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# THREE-DIMENSIONAL CONFIGURATION OF CRYPTS OF DIFFERENT TYPES OF COLORECTAL ADENOMAS

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## Abstract

The three-dimensional configuration of isolated crypts of normal human colonic mucosa and colorectal adenomas was examined by scanning electron microscopy. For isolation of the crypts, the digestion method with HCl was used for formalin fixed tissues, and the separation method with ethylenediaminetetraacetic acid (EDTA) following ultrasonication was applied to fresh tissues. In a comparative study, the NaOH cell-maceration method, which visualized the sub-basal laminal collagen sheath, was applied. The isolated crypts from the normal colon were visualized as a single straight tubule resembling a test tube. Most isolated crypts of the tubular adenomas were visualized as elongated fan-like structures with several protuberances and a few short branchings. Their average length was more than twice that of the normal colonic mucosa crypts. Most crypts of the villous adenomas were visualized as slender tubules without protuberances and short branchings, and their average length was three times that of the tubular adenoma crypts. Most crypts of the tubulovillous adenomas were long and triangular with several longitudinal folds and protuberances, and the average length was about three times that of the tubular adenoma crypts.

**Key Words**: Scanning electron microscopy, colonic adenoma, crypt isolation, tissue digestion, ethylenediaminetetraacetic acid (EDTA), three-dimension.

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## Introduction

Human colorectal adenomas are classified into three types: tubular, villous and tubulovillous. The three-dimensional structure of these adenomas has been studied by graphic reconstruction (Wiebecke *et al.*, 1974), but there has been no direct observation of the three-dimensional structure of the crypts of these adenomas.

Recently, Ohtani (1987) visualized the three-dimensional structure of the sub-basal laminal collagen sheath of the pancreas with a cell-maceration method which employs NaOH at low temperature. The three-dimensional structure of the sub-basal laminar collagen sheath of the hyperplastic and tubular adenoma has also been observed by this method (Furuya and Ogata, 1993). However, by observation of the cut surface of the adenoma, it is difficult to clarify in detail the three-dimensional structure of the crypts. Recently, Mitsushima and Inoue (1993) successfully isolated rat gastric glands with a 6N NaOH solution and observed these by scanning electron microscopy (SEM). Using ethylenediaminetetraacetic acid (EDTA), Satoh *et al.* (1985) observed isolated mucosa of small intestine in rat. In the present study, using the HCl-digestion method for formalin fixed tissues and the EDTA-ultrasonication method for fresh tissues, the crypts of the normal colon and the adenomas were isolated and their three-dimensional structure was observed by SEM. In a comparative study, the NaOH cell-maceration method of Ohtani (1987) was also applied to these tissues for observation by SEM.

## Materials and Methods

The materials were 14 normal human colonic mucosae, 26 tubular, 5 villous and 28 tubulovillous adenomas obtained at endoscopic polypectomy or at surgery. Adenomas with low- or moderate-grade dysplasia were used, while those with high-grade dysplasia as well as those of familiar polyposis, were excluded from this study.

For isolation of the crypts, the following two methods were used. In the HCl-digestion (HCl) method, tissues fixed with 3% formaldehyde in 0.01 phosphate

