Dietary heme injures surface epithelium resulting in hyperproliferation, inhibition of apoptosis and crypt hyperplasia in rat colon

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Epidemiological and animal model studies suggest that a high intake of heme, present in red meat, is associated with an increased risk of colon cancer. The aim of this study was to elucidate the effects of dietary heme on colonic cell homeostasis in rats. Rats were fed a purified, humanized, control diet or a similar diet supplemented with 0.5 mmol heme/kg for 14 days. Fecal water cytolytic activity was determined with a bioassay, and colon epithelial cell proliferation was evaluated with 3H-thymidine incorporation into DNA or by Ki-67 immunohistochemistry. Exfoliation of colonocytes was measured as the amount of rat DNA in feces, and caspase-3 expression and activity were measured to study colonic mucosal apoptosis. Dietary heme induced a >10-fold increased cytolytic activity of the fecal water and a 100-fold lower excretion of host DNA. Colonos of heme-fed rats showed injured surface epithelium and an ~25% increase in crypt depth. Finally, dietary heme doubled colonocyte proliferation, shown by all three markers, but inhibited colonic mucosal apoptosis. In conclusion, our results demonstrate that dietary heme injures colonic surface epithelium, which is overcompensated by inhibition of apoptosis and hyperproliferation of cells in the crypts, resulting in crypt hyperplasia. This disturbed epithelial cell homeostasis might explain why a high intake of dietary heme is associated with an increased risk of colon cancer.

Introduction

Colorectal cancer is a leading cause of cancer death in Westernized countries (1). Diet is one of the important environmental factors associated with increased colorectal cancer risk (2). Epidemiological and experimental studies suggest that high consumption of red meat and processed meat is associated with an increased risk of colorectal cancer (3–7). In contrast, consumption of white meat, such as poultry and fish, is not associated with increased risk (8). We and others (9,10) hypothesized that this differential effect of red and white meat is indeed associated with heme intake (22–24), we now address the question whether and how heme can disturb this balance between cell death and proliferation. Therefore, we studied whether dietary heme-induced cytolytic activity of the colonic contents indeed injured colonic epithelial cells and modulated exfoliation, proliferation and apoptosis of cells in rat colon.

Materials and methods

Animals and diets

The experimental protocol was approved by the animal welfare committee of Wageningen University and Research Centre. Eight-week-old outbred male Specific pathogen-free Wistar rats (WU; Harlan, Horst, The Netherlands) were housed individually in metabolic cages in a room with controlled temperature (20–22°C), relative humidity (50–60%) and a 12 h light/dark cycle (lights on at 0600 h). Animals were acclimatized to housing conditions for 5 days before the start of the experiment.

To study the effects of heme on the colonic epithelium, two groups of 16 rats were fed purified, humanized diets (40% energy as fat corresponding with 200 g of fat/kg diet, 20 mmol/kg of calcium) during 2 weeks as described in detail previously (12). One group was fed a control diet and the other group was fed this diet supplemented with 0.5 mmol hemin/kg diet (Sigma–Aldrich Chemie, St Louis).

Feces were quantitatively collected during days 11–14 of the experiment, frozen at –20°C and subsequently freeze dried.

Cytolytic activity of fecal water

Fecal water was prepared by reconstituting a small amount of freeze-dried feces with double-distilled water to obtain a physiologic osmolarity of 300 mOsm/l, as described earlier (9). After preparation, the fecal waters were stored at –20°C until further analysis. Cytolytic activity of fecal water was quantified by potassium release of human erythrocytes after incubation with fecal water as described previously (9) and validated earlier with human colon carcinoma-derived Caco-2 cells (25). In short, fecal water was mixed with washed human erythrocytes (final hematocrit 5%) and incubated for 15 min at 37°C. The intact erythrocytes were precipitated by mild centrifugation for 1 min at 1500g and washed twice with 154 mmol NaCl. These erythrocytes were acidified with thricloric acid (5% wt/vol) and centrifuged at high speed for 1 min at 10000g. Their potassium content was measured with an inductively coupled plasma atomic emission spectrophotometer (Varian, Mulgrave, Australia). Simultaneously, erythrocytes were incubated in 154 mmol NaCl (0% K release) and in double-distilled water (100% lysis). Finally, the cytolytic activity of the fecal waters was calculated as a percentage of the 0% and 100% lysis samples.

