Dietary resistant starch type 3 prevents tumor induction by 1,2-dimethylhydrazine and alters proliferation, apoptosis and dedifferentiation in rat colon

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Some epidemiological and experimental studies suggest that consumption of resistant starch is preventive against colon cancer. Resistant starch leads to a fermentation-mediated increase in the formation of short-chain fatty acids, with a particularly high butyrate fraction in large bowel. Butyrate is considered to be protective against colon cancer because it causes growth arrest and apoptosis and regulates expression of proteins involved in cellular dedifferentiation in various tumor cell lines in culture. We sought to investigate these processes under conditions of a carcinogenicity experiment in vivo. In the present study, 1,2-dimethylhydrazine-treated Sprague–Dawley rats were fed standard diet (n = 12) or diet with 10% hydrothermally modified Novelose 330*, a resistant starch type 3 (RS3), replacing digestible starch (n = 8). After 20 weeks tumor number, epithelial proliferation, apoptosis, immunoreactivity of carcinogenesis-related proteins [protein kinase C-δ (PKC-δ), heat shock protein 25 (HSP25) and gastrointestinal glutathione peroxidase (GI-GPx)], as well as mucin properties were evaluated in proximal and distal colon in situ. No tumors developed under RS3 diet, compared to a tumor incidence of 0.6 ± 0.6 (P < 0.05) under the standard diet. RS3 decreased the number of proliferating cells, the length of the proliferation zone and the total length of the crypt in the distal colon, but not proximal colon, and enhanced apoptosis in both colonic segments. It induced PKC-δ and HSP25 expression, but inhibited GI-GPx expression in the epithelium of distal colon. RS3 increased the number of predominantly acidic mucin containing goblet cells in the distal colon, but had no effect on the goblet cell count. We conclude that hydrothermally treated RS3 prevented colon carcinogenesis, and that this effect was mediated by enhanced apoptosis of damaged cells accompanied by changes in parameters of dedifferentiation in colonic mucosa.

Introduction

The large intestine is a complex environment in which colonic mucosa, mucus produced by the colonocytes, intestinal microbiota and their fermentation products, alimentary components and bile acids interact. Based on epidemiological data it was postulated that dietary fiber protects against the development of colorectal neoplasia (1,2). Fermentable dietary fiber, like starch, provides the majority of enzymatic substrate for saccharolytic colonic bacteria. Of particular interest is resistant starch due to its resistance to digestion in the small intestine and its use by cecal and colonic microbiota for anaerobic fermentation productive of short-chain fatty acids (SCFA), including acetate, propionate and butyrate. Butyrate is considered as an important factor in the maintenance of healthy function in colonic mucosa. Normal colonocytes gain 70–80% of their energy from butyrate (3) and therefore epithelial proliferation is supported (4). Conversely, intestinal tumor cell lines respond to butyrate with growth arrest, differentiation and apoptosis (5–10). In vitro studies demonstrated that butyrate in non-toxic concentrations causes growth arrest in the G0/G1 phase of the cell cycle resulting from induction of p21/Cip1, an inhibitor of cyclin D1 in the early G1 phase (4).

Several rodent short-term studies have demonstrated an increased acute apoptotic response to a genotoxic colorectal carcinogen (6 h post-azoxymethane application) in vivo by feeding resistant starch type 2 (11) or non-starch polysaccharides such as wheat bran (12,13). This pathway may allow removal of damaged cells prior to fixation of mutations. In vitro experiments have shown induction of apoptosis by butyrate through cytochrome c-mediated caspase activation, and partly by autophagic cell death (14). The former pathway is suggested to be executed via caspase-3-dependent activation of protein kinase C-δ (PKC-δ) (15). PKC-δ belongs to a PKC family of serin/threonin protein kinases, whose expression and activity are downregulated in colorectal cancer (16,17). PKC-δ gene expression and protein levels were found to be increased in HT-29 colon tumor cells under butyrate (18).

Heat shock protein 27 (HSP27) and its rodent homolog HSP25 can inhibit apoptosis and may subsequently facilitate malignant transformation (19,20). HSP25 is inducible by a variety of stimuli in intestinal epithelial cells, e.g. oxidative stress, cytokines, luminal bacteria and SCFA, in particular butyrate (21–23).