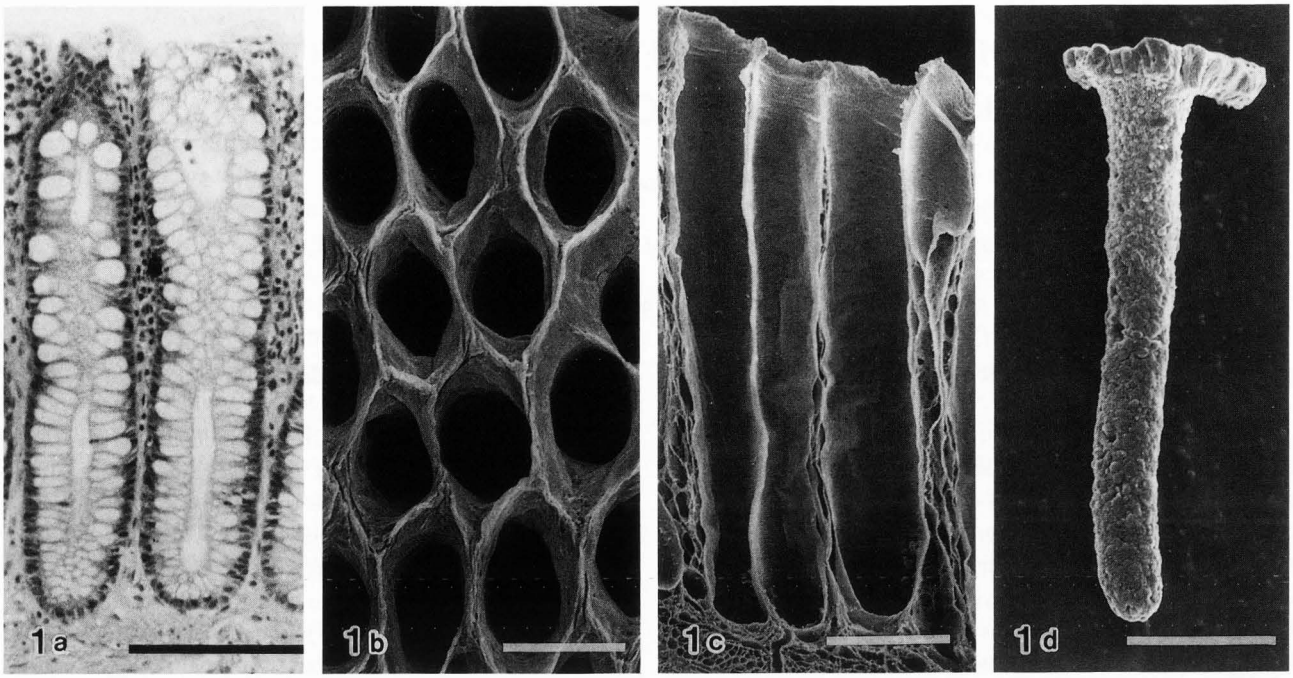


**Figure 1**. Micrographs of normal colon. (**a**) H-E stained section. A crypt of Lieberkühn is seen as a single tubule. (**b**) Scanning electron micrograph of a specimen treated with the cell-maceration method. The sub-basal laminal collagen sheath corresponding to the orifices of the crypts is round and shows regular arrangement. (**c**) A longitudinally-cut section of the cell-macerated specimen. The sub-basal laminal collagen sheath of the crypts consists of straight tubular concavities arranged in parallel. (**d**) An isolated crypt obtained by the HCl method shows a test tube-like structure without branching. Bars = 100 $\mu$m.

buffer (pH 7.4) for several months were used. The tissues were sliced at 3 mm thickness, then further fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours, and washed with phosphate buffer. The specimens were then placed in 8N HCl at 60°C for 60 to 90 minutes, and then manually agitated for 30 minutes in the same solution. They were treated for 30 seconds at 26 kHz in an ultrasonicator (UO 150 FS, Kokusai Electric Co., Tokyo, Japan), and then rinsed three times in phosphate buffer.

In the EDTA ultrasonication (EDTA) method, the fresh tissue was sliced into 2-3 mm thick specimens. The tissues were immersed in 30 mM EDTA solution in 0.1 M phosphate buffer for 30 minutes at 37°C. They were then ultrasonicated in the same EDTA solution at 26 kHz for 3 minutes. The specimens were cut perpendicularly to the luminal surface with a razor blade at 1 to 2 mm in width and then transferred into a test tube containing 0.1 M phosphate buffer, which was vigorously manually shaken to separate the crypts. The separated crypts were transferred into another test tube, were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours and then washed with phosphate buffer solution.

For observation of the sub-basal laminal collagen tissue, cellular components were removed by the NaOH cell-maceration (cell-maceration) method (Ohtani, 1987). In brief, formaldehyde fixed tissues were cut into small blocks (5 x 5 x 4 mm), and then further fixed with 2% glutaraldehyde in cacodylate buffer (pH 7.4) for 2 hours followed by immersion in 10% aqueous solution of NaOH at 20° C for 5 to 8 days. The tissues were washed several times with distilled water for 24 hours.

All specimens treated by the above three methods were immersed in 2% tannic acid solution for 1 hour and post-osmicated in 1% osmium tetroxide solution for 2 hours. They were dehydrated in a graded ethanol series, followed by replacement with t-butyl alcohol. They were then dried by the t-butyl alcohol freeze-dry method (Inoue and Osatake, 1988) in an evacuator (VFD-20, Vacuum Device, Ibaraki, Japan). Some of the dried specimens treated with the cell-maceration method were serially sliced to 200 $\mu$m in thick slices with a microslicer (DTK-1000, Dosaka EM, Kyoto, Japan). The isolated crypts and cell-macerated tissues were mounted on brass stubs with double stick tape. They were coated with gold in an ion-coater (IB-5, Eiko, Ibaraki, Japan) and observed with an SEM (S-430,