Total feces analysis

One of the important physiological functions of colonocytes is the reabsorption of minerals and water. Therefore, we measured the fecal cations sodium and
potassium as markers for colon epithelial cell function. Feces were treated with 5% trichloroacetic acid for 1 h at room temperature and centrifuged for 2 min at 14000g. The supernatants were diluted with 0.05% CaCl₂ and sodium and potassium were analyzed with an inductively coupled plasma atomic emission spectrophotometer. Fecal host DNA was quantified as a marker for epithelial exfoliation as described earlier (12,26). Briefly, fecal host DNA was extracted from freeze-dried feces and the DNA in all isolates was of good purity (A₂₆₀/A₂₈₀ ~ 1.8). The standard DNA used for quantification was isolated from rat spleen. Quantification was based on real-time polymerase chain reaction, performed with a rat-specific probe and rat-specific primers targeted to the β-globin gene sequence (26).

DNA synthesis of epithelial cells
After 14 days of experimental feeding, colon mucosal proliferation was quantified by measuring DNA replication in vivo, using 5-bromo-2'-deoxyuridine (BrdU) or 5H-thymidine incorporation into DNA. Eight rats fed a control diet and 8 rats fed a hemi diet were non-fasted injected intraperitoneally with BrdU (Sigma) at a dosage of 100 mg/kg body wt. The 16 remaining rats (8 control and 8 hemi) were injected intraperitoneally with (methyl-5H)thymidine (specific activity 925 GBq/mmol; dose 3.7 MMBq/kg body wt; Amersham International, Amersham, UK) in 154 mM NaCl. After 2 h, rats were sacrificed by CO₂ inhalation.

The entire colons of the 5H-thymidine-injected rats were excised and opened longitudinally. The colonic contents were removed and the mucosa of the proximal and distal colon was scraped, homogenized in buffer and analyzed for 5H-thymidine incorporation into DNA as described previously (12). The entire colons of the BrdU-injected rats were excised and the middle 3 cm part of the proximal and the middle 3 cm part of the distal colon were sampled and fixed in 10% neutral buffered formalin. After fixation, the colon tissues were embedded in paraffin and serially cut in 3 µm sections, which were used for all immunohistochemical analysis described below. The paraffin-embedded rat tissue sections were deparaffinized, hydrated and placed in antigen retrieval solution (10 mmol/l citrate buffer, pH 6.0) for 15 min in a microwave oven at 350 W. Endogenous peroxidase was inhibited by incubation with 3% H₂O₂ in phosphate-buffered saline (PBS) for 30 min. The sections were then incubated with anti-BrdU mouse monoclonal antibody (27) for 1 h at room temperature. Next, tissue sections were sequentially incubated with mixture one [peroxidase-conjugated goat anti-rabbit (DakoCytomation, Heverlee, Belgium) at a dilution of 1:50 in 5% normal rat serum (Sigma) and 1% bovine serum albumin (BSA) (SERVA, Heidelberg, Germany) in PBS] and with mixture two [peroxidase-conjugated goat anti-rabbit (DakoCytomation) at a dilution of 1:50 in 5% normal rat serum and 1% BSA in PBS] for 30 min. Sections were developed in staining solution (25 mg diaminobenzidine (DAB) substrate, 50 mg imidazol and 50 µl 30% H₂O₂ in 50 ml PBS) for 10 min and counterstained with hematoxylin. Colonocytes in the proximal and distal colon from 15 well-oriented crypts (longitudinal section) were counted as described above for BrdU. A cell was scored positive for BrdU when the nucleus of the cell was distinctively brown. The number of positive cells per crypt column, labeling index and the total number of cells per full-length hemi-crypt were determined. Labeling index was calculated as the ratio of the number of positive BrdU-labeled cells to the total number of crypt cells.

