Increased tight junctional permeability is associated with the development of colon cancer

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Epithelial tissues act as barriers between two fluid compartments, and the epithelial barrier function is provided by the epithelial cells and the tight junctions (TJs) that connect them. We have shown previously that chronic treatment of a cultured epithelial monolayer with phorbol ester tumor promoters induces an increase in transepithelial paracellular permeability and produces tumor-like polyps, suggesting an association between TJ permeability and tumor formation. In this study, we analyzed the association between TJ permeability and formation of tumors *in vivo*. The permeability of the TJs was assessed in normal human and rat colon epithelia and in colon tumors by measuring the transepithelial electrical resistance, the paracellular flux rate of D-[^{14}C]mannitol and the electron microscopic evaluation of the penetration of the electron dense dye ruthenium red across the TJs. By these criteria, the TJs of human colon tumors, including carcinomas and adenomatous polyps, and the TJs of 1,2-dimethylhydrazine (DMH)-induced rat colon tumors were leakier than the TJs of normal colon. Treatment of rats with the carcinogen DMH induced a progressive increase in the number of aberrant colonic crypts, considered the putative pre-neoplastic colonic phenotype while increasing TJ permeability of the colon epithelium prior to the development of tumors. These results showed that increased TJ permeability of the colon epithelium and consequently a decrease in epithelial barrier function precede the development of colon tumors.

Introduction

The integrity of the barrier function of the colon epithelium is critical for the separation of two different fluid compartments and is determined by the epithelial cells and the tight junctions (TJs). The TJs are specialized structures (1) comprised of integral proteins like occludin (2) and claudins (3), although the TJ exact composition is still being characterized (reviewed in 4,5). The TJs are connected to the actin cytoskeleton by TJ-associated proteins, including zonula occludens (ZO)-1, ZO-2 (6) and ZO-3 (7). The TJs are critical for establishing epithelial cell polarity (reviewed in 8) and for the control of the paracellular transport between the luminal and the basal-lateral fluid compartments (reviewed in 9).

Abbreviations: DMH, 1,2-dimethylhydrazine; EM, electron microscopy; RR, ruthenium red; R_t, transepithelial electrical resistance; TJ, tight junction.

Epithelial TJs are dynamic structures and subject to modulation during epithelial tissue remodeling (10), wound repair (11), inflammation (12,13) and transformation into tumors (14). The association of abnormal TJ function and epithelial tumor development has been suggested by earlier studies showing alterations in the TJ structures of epithelial cancers (15,16). *In vitro* studies using epithelial cell lines demonstrated that monolayers can be transformed into multilayered polyp-like structures by oncogenes, such as K-*ras* (17), or by phorbol ester tumor promoters (18). Epithelial multilayering was associated with increased TJ permeability (18,19), activation of protein kinase C-α (20) and phosphorylation of TJ proteins (21).

In the normal intestinal epithelium of the rat, structural modifications of the TJs are observed during mitosis (22). But cell division *per se* does not increase epithelial TJ permeability (23), suggesting that altered TJ permeability affecting the colonic epithelium may be intrinsic to disease states, including cancer. However, the functional status of the TJs and their role in the development of colon cancer is still controversial. Breakdown and internalization of TJ components have been observed in the HT29 human colon carcinoma cell line (24), but well differentiated colon carcinomas *in vivo* maintain TJ structure, cell polarity (25) and expression of TJ proteins, like occludin (26).

In this study, we analyzed the TJ permeability in human and rat colon tumors, and in the colon epithelium of the rat during the process of 1,2-dimethylhydrazine (DMH)-induced carcinogenesis. Highly permeable TJs were found in human and rat colon tumors and also in the colon of DMH-treated rats prior to the development of tumors. These results provide evidence indicating an association between increased TJ permeability of the colon epithelium and tumor development.

Materials and methods

Human colon tissues

Human colon tissues were obtained from specimens surgically removed from patients with colon cancer. Colon tissues were also obtained from non-cancer patients (diverticulosis) for epithelial electrophysiological and flux studies. Sample processing and handling were carried out following the guidelines of the Institutional Review Board of the Lankenau Hospital for the use of human tissues for research, and with the patients' consent. For the evaluation of TJ permeability, colon specimens were brought from the operating room immediately after removal and processed in the pathology laboratory with the assistance of a pathologist.