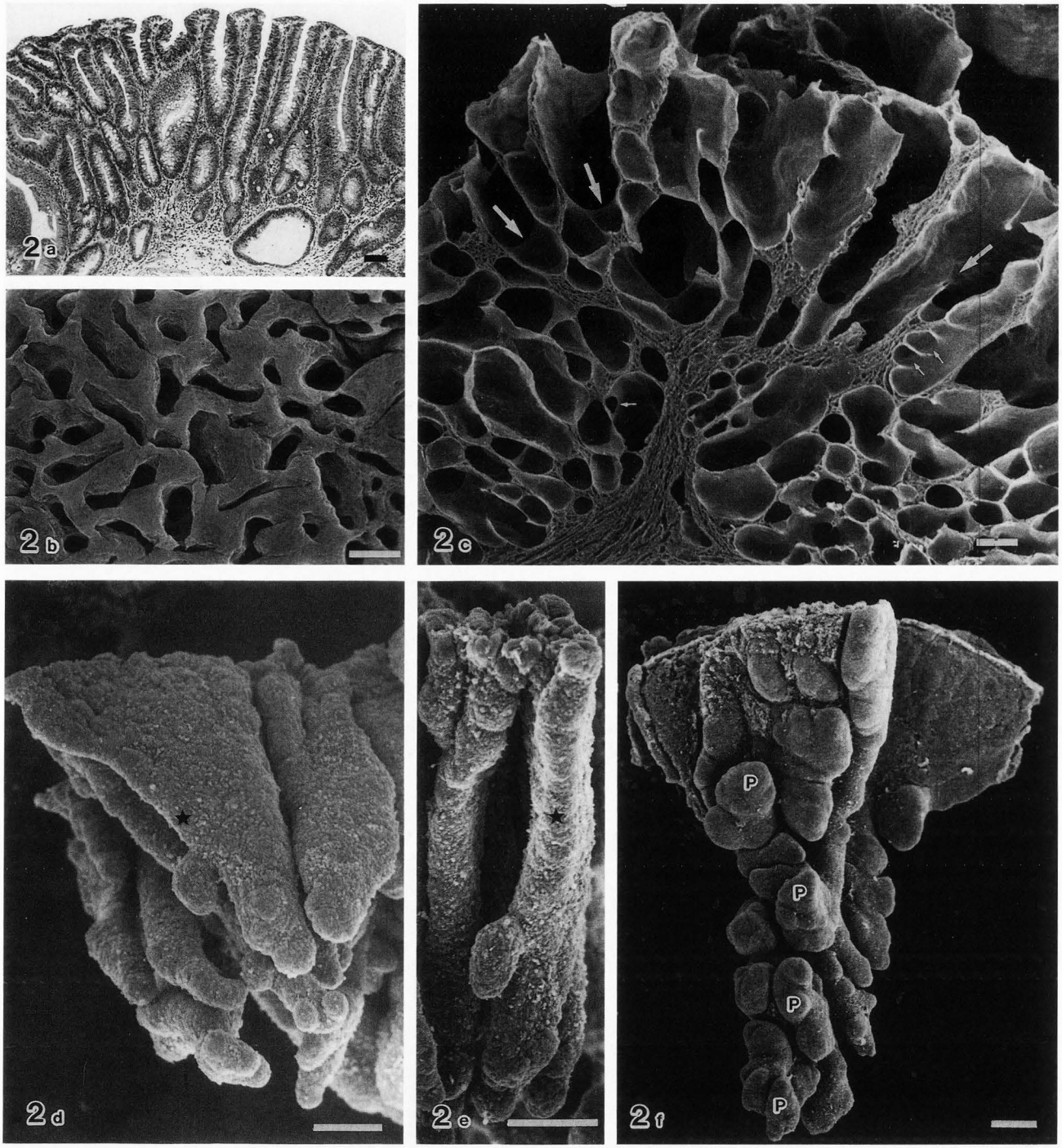


**Figure 2.** Micrographs of a tubular adenoma. (**a**) H-E stained section. Adenomatous crypts are elongated or short with tubular-shape. Some crypts have branchings or cystic dilatations. (**b**) The luminal surface of a cell-macerated specimen. Elongated and irregularly shaped crypt orifices are seen. (**c**) The longitudinally-cut section of the same specimen. Tubular or hemispherical concavities are seen. Note that the concavities widen on the luminal side. Branchings (large arrows) and small concavities (small arrows) are occasionally seen. (**d**) A cluster of crypts obtained by the EDTA method; the crypts appear to have the shape of an inverted triangle. (**e**) The 90° transversely rotated view of the crypts shown in Figure 2d. The triangular crypt indicated by a star in Figure 2d shows a plate-like configuration (star). (**f**) An isolated crypt obtained by the HCl method; this crypt has numerous protuberances (P). Bars = 100 $\mu$m.