Cytotoxic effects in the colonic mucosa are also mediated via glutathione peroxidases, which are involved in cell’s antioxidant system by reducing hydrogen peroxide and organic hydroperoxides. Gastrointestinal glutathione peroxidase (GI-GPx, GPx2), one of five selenium-dependent glutathione peroxidases (24), appears to be the major glutathione-dependent peroxidase in intestinal mucosa (25). Mice lacking GI-GPx and an additional glutathione peroxidase (cGPx, GPx1) are highly susceptible to bacteria-associated inflammation and cancer (26–28). GI-GPx upregulation is an early step during

Abbreviations: DMH, 1,2-dimethylhydrazine; GI-GPx, gastrointestinal glutathione peroxidase; HSP, heat shock protein; PKC, protein kinase C; RS3, resistant starch type 3; SCFA, short-chain fatty acids.

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malignant transformation in the colon (29,30) and seems to be an early neoplastic marker. Furthermore, GI-GPx expression can be induced by luminal microbiota (31).

Mucus plays important protective and immunological roles while providing the environment for colonic microbiota (32). Mucins are high molecular mass glycoproteins responsible for the physical properties of the intestinal mucus (33). Butyrate and mucus play an important role in the prevention of colon cancer (34). However, the role of individual glycoproteins in the risk of colon cancer is still unknown. Mucins are high molecular mass glycoproteins responsible for the protective and immunological roles of the mucus layer (33). Butyrate is a known carcinostatic agent that induces apoptosis and prevents cell proliferation (34).

Materials and methods

Animals and diets

Male Sprague–Dawley rats (Charles River, MB, Denmark) (n = 20, weight on arrival 187 ± 6 g) were housed in stainless steel cages with split bedding under a 12 h/12 h light/dark cycle and free access to chow and water. Animals were divided into two experimental groups. Control animals (n = 12) were fed a semisynthetic standard diet. Test animals were fed 10 g RS3, replacing 10 g starch/100 g chow (Table I). After a run in phase of 1 week animals received 20 mg 1,2-dimethylhydrazine (DMH) (pH 6.5) per kg body weight by s.c. injection at 1-week intervals for 20 weeks.

Tissue preparation

Animals were killed by decapitation 1 week after the last DMH injection under ether anesthesia and autopsy was performed. Colon length and weight were recorded. The first 0.5 cm of the proximal colon and the final 0.5 cm of the distal colon were removed and washed in ice-cold phosphate-buffered saline. Tissue was fixed in 4% neutral buffered formalin for 24 h and washed for 24 h under tap water. Colon specimens were dehydrated and embedded in paraffin wax. Serial tissue sections (2 μm) from eight animals per diet group were cut. After a run in phase of 1 week animals received 20 mg 1,2-dimethylhydrazine (DMH) (pH 6.5) per kg body weight by s.c. injection at 1-week intervals for 20 weeks.

Immunohistochemistry and TUNEL assay

For the detection of Ki-67, active caspase-3, PKC-δ, HSP25 and GI-GPx, immunohistochemistry was performed on sections from the proximal and distal colon. Slides were immersed in boiling Target Retrieval Solution (ChemMate®-C210, DakoCytomation, Hamburg, Germany) and incubated with primary antibody for Ki-67 (MB-5, Dianova, Hamburg, Germany), cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA), PKC-δ (C-17 sc-213, Santa Cruz Biotechnology, Santa Cruz, CA), HSP25 (Stressgen, Victoria, BC, Canada) and GI-GPx (47), diluted in antibody diluent (DakoCytomation), overnight at 4°C in a humid chamber. For active caspase-3 detection, secondary horseradish peroxidase-conjugated anti-rabbit antibody (EnVision, DakoCytomation) was incubated for 35 min at room temperature. All other primary antibodies were treated with a biotin-spacer-conjugated secondary antibody IgG (Dianova) for 30 min at room temperature, followed by incubation with a streptavidin–biotin–horseradish peroxidase complex (StreptABComplex/HRP, DakoCytomation) and visualization via diaminobenzidine (DAB, DakoCytomation).