Rat colon carcinogenesis

Male Sprague–Dawley rats, weighing 350–500 g (Buckshire, Perkasie, PA) were maintained on standard laboratory diet with free access to water. An established protocol, known to produce 70–100% colon tumors after 15–20 DMH (Aldrich, Milwaukee, WI) injections, was followed (27). The rats received weekly i.p. injections of 30 mg/kg DMH in a solution of sodium bicarbonate and 0.01 M EDTA (Fisher, Atlanta, GA), pH 8.0. Control rats were injected with vehicle alone. For evaluation of aberrant crypts, tumor formation and the permeability of the colon epithelial TJs, animals were killed by decapitation without anesthesia, because anesthetics have been observed to cause changes in TJ permeability (28). The number of DMH injections is described in each experiment.

Electron microscopic evaluation of TJ permeability in human and rat colon

The permeability of the colon epithelial TJs was evaluated using the electron-dense dye ruthenium red (RR) and electron microscopy (EM). All EM reagents were purchased from Electron Microscopy Sciences (Fort Washington, PA). Human colon specimens were received immediately after colectomy, opened and the mucosa gently washed. Sections of the tumor tissues, tumor border and normal mucosa were cut, the serosa was quickly removed and the pieces were placed in chambers specifically designed and made for EM evaluation of TJ permeability. The chambers consisted of a base and a top with a 1.13 cm^2 circular opening that exposed the apical side of the epithelium only. The tissues were placed in the chamber with the mucosa side up, sealed with rubber o-rings and the chamber closed, exposing only the mucosal surface. A fixative containing 2.5% glutaraldehyde and 0.6% RR in 0.1 M sodium cacodylate buffer, pH 7.3, was added to the mucosa for 30 min at room temperature. After washing in 0.1 M sodium cacodylate, the mucosa was fixed in 2% osmium tetroxide and RR for another 30 min. Chambers were then disassembled and the tissues processed for EM. Thin sections were photographed in a JEOL 1200 EX transmission electron microscope, and the TJ permeability to RR evaluated using multiple micrographs from each sample. Permeability to RR was determined by the presence of electron-dense deposits in the intercellular space between epithelial cells. A total of 251 TJs were analyzed from 16 different cases of human colon cancers, and 115 TJs from 10 samples of normal mucosa. Polyps were also studied, including two hyperplastic polyps, six adenomatous polyps and one villous adenoma, with a total of 140 TJs being analyzed. Analysis of TJ permeability of rat colon tissues by RR and EM was performed similarly. After measuring the transepithelial electrical resistance (R_t), samples removed from the Ussing chambers were processed for EM as described for human colon tissues.

Preparation of human and rat colon for transepithelial electrical measurements

Human colon segments of ~1.5 cm^2 were cut, and after removing the serosa and muscle layers, were prepared for transepithelial electrical measurements. The colons from DMH-treated and control rats were removed and opened longitudinally along the mesenteric border. After gently rinsing the mucosa with ice-cold Ringer's solution bubbled with 95% O_2 and 5% CO_2, the opened rat colon was placed with the mucosa down on a cooled Plexiglas plate moistened with Ringer's solution. The serosa and the muscularis propria were removed with a No. 11 scalpel blade and fine forceps. The colon was divided into four segments of ~2 cm^2, labeled as follows: segment 1, mucosa located over the distal colon lymphoid aggregate; segments 2 and 3, mucosa without lymphoid aggregates, proximal to segment 1; segment 4, mucosa overlying the mid-colon lymphoid aggregate. All four pieces were studied simultaneously in separate chambers.

The segments of human or rat colon tissue were mounted in Lucite Ussing chambers (PennCentury, Philadelphia, PA) with an aperture of 1.13 cm^2. The tissues were bathed with a circulating 285 mosml/kg Ringer's solution, pH 7.40, containing 112 mM NaCl, 5mM KCl, 1.3 mM CaCl$_2$, 1 mM MgCl$_2$, 3 mM Na$_2$HPO$_4$, 0.5 mM Na$_2$HPO$_4$, 25 mM NaHCO$_3$ and 10 mM glucose, at 37°C, and gassed with 95% O_2 and 5% CO_2.

Transepithelial electrical measurements

Transepithelial potential difference (PD) was measured continuously using voltage clamps (JWT; Biomedical Design, Mohnton, NJ) and Ag–AgCl electrodes in series with 4% agar bridges containing 1 M NaCl. The tissue was short-circuited with 1 s current pulses after correcting for fluid resistance between the agar bridges. Tissue resistance (R_t), measured in $\Omega \times cm^2$, was obtained by dividing PD by short circuit current.