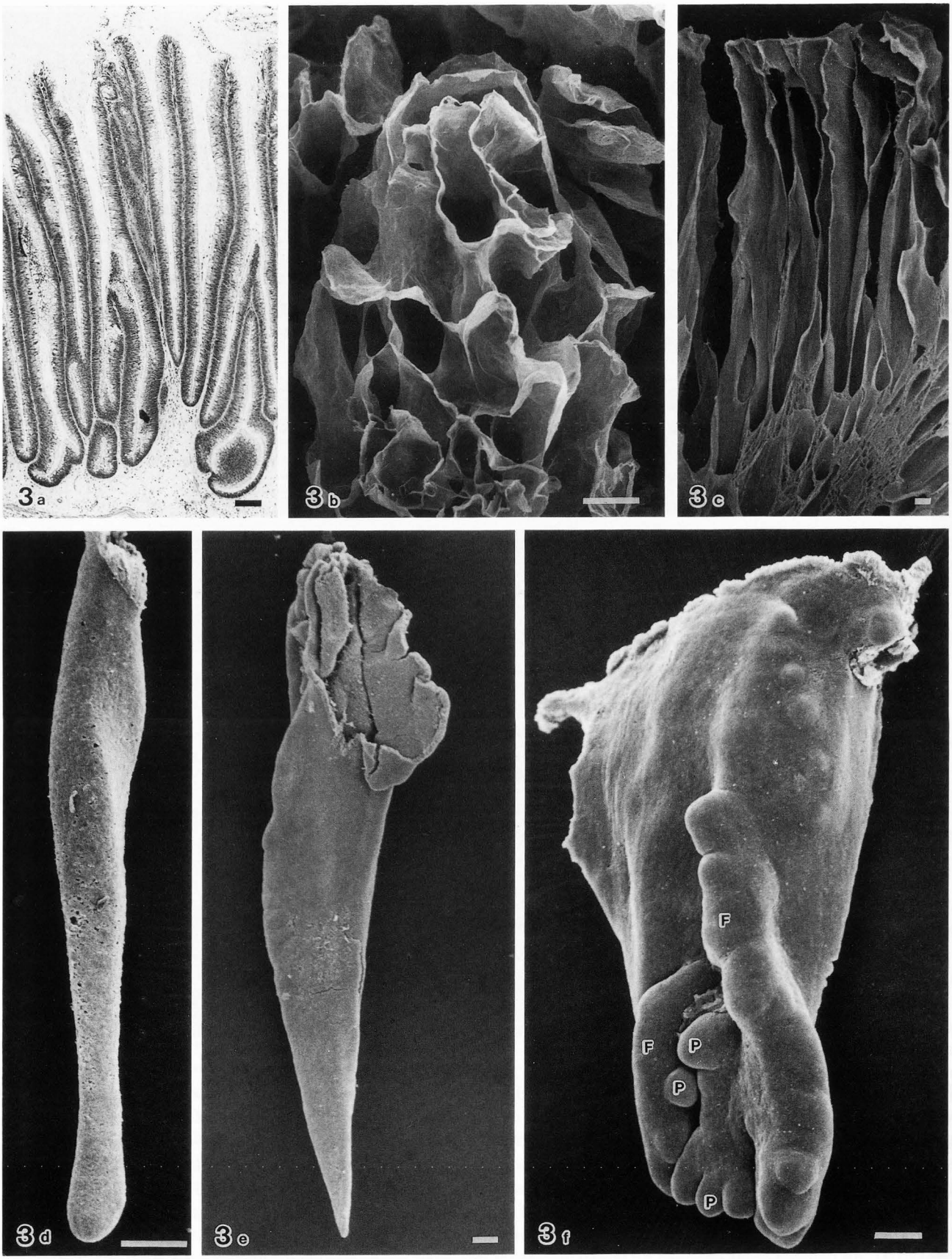
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**Figure 3 (on the facing page).** Micrographs of villous adenoma. (**a**) H-E stained specimen. (**b**) The luminal surface of a cell-macerated specimen; irregularly arranged polygonal orifices are seen. (**c**) The longitudinally-cut section; very long straight tubular concavities with parallel arrangement are seen. (**d**) An isolated crypt (HCl method) presents simple slender tube-like appearance. (**e** and **f**) Isolated crypts (HCl method). (**e**) The upper part is widened while the basal part is gradually tapered. (**f**) Crypt with several protuberances (P) and folds (F). Bars = 100 $\mu$m.

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Hitachi, Japan) operated at an accelerating voltage of 15 kV.

For light microscopic observation, a 2 mm slice from near the center of each adenoma was embedded in paraffin and cut into 4 $\mu$m sections, which were stained with hematoxylin and eosin.

## Results

### Isolated crypts

With the HCl method, single crypts were isolated (Figs. 1d, 2f, 3d-3f, 4e and 4f), while with the EDTA method, single crypts (Fig. 4d) as well as clusters of crypts (Figs. 2d and 2e) were obtained. Tiny fragments remained on the surface of the specimens prepared by the EDTA method.

### Normal colonic mucosa

The histological specimens of normal human colonic crypts were visualized as a straight tubular gland (Fig. 1a). In the cell-maceration specimens, the epithelial cells were removed and the sub-basal laminal collagen sheath was disclosed. The luminal surface presented regularly arranged round holes corresponding to the crypt orifices (Fig. 1b). In longitudinally-cut sections, the sub-basal laminal collagen sheath of the crypts formed straight tubular concavities with parallel arrangement (Fig. 1c).

The individual isolated crypt was observed as a single straight tubule resembling a test tube (Fig. 1d). The average length of the isolated crypts was 344 $\pm$ 34 $\mu$m (mean $\pm$ standard deviation, SD), and the average maximum diameter was 52 $\pm$ 5 $\mu$m (mean $\pm$ SD, n = 54).

### Tubular adenoma

Light microscopy of the tubular adenomas revealed tubules with either a regular, well-differentiated structure or considerable irregularity with some branchings (Fig. 2a). In the specimens treated with the cell-maceration method, oval or elongated orifices were irregularly arranged on the luminal surface of the adenoma (Fig. 2b). On the surface cut longitudinally to the long axis of the adenoma, the sub-basal laminal collagen sheath of the crypts presented elongated hemi-ovoid or tubular concavities which occasionally showed branchings or plicae (Fig. 2c).

Isolated crypts usually appeared as elongated fan-like configurations (Fig. 2d and 2f). Most crypts had several small protuberances and a few short branchings (Fig. 2d), but a few of them had many protuberances (Fig. 2f). Most branchings were short, but long ones were occasionally seen. The average length of the crypts was 730 $\pm$ 250 $\mu$m and the average maximum width was 350 $\pm$ 240 $\mu$m (mean $\pm$ SD, n = 65).

### Villous adenoma

Light microscopically, the villous adenomas consisted of finger-like processes (Fig. 3a). In the cell-macerated specimens, irregularly arranged polygonal crypt orifices were seen on the luminal surface (Fig. 3b). On the longitudinally-cut surface, slender tubular concavities arranged in parallel were seen (Fig. 3c).

