Surface Changes in the Descending Colon of Rats Treated with Dimethylhydrazine

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SUMMARY

Male Sprague-Dawley rats were given weekly s.c. injections of 1,2-dimethylhydrazine (21 mg/kg) for periods of up to 20 weeks. The descending colon of treated animals killed at 2 weekly intervals was examined for morphological changes, over a 30-week period, after commencement of treatment using scanning electron microscopy, light microscopy, transmission electron microscopy, and freeze-fracture techniques.

Scanning electron microscopy showed that 1,2-dimethylhydrazine treatment resulted in the progressive replacement of the normal arrangement of epithelial cells covering the luminal surface of intestinal glands with enlarged and irregularly shaped arrangements of epithelial cells, so that the entire mucosa was atypical and disorganized at 30 weeks after commencement of treatment. The changes were not readily observable using other methods of microscopy.

Multiple tumors that were apparently unrelated to sites of specific morphological change erupted into the intestinal lumen through the atypical epithelium. Tumor surface cells and normal absorptive cells were compared using scanning electron microscopy and transmission electron microscopy of thin sections and freeze-fracture replicas. The results showed that tumor cells were usually smaller, more rounded, showed less regularly shaped microvilli, and had fewer particles in the apical surface membrane than on normal absorptive cells.

INTRODUCTION

Tumors of the large intestine in DMH-treated rats and mice (3) are not seen until many weeks after the commencement of treatment, thus providing an opportunity for the observation of morphological changes in the large intestine. Hence it is possible to survey the entire spectrum of changes from the normal appearance to the development of overt cancer. An advantage of SEM is that detailed observations can be made more rapidly than by overt cancer. An advantage of SEM is that detailed observations can be made more rapidly than by

For SEM, the descending colon was removed, opened longitudinally, and pinned onto corkboard. The luminal surface was washed with Puck's Saline G, and specimens were fixed for 8 to 10 hr in 5% glutaraldehyde in 0.067 M Sorenson's phosphate buffer (pH 7.2). Specimens were rinsed in 4 changes of the same buffer over a period of 10 hr, and the descending colon was cut into lengths of approximately 1 cm, postfixed in 2% osmium tetroxide in 0.67 M Sorenson's phosphate buffer (pH 7.2) for 2 hr, and then rinsed in 4 changes of the same buffer for 30 min. The tissue was washed twice for 30 min in distilled water, dehydrated through ether a series of graded ethanol or acetone, and immersed in amyl acetate for 30 min. All procedures were carried out at room temperature on a roller turning at 4 rpm. For scanning electron microscopic examination of the basal lamina, the epithelium was removed by using the following modifications to the above procedures: glutaraldehyde fixation was omitted and instead specimens were fixed in 2% osmium tetroxide for 2 hr and then washed in distilled water for 6 hr.

All scanning electron microscopic specimens were then processed in a critical point drier (Samdri PVT-3; Tousimis Research Corp., Rockville, Md.) using dry CO2 gas, thinly coated with gold (Polaron E5000, Watford, Herts., England), and examined in a Cambridge SEM at 20 kV (Stereoscan S4; Cambridge, England).

For TEM, the descending colon was cut into 1- x 5-mm segments and fixed in a solution containing formaldehyde (3%), glutaraldehyde (4%), and trinitroresol (0.05%) in 0.05 M cacodylate buffer (pH 7.4) for a period of 6 hr at 4°. The tissue was then rinsed in 0.1 M cacodylate buffer (pH 7.4) occurring on the luminal surface of the descending colon in rats both during and after 20 weekly injections of DMH. Additional observations have been made using TEM, light microscopy, and the freeze-fracture technique.

MATERIALS AND METHODS

Male Sprague-Dawley rats (4 to 6 weeks old) were given s.c. injections of 21 mg of DMH (Aldrich Chemical Co., Inc., Milwaukee, Wis.) per kg weekly for 20 weeks. The stock solution for injections was comprised of 400 mg of DMH dissolved in 100 ml of water containing 37 mg of EDTA and was adjusted to pH 6.5 using sodium hydroxide. Treated animals were killed by decapitation at 2 weekly intervals and the descending colon was examined for morphological changes. The study was conducted over the 30-week period following commencement of treatment.