Transepithelial flux of D-[^{14}C]mannitol in human and rat colon

The basal-lateral to apical flux of D-[^{14}C]mannitol (NEN Life Sciences, Boston, MA) was measured in human and rat colon tissues placed in Ussing chambers as described above. Krebs Ringer buffer containing 1 μCi/ml D-[^{14}C]mannitol and 10 μM unlabeled mannitol was added to the basal-lateral side and the tissue was incubated at 37°C after stabilization of transepithelial electrical parameters. Two 100 μl samples were obtained for liquid scintillation counting from the apical compartment of each colon segment, 10, 20, 30 and 40 min after adding D-[^{14}C]mannitol to the basal-lateral compartment. Fluxes were expressed as d.p.m./min/cm^2 then divided by specific activity to obtain mol/min/cm^2.

Analysis of aberrant crypts

After electrical measurements, rat colon tissue segments were taken from the Ussing chambers and used for analysis of colonic crypts. The tissues were fixed overnight in Fekete's solution (70% ethanol, 3.7% paraformaldehyde and 0.75 M glacial acetic acid) and stained with 0.2% methylene blue in distilled water for 15 min. After rinsing in PBS, the tissues were dehydrated and embedded as a wholemount in Spurr's resin (Electron Microscopy Sciences) forming clear blocks 5 mm thick. Aberrant crypts were easily
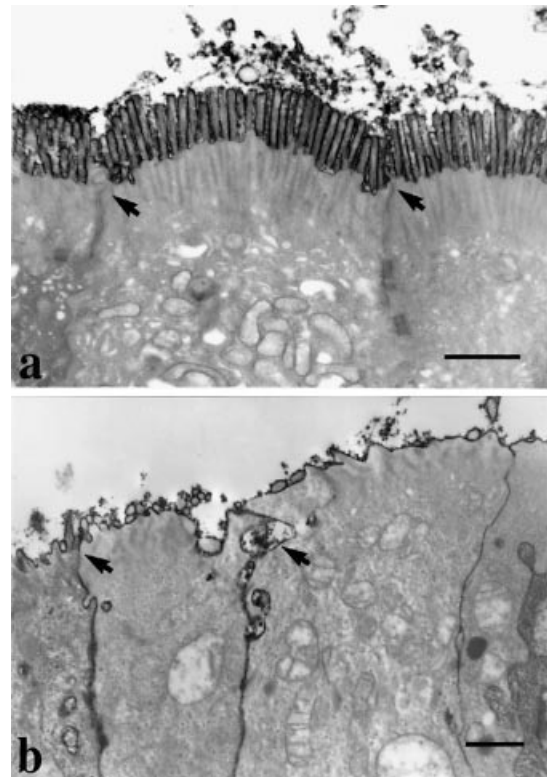

Fig. 1. EM evaluation of TJ permeability of human colonic epithelium using the electron-dense dye RR. (**a**) Absence of RR penetration across the TJ of normal human colonic epithelium (arrows). (**b**) RR penetration (arrows) across all of the TJs of a human colon cancer. Bars, 1 micron.

identified under the light microscope, following previously described guidelines (29). Briefly, the aberrant crypts had a diameter two to three times larger than a normal crypt, and were darkly stained due to nuclear stratification and increase in nuclear size. In each animal, quantitative analysis of aberrant crypts was performed for four distinct regions, each of 1.13 cm^2 in diameter, corresponding to the opening of the Ussing chambers, with a total 4.52 cm^2 surface per colon.