Most isolated crypts were visualized as a single slender tubule without protuberance or branching (Figs. 3d and 3e), but a few of them had several protuberances and longitudinal folds (Fig. 3f). In most crypts, the luminal side was extended and the basal side tapered (Fig. 3e) or slightly swollen (Figs. 3d and 3f). The average length of isolated crypts was 2,100 $\pm$ 1,070 $\mu$m and the average maximum width 370 $\pm$ 300 $\mu$m (mean $\pm$ SD, n = 43).

### Tubulovillous adenoma

Crypts of the typical tubulovillous adenomas showed long branching tubules intermingled with short tubules or broad and stunted villi (Fig. 4a). On the luminal surface of the cell-macerated specimens, the branched and distorted orifices of crypts were seen (Fig. 4b). On the longitudinally cut surface, the crypts presented as irregular tubular concavities with widened luminal side and tapered basal side (Fig. 4c). Small concavities bounded by plicae were seen in places (Fig. 4c).

Most isolated crypts were long and triangular with several longitudinal folds and protuberances (Figs. 4d-4f). However, a few of them were short and fan-like and resembled the isolated crypts from the typical tubular adenomas, while a few others were long tubules similar to the isolated crypts from the typical villous adenomas. The average length of the isolated crypts was 2,500 $\pm$ 680 $\mu$m and the average maximum diameter 1,050 $\pm$ 210 $\mu$m (mean $\pm$ SD, n = 32).

There was a statistical difference between each normal and three types of adenoma in both of their crypt length and diameter ($p < 0.001$, Student's t-test), except between the crypt length of villous and tubulovillous.

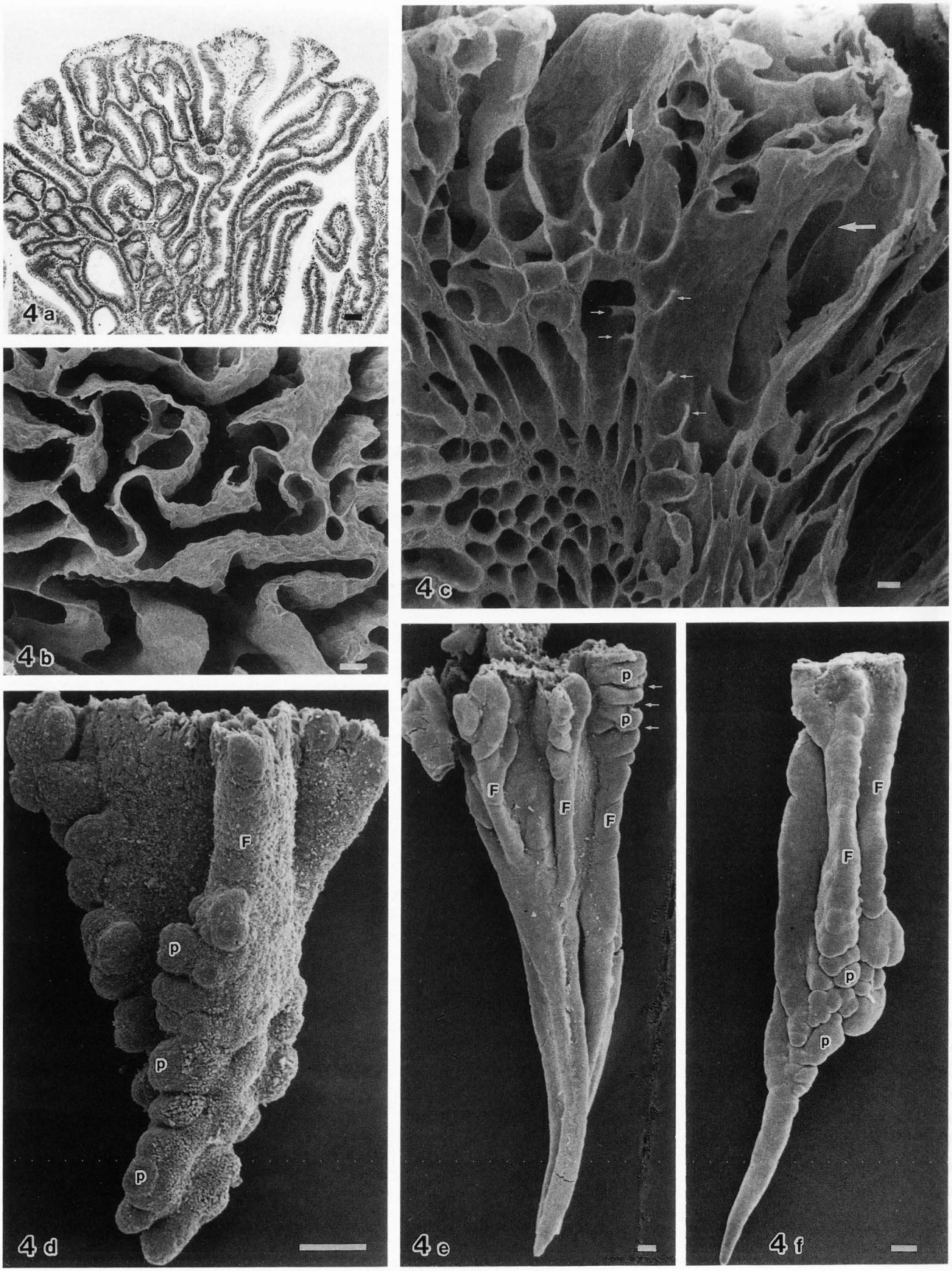
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**Figure 4 (on the facing page).** Micrographs of tubulovillous adenoma. (**a**) H-E stained specimen. (**b**) Luminal surface (cell-macerated specimen); the orifices of crypts show distortion and branching. (**c**) The longitudinally-cut section; long tubular concavities with some branchings (large arrows) and plicae (small arrows) are seen. (**d**) An isolated crypt (EDTA method) shows a longitudinal fold (F) with numerous protuberances (P). (**e** and **f**) Isolated crypts (HCl method). (**e**) The luminal side of the crypt is widened, while its basal side is tapered; F: longitudinal folds, P: small protuberances. (**f**) Note the longitudinal folds (F) with multiple protuberances (P) at the middle portion. Bars = 100 $\mu$m.