For TEM, the descending colon was removed, opened longitudinally, and pinned onto corkboard. The luminal surface was washed with Puck's Saline G, and specimens were fixed for 8 to 10 hr in 5% glutaraldehyde in 0.067 M Sorenson's phosphate buffer (pH 7.2). Specimens were rinsed in 4 changes of the same buffer over a period of 10 hr, and the descending colon was cut into lengths of approximately 1 cm, postfixed in 2% osmium tetroxide in 0.67 M Sorenson's phosphate buffer (pH 7.2) for 2 hr, and then rinsed in 4 changes of the same buffer for 30 min. The tissue was washed twice for 30 min in distilled water, dehydrated through ether a series of graded ethanol or acetone, and immersed in amyl acetate for 30 min. All procedures were carried out at room temperature on a roller turning at 4 rpm. For scanning electron microscopic examination of the basal lamina, the epithelium was removed by using the following modifications to the above procedures: glutaraldehyde fixation was omitted and instead specimens were fixed in 2% osmium tetroxide for 2 hr and then washed in distilled water for 6 hr.

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and postfixed for 2 hr in 1% osmium tetroxide. The tissue was then treated with 1% uranyl acetate in 0.2 M maleate buffer (pH 5.15) for 1 hr and dehydrated through a series of graded ethanol and embedded in Epon-Araldite. The thin sections were stained with uranyl acetate and lead citrate and examined in a Siemens 1A electron microscope at 80 kV.

For light microscopy, 1-μm sections of Epon-Araldite-embedded tissues were stained with toluidine blue.

For preparing freeze-fracture replicas, small pieces of descending colon were fixed in paraformaldehyde (2%) and glutaraldehyde (2.5%) in cacodylate buffer (pH 7.4) containing 0.02 M CaCl₂ for 1 hr at 4°C. The tissue was washed in cacodylate buffer infiltrated for 1 hr with 20% buffered glycerol at room temperature, trimmed to appropriate size, mounted on gold discs, frozen in liquid Freon 22 (chloroform-difluoromethane), and stored in liquid nitrogen. Specimens were fractured in a Balzer’s apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) at —110° and coated immediately. The replicas were cleaned in hypochlorite solution (Clorox) and collected on grids after several washes in distilled water.

**RESULTS**

**Morphology of the Colonic Mucosa**

**Normal Rats.** Macroscopically, the mucosa of the descending colon was folded into 4 to 6 parallel longitudinal elevations, with occasional areas where folds were replaced with flattened depressed areas.

Light microscopy showed that the luminal surface of the descending colon was formed by a single layer of epithelial cells in continuity with epithelial cells lining straight tubular intestinal glands. Absorptive cells were the most abundant cell type present on the surface, with occasional mucous cells containing varying amounts of secretory product (Fig. 3). Light microscopy also revealed aggregates of lymphoid tissue beneath the macroscopically flattened areas of the surface.

SEM showed the luminal surface to be formed by repeating units of groups of cells surrounding the orifice of individual intestinal glands (Figs. 2 and 3). These gland units showed little variation in size, although boundaries between adjacent gland units were not obvious. Three morphologically distinct cell types were identified on the surface. Absorptive cells having a flat or gently convex contour covered by a dense coat of long, even microvilli were the most abundant. Mucous cells were seen as single elements bulging from between absorptive cells and showing a distinctly convex surface covered with short, sparse microvilli (Fig. 4). Cells having an appearance different from either mucous or absorptive cells were seen in areas toward the periphery of gland units. These rounded cells appeared to be lying on, rather than being part of the surface mucosa, and the location and appearance of these cells suggested that they were referee absorptive and mucous cells undergoing exfoliation (Fig. 3).