Results

The permeability of the TJs was assessed in human colon surgical specimens using the electron-dense dye RR and EM. Our results showed absence of RR penetration in most of the TJs between epithelial cells of normal mucosa far from the tumors (Figure 1a), although RR was seen internalized in the cytoplasm of goblet cells (not shown). Colon carcinomas showed frequent penetration of RR across TJs, producing electron-dense deposits in the intercellular spaces between cancer cells (Figure 1b). TJs were only considered permeable if the adjacent cells did not show penetration of RR into the cytoplasm, ensuring that TJ permeability was not the result of hypoxic changes. Quantitative analysis showed that the TJs of colon cancer were significantly more permeable than those of normal mucosa. Data were expressed as means ± SEM. Only 6.7 ± 3% of the TJs between normal surface colonocytes from 10 different cases were permeable to RR, in contrast to 74.5 ± 23.7% permeable TJs between colon cancer cells from 16 different cases ($P = 0.0001$). The study of TJ permeability in human polyps was limited due to use of most tissue from polyps for diagnostic purposes. However, six different cases of adenomatous polyps had a significantly higher number of permeable TJs (40.4 ± 25%) than normal mucosa ($P = 0.002$) and the one case of villous adenoma analyzed showed 69%
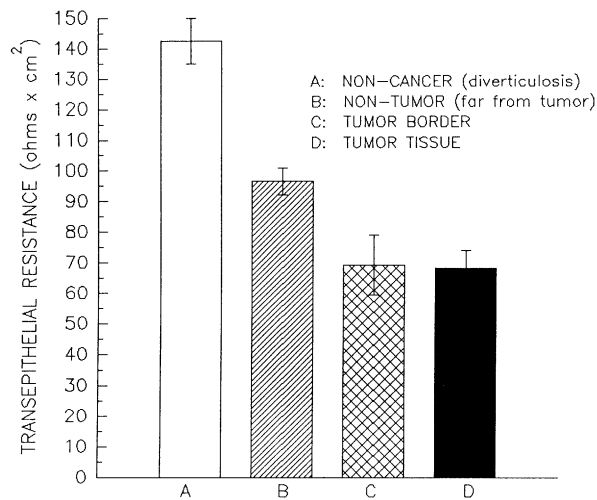
Fig. 2. R_t of human colon tissue samples expressed as means ± SD. A (empty bar) shows values from 16 samples of normal colonic mucosa from four different non-cancer surgical specimens (diverticulosis). The B, C and D values represent surgical specimens from colons with cancer. B (hatched bar) shows values from 45 samples of normal-appearing mucosa from 16 different specimens; C (crossed bar) shows values from 12 samples of tumor border from six different specimens and D (filled bar) shows seven samples of tumor tissue from five different specimens.
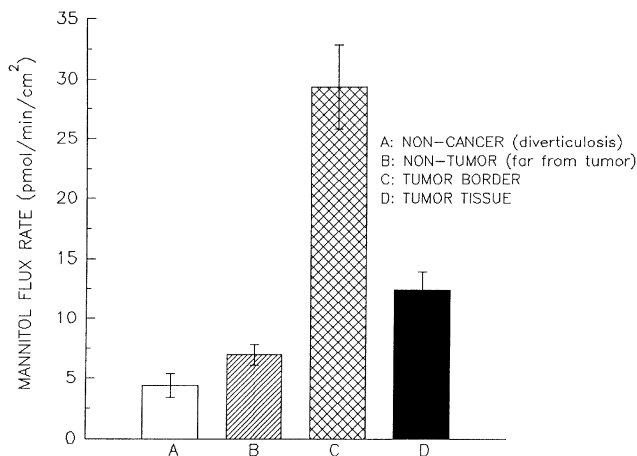

Fig. 3. Mannitol flux rates in human colon tissue samples expressed as means ± SD. A (empty bar) shows values from seven samples of normal colonic mucosa from two different non-cancer surgical specimens (diverticulosis). The B, C and D values represent surgical specimens from colons with cancer. B (hatched bar) shows values from 14 samples of normal-appearing mucosa from five different specimens; C (crossed bar) shows values from nine samples of tumor border from four different specimens and D (filled bar) shows five samples of tumor tissue from three different specimens.

of the TJs permeable to RR, similar to colon cancers. In contrast, the TJs between epithelial cells of two cases of hyperplastic polyps were not more permeable than those of normal mucosa.

The TJ permeability of normal and neoplastic human colon mucosa was also studied by measuring R_t (Figure 2) and mannitol flux rates (Figure 3). Data are expressed as means ± SD. Our results showed an R_t of 142.3 ± 29.9 $\Omega \times cm^2$ in the colon epithelium of patients without cancer. The R_t of the normal colon epithelium from patients with colon cancer was 96.5 ± 29.3 $\Omega \times cm^2$, significantly lower than in non-cancer patients ($P = 0.01$). Electrical resistance was significantly lower ($P = 0.05$) in tumor tissues (68.4 ± 14.8 $\Omega \times cm^2$) than

in normal epithelium far from the tumors. Tumor borders had R_t values similar to tumor tissues (69.3 ± 36.5 $\Omega \times cm^2$). Mannitol flux rates were significantly higher ($P = 0.003$) in tumor borders (29.3 ± 10.6 pmol/min/cm^2) than in the colon epithelium far from the tumors (6.9 ± 3.4 pmol/min/cm^2). Mannitol flux rates were also higher in tumor tissues (12.4 ± 3.4 pmol/min/cm^2) but the difference was less significant ($P = 0.073$) when compared with normal epithelium, probably due to the variable thickness and fibrosis of the invasive cancer areas, which may have presented a diffusion barrier to the flux of mannitol.