## Discussion

Using the 6N NaOH digestion method, Mitsushima and Inoue (1993) successfully isolated rat gastric glands and observed them by SEM. When we first applied this method to human colonic tissues, the well preserved crypts were partially damaged and could therefore not be successfully isolated. We then used two other methods for the isolation of the crypts from human adenomas. Evan *et al.* (1976) introduced the HCl and collagenase method to remove connective tissue elements. Later studies (Fujiwara and Uehara, 1984; Nakamura and Ymamoto, 1988) revealed that HCl alone was sufficient for this purpose. Adding manual agitation and ultrasonication during HCl digestion procedure, we successfully isolated well preserved crypts from the human colonic tissues. This method can be applied to specimens routinely preserved in 3% formaldehyde solution. Using 30 mM EDTA, Bjerknes and Cheng (1981) isolated intact intestinal epithelium from the mouse intestine, Satoh *et al.* (1985) also isolated mucosa of rat small intestine utilizing EDTA. We added the ultrasonication procedure after the EDTA treatment and successfully isolated the individual crypts and clusters of several crypts. The three-dimensional structures of the isolated crypts obtained by these two methods were basically similar. The advantage of the crypt isolation is that it allows direct observation on branchings, foldings and protrusions which are difficult to observe on the cut surface by SEM or histological methods.

In light microscope specimens, the normal colonic mucosal crypts were straight tubular glands, and the isolated crypts were test-tube shaped. This configuration coincides well with the light microscopic images.

Colorectal adenomas are classified into three types: tubular, villous, and tubulovillous adenoma (Morson and Dawson, 1979; Fenoglio-Preiser *et al.*, 1989). The histological features of tubular adenoma show that the original configuration of the crypts is maintained, but they may be irregular, elongated and branched (Morson and Dawson, 1979; Fenoglio-Preiser *et al.*, 1989). Wiebecke *et al.* (1974) described that the crypts of tubular adenoma are glandular tubes with numerous bulgings and a division of glands. SEM observations on the isolated crypts from various angles show that most crypts are rather flat and triangular and not conical in shape. In addition, numerous protuberances and several short branchings are frequently seen, while long branchings, i.e., those longer than 200 $\mu$m, are rather rare. It is possible that such findings can be established only by SEM observation of isolated crypts.

The isolated crypts of villous adenomas were slender tubular structures or elongated triangular structures with expansion of the luminal side. These crypts were about three times longer than the tubular adenoma crypts. Protuberances or branchings were occasionally seen, but they were much fewer and shorter than those in the tubular adenomas. These findings are in good accordance with the reported histological appearance in which the crypts of villous adenoma are described as foliaceous or finger-like with outward extensions from the surface (Wiebecke *et al.*, 1974).

The classification of tubulovillous adenomas as a separate entity is not completely accepted. Some authors have maintained that tubulovillous adenoma is composed of tubular as well as villous components, each contributing more than 20% to the tumor mass (Jass and Sobin, 1989). According to Fenoglio-Preiser *et al.* (1989), this adenoma is composed of blunt and short villi containing tubular structure. Most crypts isolated from a typical tubulovillous adenoma are longer than those of a tubular adenoma and are of almost the same length as those of a villous adenoma. However, the prominent feature of the crypts of tubulovillous adenoma is more numerous longitudinal folds and protuberances compared with those of a villous adenoma.

Using crypt isolation and cell maceration techniques, we revealed the direct three-dimensional configurations of normal colonic crypts and adenomatous crypts of three histological types of colorectal adenomas. Their configurations were particular to each type of adenoma however, some adenomas admixed the crypts having particularity of each type of adenoma.

## Acknowledgments

The authors are grateful to Mr. Y. Yamasaki for his skillful contribution throughout this project. We also thank Ms. M. Miyata, M. Fukui and M. Inoue for their assistance in preparing the photographs.