Spiral microorganisms having a polar flagellum were frequently seen projecting from the luminal surface (Figs. 3 and 4). These were the only microorganisms seen using SEM, although a variety of unidentified microorganisms were seen in the lumen of intestinal glands during light microscopic and transmission electron microscopic observations.

Light microscopy and TEM of specimens treated to remove surface epithelial cells showed that this treatment did not disrupt the underlying basal lamina or supporting tissues of the lamina propria. SEM of the basal lamina showed repeating units of cylindrically shaped gland forms that were similar in size throughout the descending colon. Each gland form was surrounded by 6 to 8 adjacent gland forms (Fig. 5). Higher power SEM showed the basal lamina to be a continuous smooth sheet apart from occasional small round holes and filamentous surface projections (Fig. 5).

Freeze-fracture replicas of the apical plasma membrane of absorptive cells showed microvilli that were regularly arranged with randomly distributed intramembranous particles that were more numerous on the PF face than on the EF face (Fig. 27). The close-packed microvilli prevented observation of particles on the intermicrovillous membrane.

**Morphology of DMH-treated Rats**

**Four to 8 Weeks after Commencement of Treatment.** Widespread changes in the scanning electron microscopic appearance of the luminal surface of the descending colon were not observed during the initial 4 to 8 weeks of DMH treatment. However, discrete and isolated changes were observed as early as 4 weeks after commencement of treatment when areas of mucosa were seen to be elevated and protruding into the lumen of the intestine (Fig. 6). The surface of such protuberant areas was formed by 1 to 4 gland units having an apparent increase in diameter and in surface cell numbers (Fig. 7). When 2 or more gland units were present on a protuberance, the surface contour of the units and the surface contour of individual cells upon them were often more flat than on normal gland units. However, the reverse of this observation was true when only 1 gland unit was involved. No changes in either the size of surface cells or in the ratio of mucous cells to absorptive cells present on protuberant areas were apparent (Fig. 7). The orifice of protuberant gland units was slit-like rather than round (Figs. 6 and 7). Protuberant gland units were not seen in all animals following 4 weeks of DMH treatment but after 8 weeks of treatment, there were usually several such areas in each cm of descending colon examined. SEM of the basal lamina in DMH-treated rats during the initial 4 to 8 weeks showed isolated areas of basal lamina having enlarged gland outlines on the luminal surface. These areas were clearly distinguishable from surrounding areas and occurred with a similar frequency to that of protuberant gland units (Fig. 8). When the scanning beam was projected vertically downward into these surface abnormalities, it was seen that 2 to 4 glands which were smaller than normal were present in the deeper layers and that there was a confluence of the lumens of these small glands close to the luminal surface forming a common orifice (Fig. 9). The basal lamina adjacent to the abnormalities was usually similar to that of normal glands (Fig. 8) but on occasions, smaller than normal gland units were present. The open ends of these glands were often protruded into the lumen of the intestine.
mal glands surrounded the enlarged confluent glands (Fig. 9).

Ten to 12 Weeks after Commencement of Treatment. A further morphological change was observed during this period when groups of up to 100 gland units were seen to be enlarged and to have an appearance that was distinct from both protuberant gland units and from normal gland units (compare Figs. 10 and 11). Enlarged gland units were not uniform in diameter, although surface cells were similar in morphology and distribution to those on normal gland units (Fig. 11). Enlarged gland units differed from protuberant gland units in that they were not protuberant, grouped in clusters of 1 to 4, and showed a rounded rather than slit-like gland orifice.

Protuberant gland units that were similar in morphology, distribution, and number to those present at 8 weeks remained evident (Fig. 12).

Eighteen to 24 Weeks after Commencement of Treatment. Small macroscopic tumors and occasional large tumors were evident in many animals during this period and are described under “Morphology of DMH-induced Tumors.” SEM revealed that most of the luminal surface not bearing tumors was abnormal in appearance; protuberant gland units, similar in morphology, distribution, and number to those present at 10 to 16 weeks remained evident. Most of the luminal surface that was formed by enlarged gland units similar to those that were seen initially at 10 to 16 weeks after the commencement of DMH treatment were now present as either isolated entities, small groups, or groups of up to several hundred. The intervening luminal surface was formed by gland units having an appearance similar to that seen in control rats.