Immunohistochemistry Ki-67
Ki-67 is a protein involved in the cell cycle (28). To visualize Ki-67 antigen, tissue sections were treated for optimal antigen retrieval and to quench endogenous peroxidase activity as described above. The sections were then incubated with MIB-5 (DakoCytomation) at a dilution of 1:50 in combination with 1% BSA for 1 h at room temperature. After incubation with primary antibody, tissue sections were sequentially incubated with biotinylated rabbit anti-mouse antibody (DakoCytomation) at a dilution of 1:50 in 5% normal rat serum and 1% BSA in PBS and streptavidin/ horseshadish peroxide (DakoCytomation) at a dilution of 1:300 (in 5% normal rat serum and 1% BSA in PBS) for 30 min. Sections were developed in diaminobenzidine, counterstained with hematoxylin, dehydrated mounted. For negative controls, PBS and 1% BSA replaced the primary antibodies. Colonocytes from 15 well-oriented crypts (longitudinal section) were counted as described above for BrdU. A cell was scored positive for Ki-67 when the nucleus of the cell was distinctively brown.

Histological analysis
Intercryptal surface epithelial damage was identified in paraffin-embedded colon tissue sections (3 µm). Sections were stained with hematoxylin and eosin. Only surface epithelium between two intact U-shaped crypts that extended from the basal lamina to the gut lumen was accepted as satisfactory for evaluation. We developed a three-grade classification score for injury of the surface epithelium. These grades were as follows: 0, continuous surface epithelium with normal epithelial architecture; 1, ruffled surface epithelium with disturbed epithelial architecture, i.e. decreased apical cell volume, and 2, grade 1 plus injured epithelial cells with necrotic appearance in the surface epithelium. Surface epithelial injury was calculated as the sum of the scores of 25 intercryptal surface areas per rat and averaged per group. Thus, this score varies between 0 and 50. All histological observations were done by an observer who was blinded to the origin of each tissue section.

Apoptosis
We studied colonic apoptosis by measuring caspase-3 expression. Mucosal scrapings of colon tissues were snap frozen and homogenized in N₂. A volume of 0.5 ml buffer containing 0.2 M sucrose, 20 mM Tris and 1 mM dithiothreitol, pH 7.4, combined with a protease-inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) was added to half of the lyophilized and powdered colon scraping. This homogenate was shaken for 5 min and centrifuged at 20000g for 1 min. The supernatant was saved for further processing and stored at −20°C. Protein concentration in the supernatants was determined by bicinchoninic acid reagent (Pierce, Rockford, IL).

Protein aliquots from colon mucosal extracts (15 µg protein per lane) were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membrane (Amersham). The membranes were stained with Ponceau S to confirm equal protein loading and effective transfer. No variability in transfer was observed. Destained membranes were blocked with 5% non-fat milk powder in Tris-buffered saline–Tween (TBS-T; 20 mM Tris-base, pH 7.6, 137 mM NaCl, 0.3% (wt/vol) Tween 20) with 1 h at room temperature. Blots were incubated with 1:1000 diluted caspase-3 primary antibody (clone H277, Santa Cruz Biotechnology, Heidelberg, Germany) in TBS-T with gentle agitation at 4°C overnight. Membranes were washed twice with TBS-T, blocked again with 5% non-fat milk powder in TBS-T for 30 min and then washed twice in TBS-T at room temperature. Blots were then incubated with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit (Sigma) at 1:5000 in TBS-T for 1 h at room temperature. After washing twice with TBS-T, peroxidase activity was visualized using SuperSignal enhanced chemiluminescence detection reagents (Pierce) according to the manufacturer’s instructions. The detection of the cleaved p-17 form of caspase-3 on the blots represents the active form, whereas the uncleaved p-32 form of caspase-3 represents inactive caspase-3 (29).