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## Discussion with Reviewers

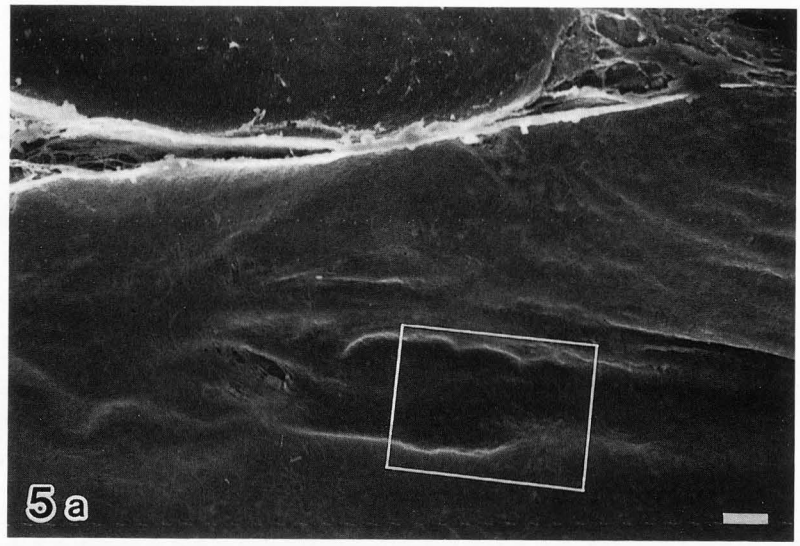
**G.M. Roomans**: In the description of the HCl method, the authors state that "tissues fixed with 3% formaldehyde in 0.01 phosphate buffer (pH 7.4) for several months were used." Do they mean that for this method, tissue has to be fixed for several months, or do they mean that despite the fact that the tissue had been fixed for several months, it could still be used.
**Authors**: We mean the latter. The tissue fixed for several months could still be used for this method. Tissues sufficiently fixed with routine formaldehyde fixation can be prepared by this method.

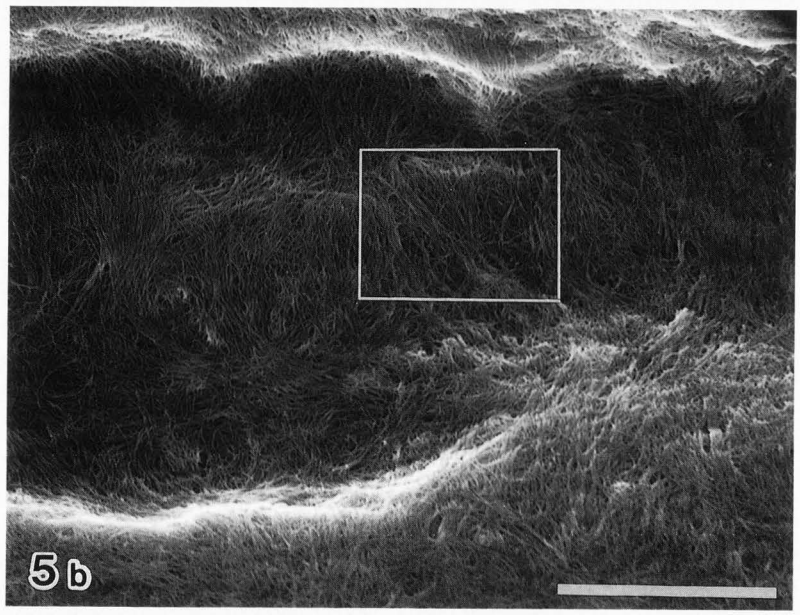
**W.H. Wilborn**: Were the findings consistent for each of the three types of adenomas?
**A.P. Evan**: Do the present observations permit the authors to distinguish three types of colorectal adenomas?
**Authors**: We obtained consistent SEM findings for each adenoma. Therefore, we can distinguish each type of adenoma in almost all cases on scanning electron micrographs. However, in some adenomas, different types of adenoma were mixed. In such cases, it was difficult to distinguish the histological types from the isolated crypts configuration.

**W.H. Wilborn**: Do you plan to use these techniques to reveal the anatomical configurations of other adenomatous tumors?
**Authors**: Yes, we are applying these techniques to the study of adenomas of the stomach and the gallbladder, and colorectal carcinomas. Our impression is that isolation of well preserved crypts is difficult in cancer other than adenoma.

**A.P. Evan**: What is the clinical outcome of the three different types of colorectal adenomas? Is there any relationship between outcome and the three-dimensional configuration of a particular type of adenoma?
**Authors**: We could not find any relationship between clinical outcome and the three-dimensional configuration.

**T. Ushiki**: The authors noted that tiny fragments remained on the EDTA-treated specimens. What are these substances?
**Authors**: Most tiny fragments on the specimens showed a thin tattered sheet-like structure at higher magnifications. We assume that they corresponded to fragmented lamina densa.