EM analysis of TJ penetration of RR also was performed in the colon epithelium and tumors of DMH-treated and control rats. The TJs of normal rat colon epithelium from control animals were not permeable to RR, including TJs of epithelia overlying lymphoid tissues of mid- and distal-colon areas (Figure 4a). After 12 weeks of DMH treatment, the TJs of the epithelium overlying the mid-colon lymphoid aggregate became permeable to RR (Figure 4b). The TJs of DMH-induced colon tumors were highly permeable to RR (Figure 4c).

Electrical measurements of the colon epithelium far from the tumors in rats treated with DMH for 18 weeks (Figure 5) showed an R_t of 111.4 ± 17.5 $\Omega \times cm^2$. A significant decrease ($P = 0.019$) in R_t, to 68.4 ± 15.5 $\Omega \times cm^2$ was observed for tumors. Tumor borders exhibited 84.1 ± 17.5 $\Omega \times cm^2$. Flux studies performed in the same experimental group (Figure 6) showed a significant increase in the flux rates of mannitol in tumor tissues ($P = 0.004$) and tumor borders ($P = 0.05$) compared with the normal epithelium.

We also analyzed the TJ permeability of the rat colon epithelium prior to the development of tumors, and correlated it with the presence of aberrant crypts. Aberrant crypts were quantified at various time points within the 18 weeks of DMH treatment and it was noted that there was a progressive increase in the number of aberrant crypts as a function of weeks of DMH injections (Figure 7). Electrical measurements were performed in four colon segments (see Materials and methods) prepared from rats without tumors, treated for 12 weeks with either DMH or vehicle alone. Figure 8 shows data from segment 2 corresponding to epithelium not associated with lymphoid tissue from distal colon, and from segment 4 corresponding to epithelium overlying the mid-colon lymphoid tissue. In vehicle-treated rats, the epithelium overlying the mid-colon lymphoid tissue (segment 4) had the lowest R_t (114.7 ± 6.7 $\Omega \times cm^2$), whereas the colon epithelium not associated with lymphoid tissues (segment 2) had 161 ± 25.1 $\Omega \times cm^2$. Values similar to those for segment 2 were observed in the other two segments, including the epithelium overlying distal colon lymphoid tissues (not shown). DMH treatment significantly decreased the R_t in all segments, to 69.4 ± 17.5 $\Omega \times cm^2$ in the mid-colon lymphoid tissue-associated epithelium, and to 109.4 ± 31 $\Omega \times cm^2$ in the epithelium not associated with lymphoid tissues. Mannitol flux rates (Figure 9) were consistent with the electrical measurements from the same experimental group, i.e. DMH treatment increased the flux of mannitol in all segments. Vehicle-treated rat colons also showed that the mid-colon lymphoid tissue-associated epithelium was more permeable than the rest of the colon.