Caspase-3 activity was measured simultaneously in the absence and presence of caspase-3 inhibitor (DEVD-CHO) (Calbiochem, Darmstadt, Germany). A volume of 10 µl of colon mucosal extract was added to 973 µl of buffer containing 50 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 0.1 M NaCl, 0.1% 3-propanesulfonate (CHAPS), 10 mM dithiothreitol, 0.1 mM ethylenediaminetetraacetic acid and 10% glycerol, pH 7.4. For incubations with the inhibitor, 7 µl of a stock solution (0.075 mM) of caspase-3 inhibitor in dimethyl sulfoxide (DMSO) was added. The slope of the reaction was calculated and the caspase-3 activity calculated. Pure aminomethylcoumarin (Calbiochem) was diluted in DMSO and used as standard in a concentration range of 0–4 µM.

Statistical analysis
All results are expressed as mean ± SEM. A commercially available package was used (Statistica 6.1, Statsoft Inc., Tulsa). Statistical significance was calculated by Student’s t-test. Differences were considered significant at P < 0.05. Associations between colonic crypt depth and labeling index, BrdU immunohistochemistry and Ki-67 immunohistochemistry were determined using the Pearson’s r correlation test.

Results
After 2 weeks feeding, food intake was slightly higher among rats fed the heme-supplemented diets. However, this did not result in different growth rates compared with control diet-fed rats (Table I). Fecal water cytoplastic activity reflects the exposure of the colonic mucosa to luminal irritants. In line with previous studies, dietary heme induces a >10-fold increase in fecal water cytoplastic activity (Table I). We studied whether this increased cytoplastic activity had any implications for one of the physiological functions of the colon mucosa. We measured the concentration of the cations sodium and potassium in the feces as a marker for the reabsorption capacity of the colon (30).
In rats fed heme, both the sodium and the potassium concentration increased in feces >10-fold and >4-fold, respectively, indicating an impaired reabsorption capacity of the surface epithelium (Table I). High luminal cytolytic activity might affect the viability of colon epithelial cells. We quantified the amount of host DNA in the feces as a marker of exfoliated epithelial cells. Table I shows that dietary heme reduced the amount of host DNA 100-fold compared with the rats on the control diet.

Histological examination showed that heme in the diet indeed resulted in increased injury of the surface epithelium (Table II). Colon samples of the heme-fed rats revealed an interrupted continuity of the surface epithelium architecture and presence of cells with a necrotic appearance compared with colon samples of control rats (Figure 1A and B). Although the mucosa was injured in the heme-fed rats, we did not observe any neutrophil infiltrations in the mucosa. A similar differential morphology was observed in the distal colon, but the injury could not be scored due to the much smaller intercryptal surface epithelium in the distal colon.

One of the mechanisms to compensate for cell loss and maintain crypt cell number is to increase proliferation. In line with previous experiments (9,12), we studied if dietary heme modified incorporation of $^3$H-thymidine into DNA of cells to quantify total DNA synthesis as a marker for proliferating cells. In rats fed heme, colonic proliferation doubled compared with the controls (Table II). Furthermore, proliferating epithelial cells in the S phase of the cell cycle were visualized by incorporation of BrdU into DNA (31). The colonic crypts of rats fed a control diet showed a normal label distribution in the bottom third of the crypt (Figure 2A). Colons of rats fed a heme diet showed an expanded proliferation compartment sometimes extending over half of the crypt depth (Figure 2B). Quantitatively, this resulted in a doubled labeling index in the proximal and distal colon of heme-fed rats in line with the $^3$H-thymidine results (Table II). Dietary treatment with heme also induced hyperplasia with an ~25% increase in number of cells in the crypts of proximal and distal colon (Table II). In addition, colonic crypt depth and labeling index correlated highly (0.82, $P < 0.005$).

