for 11 days. The proximal ends of the duodenal loops were used for this study. The excised duodena were cultured as described previously (10). CB was dissolved in dimethylsulphoxide (DMSO) and added to the culture medium to achieve a final concentration of 2 μg/ml which induced many long processes over the epithelial cell sheets. As a control, and equal volume of DMSO solution was added to the culture medium. Colchicine was added to CB-added or DMSO-added media, respectively, to achieve final concentrations of 0.5, 1, 2, 4, 10, 30, 60, 90, 120 and 180 μg/ml. All fragments were cultured for 2h.

Scanning electron microscopy:
All preparations were fixed in 2.5% glutaraldehyde buffered in 0.1M phosphate buffer, pH 7.2 for 1-2h at 4°C, and postfixed in 1% OsO₄. 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 vapor coater (Eiko IB-3) and examined under a scanning electron microscope (SEM) (JSM-35).

Immunohistochemistry of actin:
The antiserum of actin used in the present study was produced by Dr. Noboru Yamamoto (Kitasato University). This was raised in rabbits by injection of pure actin from chicken gizzard and has been used in several immunohistochemical studies (13,14). Fluoresceiniso-thiocyanate (FITC)-labeled goat anti-rabbit IgG, FITC-labeled rabbit anti-sheep IgG and rabbit anti-chicken IgG were obtained from Miles Yeda, Kiryat Weizmann, Rohovot, Israel. Normal swine serum was one of the Dako PAP kit of reagents purchased from Dako Corporation, Santa Barbara, California, USA.

All preparations were fixed in 4% paraformaldehyde solution, pH 7.2, for 2h at 4°C, dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Sections (2μm) on glass slides were deparaffinized and indirect immunofluorescent staining was then carried out (12). The preparations were exposed to normal swine serum for 30min and then to actin-antiserum diluted 1/1,000 overnight at room temperature. FITC-labeled goat anti-rabbit IgG was then applied at a dilution of 1/16 for 30min at room temperature. The preparations were rinsed, hydrated, mounted in entellan and examined under a Zeiss fluorescence microscope.

As controls, sections were exposed to rabbit anti-chick IgG instead of primary antigen, and the sections were exposed to FITC-labeled rabbit anti-sheep IgG instead of FITC-labeled goat anti-rabbit IgG. As a result, specific reactions were confirmed in this study.

At the same time, to investigate cell division, sections were immunostained by the Dako PAP kit method and counterstained with Mayer's hematoxylin or stained with Hematoxylin and eosin.

RESULTS

Scanning electron microscopic studies:
In the presence of both CB and colchicine the induction of long processes by CB was influenced by the concentration of colchicine as shown Table 1 and Fig.1. When the concentration of colchicine was 0.5 or 1 μg/ml, the induction of long processes was observed in the same way as in the case of 2 μg/ml of CB alone (Fig. 1a) (a dose of 1 μg/ml gradually decreased the induction), but doses of colchicine from 2 to 30 μg/ml greatly decreased the production of long processes (Fig. 1b). Again long processes were induced at a concentration of 60-120 μg/ml (Fig. 1c) and obviously decreased in number at 180 μg/ml (Fig. 1d).

The inducitonal localization of long processes too showed two patterns according to the concentration of colchicine. In the case of 0.5,1 and 60 μg/ml, localization of the induction was the same as that of CB alone as shown next. The induction of long processes was observed.
Table 1  Effects of colchicine on the induction of long microvillous-like processes by cytochalasin B in cultured chick embryonic duodenae.

<table>
<thead>
<tr>
<th>µg of colchicine/ml medium containing 2µg/ml CB</th>
<th>effects of colchicine</th>
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<tr>
<td>0, 0.5</td>
<td>++ ~ +++</td>
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<tr>
<td>1</td>
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<td>2, 4, 10, 30</td>
<td>0 ~ +</td>
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<tr>
<td>60, 90</td>
<td>+ ~ ++</td>
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<tr>
<td>120</td>
<td>++ ~ ++</td>
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<tr>
<td>180</td>
<td>0 ~ +</td>
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Long processes by CB were absent (0), slight (+), moderate (+++) or marked (+++).

Fig. 1 Scanning electron micrographs on the effects of colchicine on the induction of long processes by CB. The luminal surfaces of the duodenal fragments were cultured in the following media: (a) 2 µg/ml of CB alone, (b) 10 µg/ml of colchicine + CB, (c) 120 µg/ml of colchicine + CB and (d) 180 µg/ml of colchicine + CB. (lr): Low and wide previllous ridge. (hr): Higher previllous ridge. (a,b,c,d) × 1,350
on the whole surfaces of low and wide prevailous ridges, and the whole slopes and feet of higher and slender ridges. The top or upper slopes of higher and slender ridges did not produce long processes (10). On the other hand, in the case of 90-120 µg/ml, long processes also frequently appeared on the top and upper slopes of higher and slender ridges in addition to the parts shown in Fig. 1a.

The SEM image of the groups from 2 to 30 µg/ml in Fig. 1b was similar to the normal image. However, at the higher colchicine concentrations, the appearance of bulges was gradually observed on the surface of epithelial cells (10). In the 180 µg/ml group especially, cellular borders became unclear and the tissues became flattened.

In the case of colchicine + DMSO, all concentrations failed to induce long processes and the pictures were similar to Fig. 1b, but the more the concentration of colchicine increased, the more the tissues became flat in the same way as in the case of both CB and colchicine treatment.