DNA strand breaks were detected by TUNEL assay (TACS®-XL-DAB Trevigen, Inc., Gaithersburg, MD). Four micrometer tissue sections were pretreated with protease K (1:50 dilution) at 37°C for 45 min and the assay was continued according to the manufacturer’s instructions.

Evaluation of proliferation, crypt length and apoptosis

Immunohistochemically detected Ki-67-positive cells, representing proliferation, were counted in 50 crypt columns per specimen. Only crypts with an open longitudinal crypt axis were analyzed. Their lengths and that of their proliferative zone were measured. The crypt length was determined as a distance (μm) between the basal side of the lamina epithelialis at the bottom of the crypt and the apical side of the lamina epithelialis at the top of the crypt. The proliferative zone was defined as the distance (μm) between the furthest positive Ki-67 cell in the bottom–top-direction in the crypt. Apoptotic cells, detected by active caspase-3 and TUNEL assay, were counted in four crypt quarters (from the lumen to the base of the crypt) of all longitudinally open crypts per tissue section (100–250) and expressed per 50 crypts.

Evaluation of PKC-δ, HSP25 and GI-GPx immunoreactivity

The immunoreactivity was semiquantitatively described with score points standing for a product of occurrence and intensity of the staining. The occurrence was assessed as percentage of positive cells among all cells of the same cell type (0 = no positive cells; 1 = 1–10%; 2 = 11–50%; 3 = 51–75%; 4 = 76–100%). The intensity of the staining was evaluated in three grades between negative and intense (0 = negative; 1 = weak; 2 = moderate; 3 = intense). The crypts were divided into quarters from the lumen to the base of the crypt. HSP25-immunoreactive epithelial cells were counted in all longitudinally open crypts per tissue section (100–250) and expressed per 50 crypts.

Mucin histochemistry

To allow for evaluation of the mucin composition in the goblet cells, serial sections were stained by Periodic Acid-Schiff (PAS) reaction for neutral mucins (magenta), 1% alcian blue (AB), pH 2.5, for detection of acidic sialo- and sulfomucins. Dual staining allowed for determination of mucin type predominance. Goblet cells staining more than 50% of the vacuoles deep purple contained predominantly neutral mucins, whereas those staining more than 50% blue were classified as acidic mucins. The same criteria were used for HID/AB staining. Thirty well orientated, longitudinally open crypt columns per specimen were counted.

Table I. Composition of experimental diets (g/100 g dry matter)

<table>
<thead>
<tr>
<th>Components</th>
<th>Standard group</th>
<th>RS group</th>
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</thead>
<tbody>
<tr>
<td>Starch a</td>
<td>63</td>
<td>53</td>
</tr>
<tr>
<td>Casein b</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Sunflower oil c</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Cellulose d</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>RS3</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mix e</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin mix f</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*aWaxy maize starch (National starch and Chemical Company, Bridgewater, USA) not containing any resistant starch.
*bDauermilchwerk Peiting GmbH, Landshut, Germany.
*cThomy GmbH, Karlsruhe, Germany.
*dDauermilchwerk Peiting GmbH, Germany.
*eNovose 330 (National Starch & Chemical, Bridgewater, USA), hydrothermally treated.
*fAltrunon GmbH, Lage, Germany.
Microscopy
Tumor scoring was performed using a stereomicroscope (SZH10, Olympus). All further microscopy and morphometry were done with the aid of a light microscope (Eclipse E1000, Nikon) in combination with a camera CCD-1300CB (Vosskuehler, Germany) and digital analysis software Lucia Image 4.61 (Nikon).

SCFA analysis
The SCFA were analyzed in contents of the proximal and distal colon by gas chromatography as described by Sembries et al. (48)

Statistical analysis
Data are presented in box plots, showing the distribution of the values within diet groups, by SPSS software, version 8.0, or as mean ± standard deviation (SD). Fisher’s exact test was used for the analysis of tumor incidence and Mann–Whitney U-test to determine the statistical significance of histological values.