Finally, colonocyte proliferation was also measured by demonstration of Ki-67 expression. This protein is ubiquitously expressed in G1, S and G2 phase of the cell cycle but not in the G0 phase (28). The effect of dietary heme on the proximal and distal colonic labeling index measured with Ki-67 immunohistochemistry correlated with the results of the BrdU immunohistochemistry ($r = 0.74$, $P < 0.005$) and corresponded with results from the $^3$H-thymidine assay (Table II).

Another mechanism that maintains crypt cell number is modulation of intraepithelial apoptosis. We studied apoptosis by measuring caspase-3 in colon mucosal scrapings. Caspase-3 is one of the last downstream caspases, which executes the apoptotic process (32,33). Western blotting of homogenates of control colon mucosa demonstrated expression of active caspase-3 (P17), whereas expression of inactive caspase-3 (P32) was low (Figure 3, inset). Colon mucosa of the heme-fed rats expressed higher levels of inactive caspase-3 and the expression of active caspase-3 was much lower compared with mucosa of control diet-fed rats. In addition to these results, we also measured the activity of caspase-3 in colon mucosal scrapings. Dietary heme reduced caspase-3 activity almost completely compared with control rats (Figure 3). We verified that the observed activities were specific for caspase-3 by preincubation of the samples with the

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**Table I.** Effect of dietary heme on food intake, animal growth, cytolytic activity of the luminal contents, fecal sodium and potassium concentrations and fecal host DNA

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Control (g/day)</th>
<th>Heme (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food intake</td>
<td>18.2 ± 0.4</td>
<td>19.2 ± 0.4a</td>
</tr>
<tr>
<td>Growth</td>
<td>3.2 ± 0.3</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Luminal cytolytic activity</td>
<td>9 ± 5</td>
<td>97 ± 1a</td>
</tr>
<tr>
<td>Fecal sodium (µmol/day)</td>
<td>48 ± 6</td>
<td>487 ± 18a</td>
</tr>
<tr>
<td>Fecal potassium (µmol/day)</td>
<td>66 ± 6</td>
<td>280 ± 14a</td>
</tr>
<tr>
<td>Fecal host DNA (µg/day)</td>
<td>19 ± 3</td>
<td>0.1 ± 0.1a</td>
</tr>
</tbody>
</table>

All data represent mean ± SEM (n = 16).

*Significantly different from their respective controls at $P < 0.05$ to $P < 0.001$ by Student’s $t$-test.

**Table II.** Effects of dietary heme on morphology and proliferation of colonic epithelium

<table>
<thead>
<tr>
<th>Proximal colon</th>
<th>Control</th>
<th>Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injury of intercryptal surface epithelium</td>
<td>10 ± 3</td>
<td>35 ± 4a</td>
</tr>
<tr>
<td>Crypt depth (cells/hemi-crypt)</td>
<td>43 ± 1</td>
<td>55 ± 2a</td>
</tr>
<tr>
<td>$^3$H-thymidine (dpm $^3$H/µg DNA)</td>
<td>43 ± 3</td>
<td>85 ± 4a</td>
</tr>
<tr>
<td>BrdU (LI)</td>
<td>7 ± 1</td>
<td>15 ± 1a</td>
</tr>
<tr>
<td>Ki-67 (LI)</td>
<td>10 ± 1</td>
<td>20 ± 2a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distal colon</th>
<th>Control</th>
<th>Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crypt depth (cells/hemi-crypt)</td>
<td>55 ± 3</td>
<td>70 ± 3a</td>
</tr>
<tr>
<td>$^3$H-thymidine (dpm $^3$H/µg DNA)</td>
<td>46 ± 6</td>
<td>92 ± 5a</td>
</tr>
<tr>
<td>BrdU (LI)</td>
<td>10 ± 3</td>
<td>22 ± 2a</td>
</tr>
<tr>
<td>Ki-67 (LI)</td>
<td>16 ± 2</td>
<td>23 ± 2a</td>
</tr>
</tbody>
</table>

Injury scores were assigned to intercryptal surface epithelium according to the criteria described in the Material and Methods. All data represent mean ± SEM (n = 8), dpm, disintegration per minute; LI, labeling index; % positive cells of the total number of crypt cells.