Twenty-six to 30 Weeks after Commencement of Treatment. Tumors were present in all animals examined and are described under “Morphology of DMH-induced Tumors.” SEM of the luminal surface not bearing tumors showed that virtually the entire surface was abnormal in appearance, being formed by enlarged gland units having similar characteristics, to those described after 10 to 16 and 18 to 24 weeks of DMH treatment and also containing protuberant gland units similar in morphology, number, and distribution to those described at 10 to 24 weeks of treatment (Figs. 13 and 14).

SEM of the basal lamina beneath this abnormal epithelium showed that the cylindrical gland forms seen in normal animals had been replaced with enlarged and pleomorphic gland forms, so that rectangular, triangular, round, and all intermediate forms were evident (compare Figs. 15 and 16). However, the basal lamina remained as a continuous sheet similar to that described in normal tissue.

Morphology of DMH-induced Tumors

In the majority of animals, small macroscopic tumors were present following 20 weeks of DMH treatment; at 30 weeks all animals examined bore tumors and in some animals up to 6 tumors were present. Light microscopy showed most tumors to be well-differentiated adenocarcinomas (Fig. 17).

The smallest tumors identified were usually seen as plaques measuring approximately 1 × 1 mm and having a central, crater-like area. Larger tumors sometimes retained this appearance (Fig. 19), but more often were round projections attached to the intestinal wall by a stalk that varied both in length and in diameter (Fig. 18).

Malignant surface cells were first observed in the central, crater-like area of small plaques (Fig. 20) and SEM and TEM showed them to be clearly different in appearance from cells seen previously, being smaller and showing a bulging surface contour that was usually covered with short microvilli distinctive for each cell (Figs. 21 and 23). Other malignant cells showed a smooth surface (Fig. 22) and occasionally, cells having both microvillous surface areas and smooth surface areas were seen. The surface coat seen on absorptive cells was absent on tumor cells and borders between adjacent cells were obvious (Fig. 22).

The scanning electron microscopic appearance of malignant surface cells appeared to be unrelated to macroscopic tumor size and form, and a detailed classification of DMH tumors has not been undertaken at this time. SEM of the surface of large tumors projecting furthest into the intestinal lumen showed occasional areas lacking the usual tumor surface cells, showing instead a loose network of large spindle-shaped cells having an appearance suggestive of fibroblasts.

In most animals the junction between malignant and nonmalignant cells at the periphery of tumors was obvious. In some animals peripheral areas showed funnel-shaped gland units having surface absorptive cells more convex than seen previously and occasional mucous cells (Fig. 24). In other peripheral areas malignant cells were seen between absorptive and mucous cells (Fig. 22).

Specimens of descending colon containing tumors were treated in order to expose the basal lamina (Fig. 25). SEM showed the basal lamina beneath tumor cells to be different in appearance to that seen previously beneath nonmalignant cells. The basal lamina was seen as a collection of filamentous material (Fig. 26) that was sometimes arranged as interconnecting cords (Fig. 25) and that had a roughened surface. The arrangement of the cords was similar to the arrangement of epithelial cells seen in well-differentiated tumors using light microscopy. The demarcation between basal lamina of tumors and that of the adjacent epithelium was abrupt and obvious (Fig. 26). TEM showed that the basal lamina beneath malignant colonic epithelial cells was uneven in thickness and separated from the basal plasma membrane by a distance that was variable. In addition, sections of the basal lamina were occasionally overlapping, forming areas of double thickness, and on other occasions the basal lamina was discontinuous, especially over areas where the basal plasma membrane projected into the lamina propria.