Results

Weight progression
There was no significant influence of RS3 in the diet on the weight progression through the entire experimental period (Figure 1).

Tumor data
Six of eleven animals (a further animal was killed after 12 weeks after developing a cyst at the site of injection) in the standard group developed tumors as compared to zero of eight animals in the RS3 group \(P < 0.05\) (Table II). Six tumors were histologically classified as adenocarcinoma and one as adenoma. They were located in the late proximal and early distal large intestine (1 tumor after 30%, 1 after 40%, 1 after 50%, 3 after 60% and 1 after 70% of the length of the large intestine).

Proliferation and crypt length
The proximal segment of the colon showed no significant change in the number of proliferating cells, as assessed by Ki-67 staining, with RS3 compared to standard diet (Figure 2A and B). In the distal colon of the RS3 group there were significantly fewer Ki-67-positive cells per crypt present than in the standard group. The decreased proliferation in the RS3 group was accompanied by a significantly decreased proliferative zone (Figure 2C) and crypt length (Figure 2D) compared with the standard group.

Apoptosis
The frequency and distribution of the apoptotic cells were detected by the active caspase-3 immunohistochemistry (Figure 3A and C) and TUNEL assay (Figure 3B and D). Both methods showed markedly increased apoptotic activity in both the proximal and distal colonic segments of the RS3 group compared with the standard diet group. This was the case for active caspase-3-positive cells in the first and second crypt quarter in the proximal colon and in the second and third crypt quarter in the distal colon as well as for the TUNEL-positive cells in the first crypt quarter in both colonic segments. Unlike these findings, the RS3 group showed a decreased number of apoptotic cells detected by the TUNEL assay in the third crypt quarter in the distal colon, compared with the standard diet group.

PKC-δ expression pattern
PKC-δ immunoreactivity was primarily detected in epithelial cells (Figure 4). It was located in the luminal crypt quarter, diffusely distributed in cytosol, apical of the nucleus (Figure 4A). The immunoreactivity was slightly stronger in the distal than in the proximal colon (Figure 4D). The PKC-δ expression strongly decreased in poorly differentiated tumor epithelium (Figure 4C). Under the RS3 diet the immunoreactivity of the epithelial PKC-δ was significantly increased in the first crypt quarter in the distal, but not in the proximal colon (Figure 4D). In this diet group the staining was more continuous and localized apical as well as basal of the nucleus (Figure 4B). The immunoreactivity in lamina propria cells was very weak, cytosolic and irrespective of the colonic segment, diet and neoplastic features.

HSP25 expression pattern
Cytosolic HSP25 immunoreactivity was detected in cells of all layers of the colon wall in both colonic segments excluding the lamina epithelialis in proximal colon. Strongest HSP25 protein expression was found in submucosal blood vessels and in the lamina muscularis mucosae (Figure 5A). There was a slight increase in HSP25 expression in stroma cells, mainly in blood vessels, and in some epithelial cells in tumor compared with the morphologically normal mucosa. Under RS3 diet the number of HSP25-positive epithelial cells (Figure 5B) was significantly increased in the first crypt compartment in the distal colon compared with the standard diet (Figure 5D). In the proximal colon RS3 diet increased the expression of HSP25 significantly in lamina propria cells (Figure 5C).

GI-GPx expression pattern
Expression and cellular distribution of GI-GPx differed between the proximal and distal colon. The enzyme was

Table II. Incidence and multiplicity of tumors in the colon of DMH-treated rats

<table>
<thead>
<tr>
<th>Diet group</th>
<th>No. of animals</th>
<th>No. of tumor bearing animals</th>
<th>No. of tumors/animal</th>
<th>No. of tumors/tumor bearing animal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>12(^a)</td>
<td>6</td>
<td>0.6 ± 0.6</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>RS</td>
<td>8</td>
<td>0(^a)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)One animal was killed after 12 weeks.