*Significantly different from control at $P < 0.05$ to $P < 0.001$ by Student’s $t$-test.

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**Fig. 1.** Representative sections of rat colonic mucosa stained with hematoxylin and eosin after 14 days feeding of control or heme diet. (A) Proximal colon of control rat showing normal colon epithelium. (B) Proximal colon of heme-fed rat showing a ruffled surface epithelium (arrow) and cells with a necrotic appearance (asterisk).
half the crypt. (57x359) Proximal colon of heme rat showing increased label distribution covering half the crypt.

The mechanisms underlying the association between colon cancer risk and consumption of red meat are not clear. However, recent epidemiological studies showed that the intake of heme might be associated with increased colon cancer risk. In line with previous studies, dietary heme increased cytolysis of fecal water (9,12,13). This may well be an initiating event by which heme affects cell turnover. Histological examination of the colonic tissues demonstrated that the much higher concentration of luminal irritants in rats fed heme indeed increased injury to the colonic surface epithelium, as indicated by a morphologically interrupted surface epithelium and presence of cells with a necrotic appearance. Microscopic analyses of the colon samples did not show any sign of heme-induced inflammation or neutrophil infiltration, indicating that the epithelial permeability barrier is not compromised within this 2 weeks dietary intervention. We speculate that cytolytic heme and its metabolites injure the surface of the colonocytes by lysis of their apical membrane, followed by leakage of their cellular content and the extrusion of the necrotic cell into the lumen. The continuity of the intestinal barrier remains intact during this process of extrusion/shedding as has been shown in several studies (34–36). Thus, cellular debris/antigens do not pass the epithelial barrier and influx of neutrophils does not occur. An important function of the surface colonocytes is reabsorption of water and minerals via the apical membrane (30). The 10-fold and 4-fold increased concentrations of fecal sodium and potassium in rats fed heme indicate that reabsorption was drastically inhibited, which might imply severe injury to the apical membrane of the surface colonocytes, supporting our histological findings.

The heme-induced cytolysis of the colonic contents may also explain the >100-fold decrease in fecal excretion of host DNA. Senescent enterocytes are normally shed (exfoliated) from the epithelial surface into the fecal stream after their life cycle of 3–5 days (18,36,37). As these cells go into apoptosis, their DNA becomes fragmented because a caspase-activated DNase only cuts internucleosomal linker DNA (38). The resulting fragments are apparently of sufficient size to be detected by the small-size target sequence (85 bp) of the rat β-globin gene in our real-time polymerase chain reaction (26).

Fig. 3. Effect of dietary heme on colonic mucosal apoptosis. The caspase-3 activity of rats fed the control or heme diet for 2 weeks. Colonic mucosa was scraped, homogenized and centrifuged. Caspase-3 activity in the supernatant was determined using ac-DEVD-pNA as substrate. Results from $n = 3$, mean ± standard error. Inset: Western blot of caspase-3 in mucosal scraping. C represents colon mucosa of rats fed a control diet and H represents colon mucosa of a heme-fed rat. P17: active caspase-3, P32: inactive caspase-3. Significantly different from control at $P < 0.001$ by Student’s t-test.

caspase-3 inhibitor DEVD-CHO. Almost no caspase-3 activity was detected in colon mucosa of control or heme-fed rats in the presence of the inhibitor indicating the specificity of the activity method (data not shown). To test whether colon homogenates of heme-fed rats contained inhibitors of caspase-3, equal amounts of homogenates of control and heme colon were mixed and assayed for caspase-3 activity. No decrease in activity was observed in the mixture compared with the activity of control homogenate only, showing the absence of inhibiting compounds.