Discussion

In vitro models of epithelial neoplasia have shown that there is an association between increased tumor promoter-induced
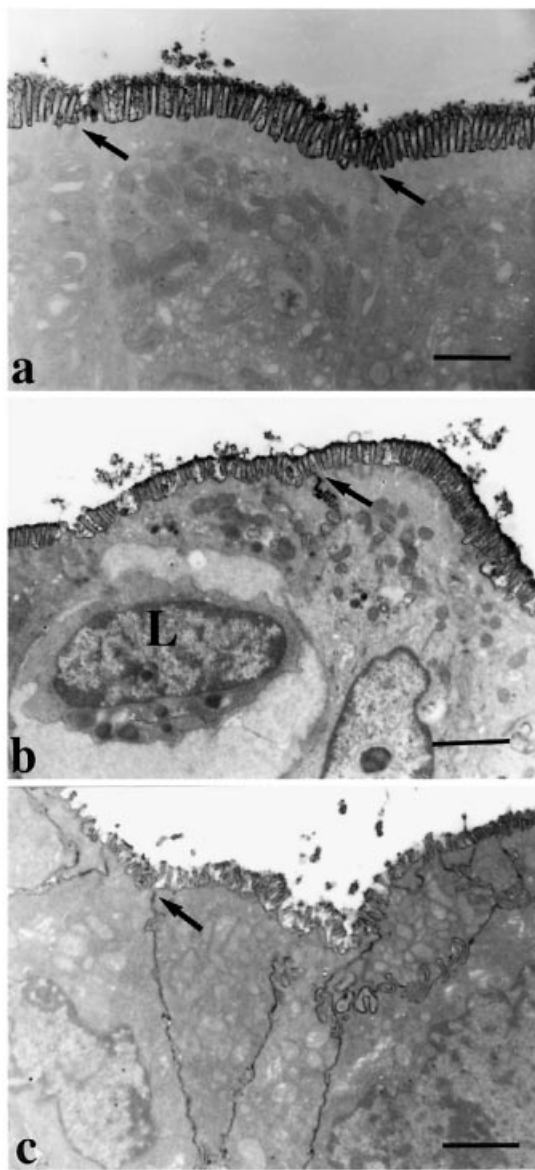
Fig. 4. EM evaluation of TJ permeability of rat colonic epithelium using the electron-dense dye RR. (**a**) Absence of penetration of RR across the TJs (arrows) of normal epithelium overlying the mid-colon lymphoid tissue from an untreated rat. Bar, 1 micron. (**b**) Penetration of RR across some of the TJs (arrow) of the epithelium overlying the mid-colon lymphoid tissue from a rat treated for 12 weeks with DMH. Bar, 2 microns. (**c**) Penetration of RR (arrow) across all of the TJs of a DMH-induced colon tumor. Bar, 2 microns.

Fig. 5. R_t of colon epithelia and tumors from DMH-treated rats expressed as means $\pm$ SD. A (empty bar) shows values from eight samples of non-tumor epithelia from two different rats. B (crossed bar) shows values from nine samples of tumor borders from five different rats. C (filled bar) shows values from nine samples of tumor tissues from five different rats.

Fig. 6. Mannitol flux rates of colon epithelia and tumors from DMH-treated rats expressed as means $\pm$ SD. A (empty bar) shows values from eight samples of non-tumor epithelia from two different rats. B (crossed bar) shows values from nine samples of tumor borders from five different rats. C (filled bar) shows values from nine samples of tumor tissues from five different rats.

TJ permeability (30), transepithelial flux of growth factors (31) and development of epithelial tumors (18,19). In the colon, cancer is caused by genetic alterations inducing epithelial cell transformation (32), followed by increased proliferation of transformed cells into tumors. Although cell proliferation is a key step in colon tumorigenesis in rodents (29,33,34) and humans (35,36), the mechanisms necessary for the growth of transformed epithelial cells are poorly understood. It is known that colon carcinogens, such as azoxymethane, can act as tumor promoters and stimulate cell proliferation by activation of EGF receptor tyrosine kinase (37). In DMH-induced colon carcinogenesis, tumors develop more often in areas of the colon containing high numbers of bifurcated (38) and aberrant crypts (39), which are known to express elevated levels
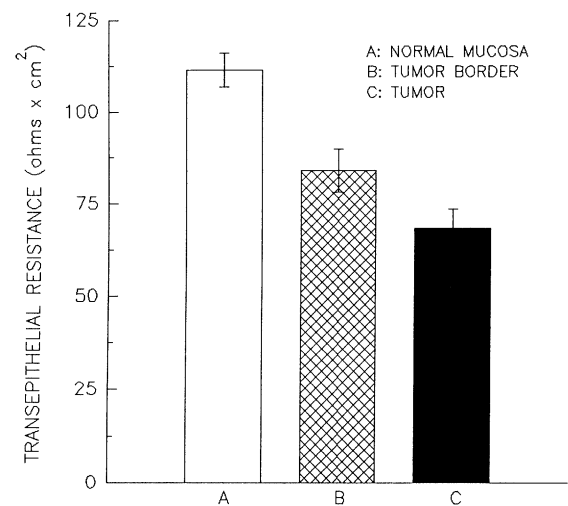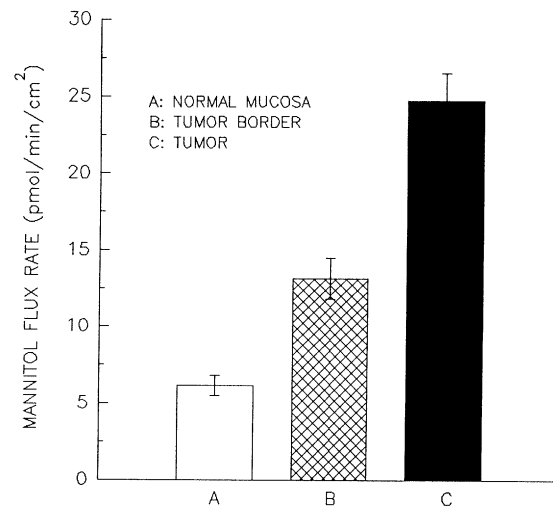
of growth factors (40) and to be highly proliferative (41). Furthermore, the presence of lymphoid tissues associated with the epithelium appears to induce epithelial cell proliferation (42), aberrant crypt development (43–45) and formation of tumors (46).