\(^P < 0.05\), compared with the standard diet (Fisher’s exact test).
most abundant in the distal colon in cytosol of luminal epithelial cells (Figure 6A and B). In proliferating cells, GI-GPx was also detected in the nuclei—in the mid crypt zone in the proximal colon and in the crypt base in the distal colon (Figure 6A and D). In the proximal colon an additional faint membrane-bound staining in the crypt base was detectable. Tumors showed a higher cytosolic and nuclear GI-GPx expression than morphologically normal crypts (Figure 6C). RS3 diet significantly decreased cytosolic GI-GPx expression.
immunoreactivity in the third and fourth crypt quarter of the distal colon (Figure 6E). Nuclear immunoreactivity was decreased as well and restricted to the third and fourth crypt compartment in this colonic segment (Figure 6F).

**Goblet cell count and composition of the mucins in the goblet cells**

RS3 diet did not affect the goblet cell count in either colonic segment, independent of whether the values were expressed per crypt or per 100 µm crypt length (proximal colon, standard diet: 24.1 ± 1.9 (mean ± SD), RS3 diet: 24.4 ± 3.9; distal colon, standard diet: 29.8 ± 3, RS3 diet: 27.8 ± 1.7 goblet cells per crypt; proximal colon, standard diet: 17.7 ± 0.9, RS3 diet: 18.6 ± 1.4; distal colon, standard diet: 13.4 ± 0.8, RS3 diet: 14.5 ± 1.2 goblet cells per 100 µm crypt length). The composition of the mucins was not affected by the RS3 diet in the proximal colon. However, in the distal colon significantly fewer goblet cells containing neutral mucins in favor of those with acidic mucins were observed in the RS3 compared with the standard group (Figure 7, *P < 0.05). This distribution change was not affected by a slightly different goblet cell count between the two diet groups since this could be confirmed when the goblet cell count was set to 100% (*P = 0.001). We could also observe that in the distal colonic segment of both groups significantly more acidic mucins containing goblet cells were detected than in the proximal colon (Figure 7, *P < 0.05). When differentiating the acidic mucins, the crypts in the standard and RS3 group contained 59.5 ± 7.9% and 66.5 ± 6.4% (mean ± SD) sulfomucin-positive goblet cells in the proximal colonic segment in contrast to 98.5 ± 1.2% and 99.8 ± 0.3% (mean ± SD) in the distal colon. There were no significant differences between the diet groups.

**Luminal butyrate and total SCFA**

Total SCFA and butyrate concentrations in intestinal contents of the proximal and distal colon showed no significant differences between the diet groups. However, the values scattered over a wide range within each dietary group and colonic segment (Figure 8).

**Discussion**

We found that hydrothermally treated RS3 prevents the development of tumors in colon of rats treated with the genotoxic colon carcinogen DMH. Perrin *et al.* (45) observed a decreased number of early preneoplastic lesions, aberrant crypt foci, after azoxymethane application by feeding a diet containing 19% commercially available RS3 (retrograded amylose corn starch, Cerestar) with 30% indigestible starch. In our study the modified Novelose 330 provided 10% of the diet, but contained 75% resistant structures due to hydrothermal treatment (43). In further studies, other types of RS (49–52) and other butyrogenic sources such as soluble non-starch polysaccharides (53–56) were used. These substrates differ in fermentation properties and in many studies the level of resistant structures is not clear. This complicates comparison between studies and may explain some controversial results. While Young *et al.* (49) found increased incidence, multiplicity and size of tumors with resistant starch type 2 from potato starch in chemically induced colon cancer in rats, Thorup *et al.* (51) observed a lower number of aberrant crypt foci, using also resistant starch.
type 2 from potato starch. Nevertheless, in the majority of the studies some protection has been detected with resistant starches and other soluble non-starch polysaccharides.

Generally, these anti-carcinogenic effects of functional dietary fiber, including resistant starch, were attributed to a fermentation-mediated increase in luminal SCFA, particularly butyrate. However, it is difficult to correlate the preventive effect to the luminal butyrate concentration as various in vivo studies showed (55,56). It should be considered that butyrate concentration in the colonic content is a result of its production and absorption. It may also vary with time and these temporal changes may be affected by the eating behavior of the individual animals.