Freeze-fracture replicas of the apical plasma membrane of malignant surface cells showed microvilli that were less frequent, less regular in size and shape, and having fewer intramembranous particles than comparable microvillous membranes of control animals (Fig. 28). Particle distribution on the intermicrovillous plasma membrane was similar to that on microvilli. Other replicas showed stubby bifurcated microvilli projecting from an apical plasma membrane.
that was almost devoid of particles (Fig. 30). Simultaneous cleavage of both apical and lateral plasma membranes of malignant cells (Fig. 29) occurred frequently in contrast to the cleavage pattern seen in nonmalignant cells. Differences in lateral plasma membrane particle distribution have been observed between normal absorptive cells and malignant surface cells, but insufficient replicas have been examined to fully describe these differences.

DISCUSSION

The results show that weekly injections of DMH produce a series of morphological changes in the descending colon and that these changes become more pronounced with increasing duration of treatment. Thus after 4 weeks of treatment, the colon showed abnormalities that were isolated, involved only a few intestinal glands, and had a frequency similar to previous light microscopic reports of abnormalities in mice (9, 10). As the duration of treatment increased, normal gland units were progressively replaced with enlarged gland units, and 26 weeks after treatment the entire mucosa was abnormal in appearance. Malignant change in the descending colon of DMH-treated rats was not observed to be related to specific sites of morphological change. Equally, malignant change did not occur de novo in a normal mucosa but rather in a mucosa showing a generally disordered surface appearance. Although there have been few reports on the scanning electron microscopic appearance of normal human colonic mucosa (4, 6), the typical, funnel-shaped glands seen adjacent to DMH-induced tumors have an appearance similar to that reported in some specimens of human colonic epithelium obtained at operation from tumor-bearing patients (4).

In DMH-treated animals it has been shown that cell numbers in the intestinal glands are increased (11) and that the ate of cecal tumor formation is increased at sites of suture-induced chronic inflammation (8). These findings and the timing of this paper that morphological change precedes tumor formation in DMH-treated animals may indicate that the modality of DMH treatment is to progressively disrupt the factors influencing cell proliferation in normal animals so such an extent that some local, nonspecific stimulus to cell proliferation, such as suture placement, can trigger a cell into undergoing malignant change. It is possible that carcinogens may act similarly upon human colonic epithelial cells and that the placement of sutures in "normal" colonic tissues, some distance from a resected tumor, may trigger malignant change in a manner similar to that proposed for DMH-treated rats and produce the "suture line" tumors reported in the human colon (7). It is also possible that undetected proliferative and morphological changes are present in the intestinal epithelium in humans reported to have multiple colonic tumors (2).

TEM has shown that the basal lamina beneath malignant cells in human colonic adenocarcinomas is variable in thickness, has a wavy outline, and is sometimes discontinuous (5). Although a basal lamina having similar transmission electron microscopic characteristics was seen beneath malignant colonic epithelial cells in this study, SEM showed more clearly that this basal lamina was different from and demarcated from the surrounding nonmalignant tissues. Although the significance of this finding is not clear, it could be postulated that malignant epithelial cells can more easily invade the surrounding nonmalignant tissues through a discontinuous basal lamina.

In view of the probable role of the cell surface in regulating cellular proliferation and interactions, it seems likely that there are morphological differences between the plasma membranes of malignant and nonmalignant cells. The freeze-fracture observations in this study have shown morphological differences between the apical plasma membranes of normal and DMH-induced malignant cells, and further freeze-fracture studies are being conducted to investigate possible structural differences between other cell membranes.

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REFERENCES

Figs. 1 to 5. Descending colon of normal rats. 
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Electron Microscopy of the Colon in DMH-treated Rats

[Images of electron microscopy of the colon in DMH-treated rats, labeled 1 to 6]
Electron Microscopy of the Colon in DMH-treated Rats

Figure 25: Image of a colon tissue sample.

Figure 26: Detail of the colon tissue sample.

Figure 27: Magnified view of colon tissue with asterisks indicating specific areas.

Figure 28: Close-up of colon tissue with asterisks highlighting structural features.

Figure 29: Labeled image showing colon tissue with "L" indicating a specific label.

Figure 30: Detailed view of colon tissue with "L" and "C" labels.

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