Although the role of colon epithelial barrier function in tumor development is not clear, early studies have suggested an association between the electrical properties of the colon epithelium and the development of colon tumors. Alterations in the Na^+–H^+ exchanger (47) and in ionic conductance across the colon epithelium have been shown to precede the formation of colon tumors in DMH-treated rats and susceptible strains of mice (48,49). In this study, we observed an increase in TJ permeability of human colon cancers and DMH-induced rat colon tumors. More importantly, increased TJ permeability was found in human colon polyps and in the pre-neoplastic
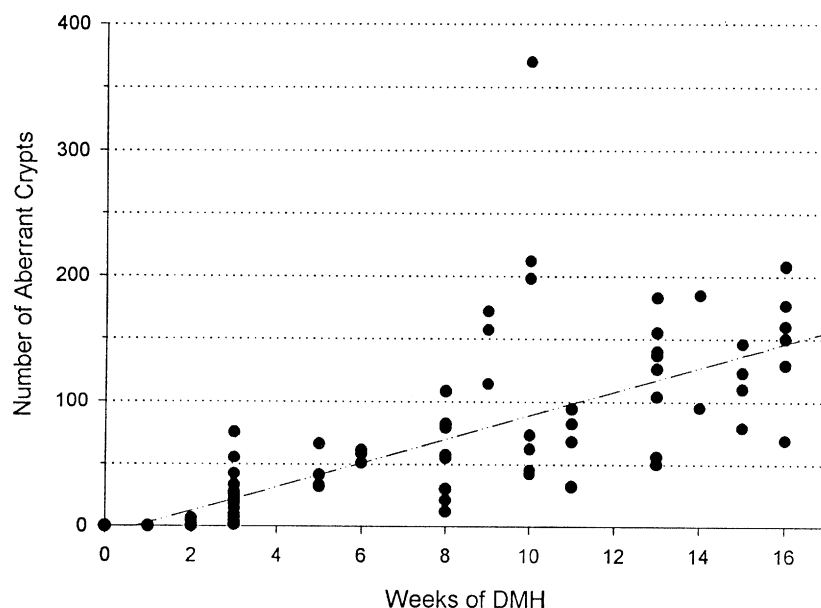
Fig. 7. Quantitative analysis of the number of aberrant crypts in rat colon mucosa as a function of weeks of DMH treatment. Each value represents the number of aberrant crypts in a 4.52 cm^2 colon surface area.
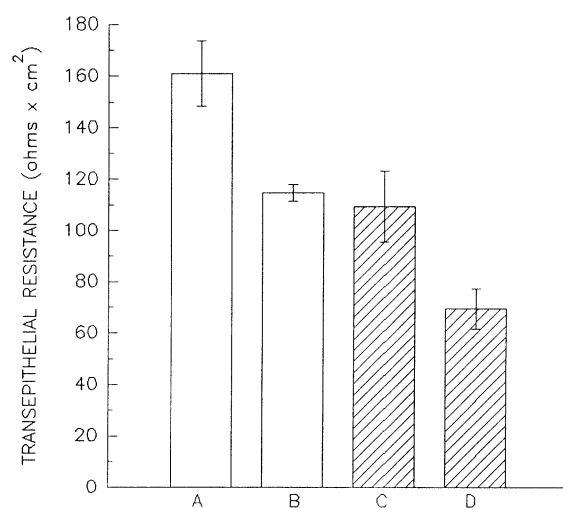