Butyrate is involved in the homeostatic maintenance of the colonic mucosa. It inhibits the proliferation of various neoplastic colonocytes (4). Our results demonstrate that similar effects occur in vivo under RS3 diet. They include reduced proliferation, smaller proliferation zone and shorter crypts in morphologically normal mucosa of the distal colon. The growth arrest leading to decreased proliferation could enhance DNA repair. Similar studies with other butyrogenic sources showed variable results regarding proliferation rate. Soluble dietary fiber, such as the fructans, reduced proliferation under conditions of carcinogen induction and displayed preventive effects (53). A RS3 did not affect proliferation although it decreased the number of aberrant crypt foci (45), and a resistant starch type 2 caused increased proliferation correlating with other tumor-promoting effects (49).

The effects of the chemical carcinogens DMH and azoxymethane upon colonic proliferation are well described. They include an increased proliferation rate, increased size of proliferation zone and deeper crypts (57,58). In normal colonic epithelium, luminal butyrate would mediate an increase in proliferation in the crypt (4), but under hyperproliferative conditions this is not expected to occur. The protective effect found in our study as a result of RS3 diet is hypothesized to arise via counteraction of hyperproliferative effects in the damaged colonic mucosa.

In previous studies it was shown that butyrate could induce apoptosis in various colon carcinoma cell lines (10,15,59–61). In our study apoptosis was significantly increased in both colonic segments of the RS3 group, contributing to antitumorigenic effects. Few in vivo studies on tumorigenesis with intervention by RS3 or other butyrogenic diets have rarely investigated the mucosal apoptotic response. Femia et al. (53) did not observe a significant increase in apoptosis after feeding of azoxymethane-treated rats with fructans for 31 weeks. On the contrary, increased apoptosis was detected with diets enriched in fructans only 24 h after DMH treatment (62). Along with proliferation, the treatment of rodents with a genotoxic carcinogen influences apoptotic cell death in the colonic crypts. Cells in the lower half of the crypt are most susceptible to acute apoptotic response 6–8 h after genotoxic treatment (13) as a physiological removal of damaged cells prior to mutation accumulation. In this context, studies with butyrate-producing fiber including resistant starch type 2 (11) and non-starch polysaccharides as wheat bran (12,13) demonstrated an increased acute apoptotic response after a genotoxic treatment. Although we did not study the acute response phase as tissue was harvested 7 days following the final DMH injection, apoptosis was still significantly increased. The majority of apoptotic cells were localized in the first and second crypt quarter.

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**Fig. 5.** Influence of RS3 on HSP25 expression pattern in the proximal and distal colonic mucosa of DMH-treated rats (n = 4). (A) Typical immunostaining against HSP25 in morphologically normal distal colon in the standard diet group showing immunoreactivity in cells of the lamina propria and lamina muscularis mucosae. (B) Discrete HSP25-positive epithelial cells within luminal mucosa under RS3. (C) HSP25 immunoreactivity in the lamina propria cells (evaluated semi-quantitatively in score points sp) and (D) the number of HSP25-positive epithelial cells (crypts were divided in quarters from the lumen to the base of the crypt) in the standard (ST) and RS3 diet group (RS) (*p < 0.05, compared with ST). The box plots are explained in the legend to Figure 2.
from the luminal side. Since this is the place where apoptosis normally takes place, the increase could implicate that RS3 ensures the reestablishment of homeostasis of the colonic mucosa by removal of damaged cells.

Highly associated with growth suppression (63) and apoptosis induction (64) is PKC-\(\delta\). We could show a significantly higher PKC-\(\delta\) expression with the modified RS3 in morphologically normal distal colonic mucosa. On the contrary, without RS3, in tumor epithelium from the standard group markedly lower levels of PKC-\(\delta\) were observed than in morphologically normal colonic mucosa. PKC-\(\delta\) is known to be downregulated in human colorectal tumors (16,17). These data emphasize the beneficial effects of the RS3 diet and implicate that RS3 in vivo exerts effects comparable to butyrate in vitro.