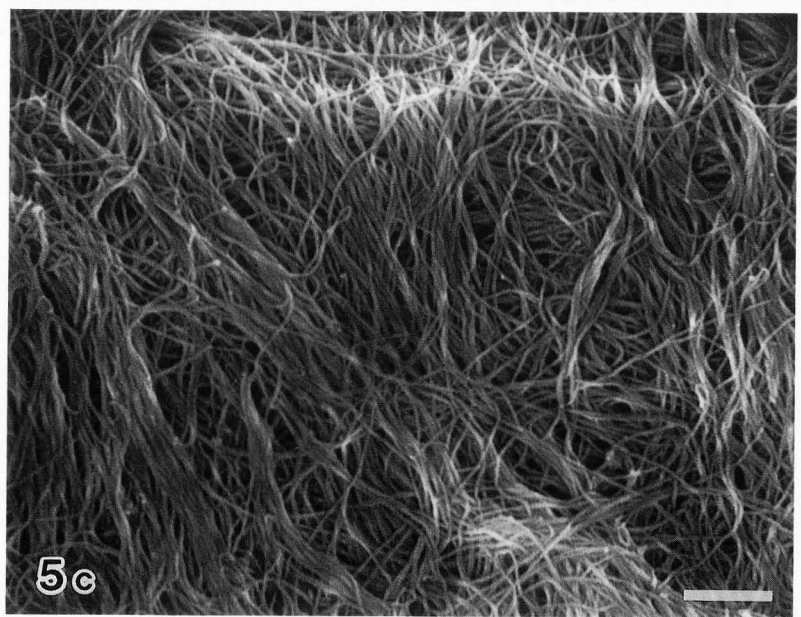




**Figure 5.** Scanning electron micrographs of the surface of cell-maceration specimen. (**a**) At low magnification; bar = 10 $\mu$m. (**b**) The area enclosed by rectangle in Figure 5a has been enlarged; bar = 10 $\mu$m. (**c**) The area enclosed by rectangle in Figure 5b has been further enlarged; bar = 1 $\mu$m.

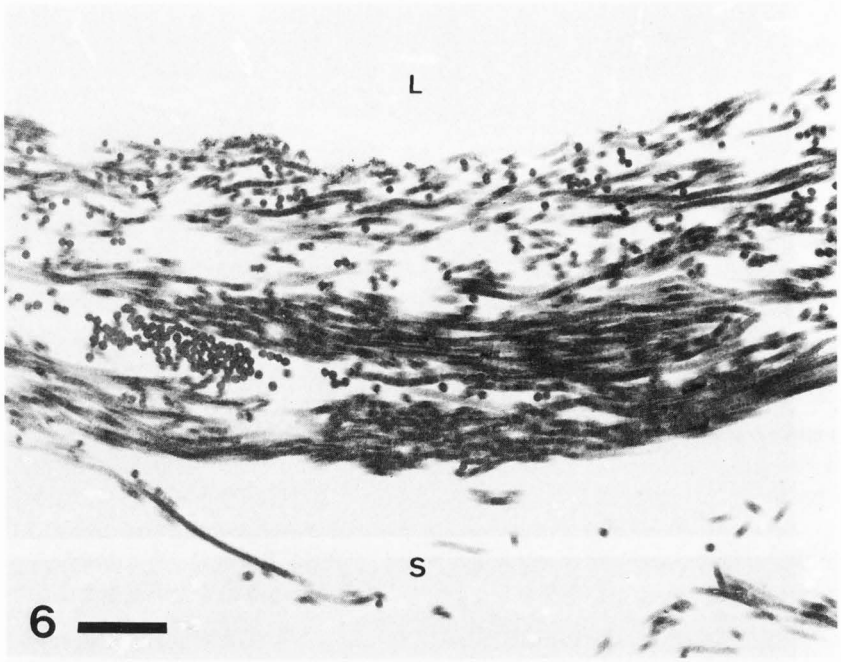



**Figure 6.** A transmission electron micrograph of cell-maceration specimen. Note that the basal lamina is absent on the luminal side surface (L). S: stromal side. Bar = 1 $\mu$m.

**T. Ushiki**: The authors treated the specimens with 8N HCl for a rather wide range of time (i.e., 60-90 minutes). Do they mean that we can obtain constant results within this range?

**Authors**: The suitable time for isolation differed from tissue to tissue. Therefore, we frequently checked the degree of isolation under a dissection microscope during the 8N HCl procedures. For colorectal adenomas, the suitable time ranged from 60 to 90 minutes.

**K.A. Warfel**: The authors repeatedly refer to the appearance of the "sub-basal collagen sheaths," but what the low and intermediate magnification illustrations show is smooth basal lamina and not a fibrillar sub-basal laminal "pericryptic" collagen network. The collagen network requires a much higher magnification to be visualized.

**Authors**: We agree that higher magnification is needed to visualize the collagen network and are including three additional scanning electron micrographs (Figs. 5a-5c) and one additional transmission electron micrograph (Fig. 6). The surface of cell-maceration specimen appears similar to smooth basal lamina at low magnification (Fig. 5a), but at higher magnification it is clear that we are observing the collagen network of the sub-basal laminal collagen sheath (Figs. 5b and 5c). TEM observations on cell-maceration specimen also revealed the absence of the basal lamina (Fig. 6).

**K.A. Warfel**: Normal colon has epithelial cell processes extending into the lamina propria of the luminal surface but not crypts. There are luminal basal lamina fenestrations associated with at least some of these. Alterations in epithelial-connective tissue physical interactions are important in function, including differentiation and cell proliferation, among others. High magnifications illustrations showing the status of these fenestrations (present/absent?) would be a nice addition to the paper.

**Authors**: We are also interested in basal lamina fenestrations reported by Warfel and Hull (1988). We have also studied these structures in normal colon and colorectal adenomas by SEM; parts of the results have already been published (Araki *et al.*, 1992, 1995).

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