**Immunoreaction of actin: Epithelial cells:**
In the epithelial cells after both DMSO and colchicine treatment (control groups), actin immunoreactive portions were seen on the apical and lateral surfaces of epithelial cells as shown Fig. 2a although they were not so strongly immunostained. This result was the same as that of epithelial cells producing no long processes due to both CB and colchicine treatment. On the other hand, in the epithelial cell sheets induced to produce long processes by both CB and colchicine treatment, the apical surfaces were strongly and widely immunostained as shown in Fig. 2b. In this case, actin of core microfilaments in the long processes induced by CB seemed to be immunostained.

**Mesenchymal layer:**
Actin positive dots were often observed in the cells of specimens cultured in medium containing CB and colchicine. In addition, morphological alterations such as obvious relaxation were observed in the same experimental groups (Fig. 2a and 2b).

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**Fig. 2** Immunostained micrographs with antiserum of actin. (a,b) Immunofluorescent staining patterns of the fragments cultured in 120 µg/ml of colchicine + DMSO(a) and 120 µg/ml of colchicine + CB(b). ×240 (c) Immunostaining pattern of the fragment cultured in 120 µg/ml of colchicine + CB, counterstained with hematoxylin. × 500 (ls): Luminal surface. (†): Pictures of cell division.
Muscle layer:
The muscle layer was strongly immunostained in all specimens (Fig. 2a and 2b).

Cell division:
Many epithelial cells showing blockage of nuclear division elicited by colchicine treatment were observed in all groups containing colchicine as shown in Fig. 2c.

In addition, in the 180μg/ml group, histological damage such as cellular shrinkage or expansion and an increase of vacuoles in the cells, was marked.

DISCUSSION
In this study, it was clarified that the induction of long processes (polymerization of core actin microfilaments) by CB and its inductive localization were influenced by the concentration of colchicine as shown in Table 1 and Figs. 1 and 2b. It was suggested that the interaction between actin and tubulin in cells was close and complicated.

In a recent study, the author compared epithelial cells of the upper and top cells of higher ridges (epithelial cells producing no long processes in the presence of CB) with epithelial cells of low and wide previllous ridges and under slopes and feet of higher ridges (epithelial cells induced to produce long processes by CB) on transmissional electron micrographs (10).

Some morphological differences were recognized between the two sets of cells such as the direction of the intracellular organelles, cellular interdigitation, size and form. It seemed that these differences depend mainly on the differences in intracellular arrangement or amount of actin and tubulin and also on the difference in the ability of cell division (10).

In addition, it is known that colchicine inhibits the polymerization of tubulin or depolymerizes microtubules and that the degrees of inhibition or depolymerization differ according to the kinds of cells, local parts of microtubules in the cells (kinds of microtubules) and the concentration of colchicine (6).

In this study too, all concentrations of colchicine seemed to block the nuclear division of the proliferating cells (Fig. 2c), but the effects of colchicine on the other cells (non-proliferating cells) clearly differed according to cellular localizations on the previllous ridges and concentration of colchicine.

At colchicine concentrations of 2 to 30 μg/ml, cytoplasmic tubulin scheduled for mitotic spindles may be affected at first (6). The resulting cellular milieu seemed to result in the disappearance of the ability of cell division, so that the induction of long processes (formation of core microfilaments) by CB also seemed to be inhibited.

At a concentration of 0.5 μg/ml, alteration of tubulin or microtubule arrangements in the cells seemed too slight to inhibit the production of long processes by CB.

Furthermore, it seemed that higher concentrations of colchicine (60-120 μg/ml) affected various kinds of microtubules in the epithelial cells and resulted in a great deal of intracellular tubulin. This large amount of tubulin seemed to act as an initial signal of cellular proliferation as shown in the reports by Crosin et al (5) and Otto et al (11). As a result, cells with the possibility of cell division seemed to produce long processes in the presence of CB unlike under normal condition.

In this study, the production of long processes by CB were observed not only on the surfaces of the low and wide ridges and under slopes and feet of higher ridges, but also on the surfaces of the top and upper slopes of higher ridges at concentrations of 90-120 μg/ml.

In the case of colchicine concentrations of 90-120 μg/ml, even morphologically supported microtubules of the structurally stabilized cells seemed to be depolymerized, so that such structural stabilized epithelial cells with no ability to proliferate in the normal developmental status, seemed to have sufficient tubulin to trigger cell division. However, details of this new ability of cell division were not clear.

At a colchicine concentration of 180μg/ml, the failure of induction of long processes and the remarkable damage to tissue seemed to be related to extreme depolymerization of microtubules for morpholaxis or secondary membranous alteration (7).

On the other hand, the refractory behavior of organs to colchicine in this study showed a stronger tendency than sensibility in the case of cell culture (6).

This study seemed to reconfirm the results of my previous suggestion that the appearance of long processes was related to the possibility
of cell proliferation in epithelial cells of previllous ridges of chick embryonic duodenum. In addition, CB and colchicine were reappraised as complicated but useful and interesting tools to clarify the mechanism of morphogenesis or mechanism of cell division under appropriate conditions.

In the future, immunostaining using antiserum of tubulin, detailed transmissional electron microscopic observations and quantitative research on cell division are necessary to study the interactions between actin and tubulin with respect to the ability of cell division and production of long processes as suggested in this study.

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REFERENCES