Discussion

This study shows that dietary heme disturbs epithelial cell homeostasis in the colon of the rat. Cytolytic heme and/or its metabolites injure colonic surface epithelial cells and increase necrotic cell death. This heme-induced loss of cells triggers a compensatory epithelial hyperproliferation and inhibits mucosal apoptosis resulting in crypt hyperplasia.

The mechanisms underlying the association between colon cancer risk and consumption of red meat are not clear. However, recent epidemiological studies showed that the intake of heme might explain this association (22–24). In addition, human intervention studies suggested that endogenous nitrosation arising from ingestion of heme may account for the increased risk associated with red meat consumption (10). Here, we offer an alternative explanation as to why intake of heme might be associated with increased colon cancer risk. In line with previous studies, dietary heme increased cytolysis activity of fecal water (9,12,13). This may well be an initiating event by which heme affects cell turnover. Histological examination of the colonic...
blotting showed that colon mucosa of rats fed the control diet expressed the active form of caspase-3 and this corresponded with caspase-3 activity measured in the colon mucosa. This activity is similar to that reported earlier (41,42). In contrast, active caspase-3 expression in colons of heme-fed rats was low, which corresponded with the very low activity in the colon mucosal scrapings. However, regarding the detrimental effects of dietary heme, it is of relevance that inhibition of apoptosis is an important step in the carcinogenesis cascade (43).

In steady state, epithelial cell death and cell proliferation are balanced to maintain tissue homeostasis. Compared with rats fed the control diet, dietary heme increased colonic epithelial proliferation and thus cell death, ~2-fold. In previous studies, we showed already that heme induced colonic proliferation in rats, using incorporation of 3H-thymidine into DNA of cells as a marker of proliferation (9,12). Although this provided a quick and reproducible answer on the question whether diet modulates colonic proliferation, it did not give information on proliferation of non-epithelial versus epithelial cells or changes of the proliferation compartment within the crypt. Here, we demonstrated with BrdU and Ki-67 immunohistochemistry that heme-induced hyperproliferation is indeed limited to the epithelial cells in the crypt proliferation compartment. This supports our previous conclusions on dietary heme-induced effects on colonic epithelial proliferation. Thus, measurement of 3H-thymidine incorporation into DNA provides a fast and reproducible screening method for the effects of diet on proliferation in the colonic epithelium.

Finally, the heme-induced hyperproliferation and decreased apoptosis of colonic epithelial cells resulted in hyperplasia in the colonic crypts. In line with our results, Newmark et al. (44,45) showed in mice and rats that feeding a Western diet (low calcium and vitamin D, high fat and phosphate) without carcinogen treatment resulted in hyperproliferation of colonic epithelial cells and hyperplasia of colonic crypts. Long-term treatment of mice with this Western diet even depleted apoptotic cells in the colonic crypts and resulted in crypt dysplasias and the formation of tumors (45,46). This corroborates our finding that diet-induced modifications of the composition of the luminal contents can disturb the balance in colonic epithelial cell turnover and thus induce crypt hyperplasia. From the Ki-67 data in Table II, it can be calculated that ~60% of this increase in crypt cell number is due to proliferating cells and ~40% to G0 cells. We speculate that this increase in G0 cells is due to the observed inhibition of apoptosis. Similar results were obtained in the muc 2/- mice by Velchik et al. (47). The colonic epithelium of these mice is less protected against luminal contents because the mucous layer is deficient. They also showed an increased cell proliferation and a decreased apoptosis, which resulted in an increased crypt depth. Hyperplasia and a disturbed cell turnover are considered early risk markers of colon cancer (16,48).

In conclusion, we demonstrated that dietary heme disturbs colonic epithelial homeostasis in rats. This was due to a heme-induced injury of surface colonocytes resulting in hyperproliferation, hyperplasia of colonic crypts, inhibition of apoptosis and decreased fecal excretion of host DNA. Further mechanistic studies of the dietary modulation of this heme-induced aberrant cell turnover in colonic crypts may provide new leads for prevention of colon cancer.

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