Fig. 8. R_t of the colon epithelium of rats injected for 12 weeks with DMH or vehicle alone. Values are expressed as means $\pm$ SD. Open bars show R_t values from five samples of segment 2 (see Materials and methods), representing epithelia not associated with lymphoid tissue from vehicle control (A) or DMH (B)-treated animals. Hatched bars show R_t values from four samples of segment 4 (see Materials and methods), representing epithelia overlying the mid-colon lymphoid tissue from vehicle control (C) or DMH (D)-treated animals.
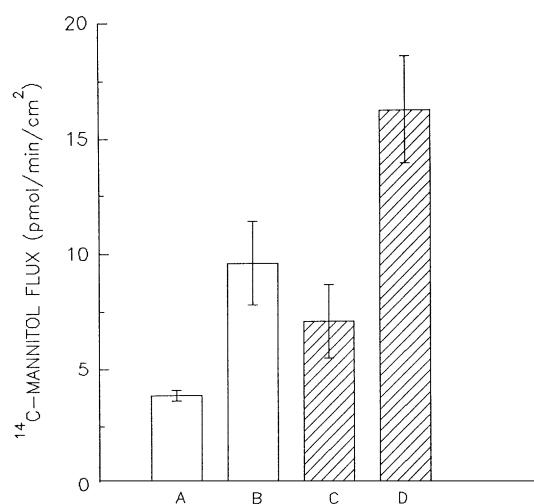

Fig. 9. Transepithelial mannitol flux rates across the colon epithelium of rats injected for 12 weeks with DMH or vehicle alone. Values are expressed as means $\pm$ SD. Open bars show R_t values from five samples of segment 2 (see Materials and methods), representing epithelia not associated with lymphoid tissue from vehicle control (A) or DMH (B)-treated animals. Hatched bars show R_t values from four samples of segment 4 (see Materials and methods), representing epithelia overlying the mid-colon lymphoid tissue from EDTA (C) or DMH (D)-treated animals.

colon of DMH-treated rats. The number of human colon polyps studied was limited, but it is significant that the polyps with more permeable TJs were those more frequently associated with colon cancer, including villous adenoma and adenomatous polyps. In contrast, hyperplastic polyps, which do not increase the risk of colon cancer, had mostly impermeable TJs, similar to normal colon epithelium.

In control rats, the epithelium overlying the mid-colon lymph tissue was the most permeable area of the colon, as indicated by electrical measurements, although the TJs of this region were not penetrated by the electron-dense dye RR, suggesting that the TJs were permeable to ions but not to macromolecules. Upon DMH treatment, this region became

permeable also to macromolecules and had the highest tumor frequency in our study (not shown). The colonic mucosa overlying lymphoid tissues has been reported as an area of high frequency for preneoplastic changes (43–45) and tumor formation (46) in DMH-treated rats. Those reports are consistent with our proposal of increased TJ permeability and increased risk of tumor development. However, the molecular mechanisms determining this association and the degree of TJ permeability between the epithelial cells that constitute aberrant crypts remain unknown.

In the normal colon epithelium, growth factor receptors are secluded in the basal-lateral compartment of the epithelial cells (50), limiting the access and growth activation by luminal

growth factors (51). Luminal growth factors alone do not increase the epithelial TJ permeability (23) or induce tumor formation (39), but an event such as exposure to a carcinogen may in fact make receptors available to luminal growth factors, and facilitate tumor growth. We propose that in DMH-treated rats, activation of growth factor receptors induces the formation of tumors as a result of increased TJ permeability of transformed cells. It is known that phorbol ester-induced TJ permeability in cultured cells allows the paracellular flux of solutes as large as 2×10^6 mol. wt (52). As a result, permeable TJs can facilitate the binding of apical growth factors to basal-lateral receptors (53), including those secreted by the cancer cells themselves, such as insulin-like growth factor-I (54). In turn, apically secreted growth factors also can increase the TJ permeability of colon cancer cells (55) and further activate cell growth.

Diseases of the colon characterized by increased TJ permeability, like Crohn's (56) and ulcerative colitis (57,58) are typically associated with a high frequency of genetic alterations and colon cancer. We postulate that an increase in TJ permeability constitutes a critical change for determining the growth of transformed epithelial cells into tumors, acting as an epigenetic tumor promotional event in colon cancer.

Acknowledgements

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