The PKC-\(\delta\) immunoreactivity was markedly stronger in the first quarter of the crypts. This is the crypt compartment where apoptosis was detected by TUNEL assay. In vitro PKC-\(\delta\) is involved in mitochondria-mediated apoptosis induction. It is cleaved to an active catalytic fragment by the caspase-3 followed by a further activation of caspase-3 (64). Interestingly, although PKC-\(\delta\) is proposed to be activated by caspase-3 under butyrate in human adenoma cells (15), we found almost no colocalization of the PKC-\(\delta\) and active caspase-3. The localization along the crypt clearly differed between these enzymes. The increase in the active caspase-3 in the RS3 group was highest in the second and third crypt quarter, while PKC-\(\delta\) was located in the first crypt quarter in the distal colon. Furthermore, we observed active caspase-3 expression in a small number of individual cells, whereas increased PKC-\(\delta\) immunoreactivity was continuously present in all crypts. Therefore, active caspase-3 and PKC-\(\delta\) may not be involved in the same apoptotic pathway in vivo, except possibly in the first compartment.

Butyrate can induce HSP25/27 expression in vitro and in vivo (23,65,66). HSP expression can modulate the fate of cells in response to stress or a death stimulus. RS3 diet resulted in

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**Fig. 6.** Influence of RS3 on GI-GPx expression pattern in the proximal and distal colonic mucosa of DMH-treated rats (\(n = 4\)). (A) Typical immunostaining against GI-GPx in morphologically normal distal colon in the standard diet group showing (B) a cytosolic localization of the enzyme in luminal epithelial cells and (D) a predominantly nuclear staining in cells of the crypt base. (C) In tumor epithelium cytosolic and nuclear GI-GPx immunoreactivity was increased. (E) Cytosolic and (F) nuclear GI-GPx immunoreactivity evaluated semiquantitatively [in score points (sp)] in four crypt quarters (from the lumen to the base of the crypt) in the standard (ST) and the RS3 diet group (RS) (*\(P < 0.05\), compared with ST). The box plots are explained in the legend to Figure 2.
upregulation of HSP25 in single epithelial cells exclusively in the first crypt compartment in distal colon. The number and localization of HSP25-positive epithelial cells we detected after feeding RS3 do not support the hypothesis that butyrate-producing fiber improve the general defense against oxidative injury via HSP25 in the luminal epithelium: pectin-enriched diet, without carcinogen treatment, resulted in a continuous upregulation of HSP25 in all luminal epithelial cells (23). Our results suggest it is more likely that upregulated HSP25 in the epithelium is involved in single cellular, e.g. apoptotic processes, rather than in a general barrier function. Other studies showed that HSP27 interacts with released cytochrome c in the cytosol, preventing formation of the apoptosome and subsequently inhibiting cleavage of procaspase-9 (67,68). Interestingly, HSP25 upregulation was restricted to discrete cells in the first crypt compartment whereas active caspase-3 was mainly confined to single cells in the second and third crypt compartment in distal colon, making possible that HSP25 indeed could inhibit activation of caspase-3 in vivo. We also detected an upregulation of HSP25 in lamina propria cells reflecting that RS3 not only affects gene expression of epithelial cells, but also of connective tissue cells. However, HSP25 upregulation might have different effects in epithelial and lamina propria cells, since lamina propria cells already showed a strong HSP25 expression. Whether the induction of HSP25 is mediated only by a higher butyrate level or also by changes in composition of colonic microbiota remains to be further investigated.

GI-GPx expression, suggested to play a role in mucosa homeostasis and to protect from ileocolitis and cancer (26), was also influenced under RS3 diet in distal colon. This is the first report demonstrating changes in the expression level of GI-GPx in situ after dietary intervention. We and others could detect an upregulation of GI-GPx already in very early stages of malignant transformation in colorectal tumors in humans (29,30) and also in the rat tumor model used. Even though subcellular localization of the enzyme slightly differed in man and rat, nuclear and diffuse cytosolic fraction show similar distribution pattern in morphologically normal distal mucosa and neoplastic tissue. It was supposed that increased GI-GPx may reflect increased cell proliferation (27). Interestingly, RS3 decreased the cytosolic GI-GPx fraction in the third and fourth crypt quarter and restricted the nuclear fraction to these compartments in morphologically normal appearing mucosa in distal colon. This probably reflects the decrease in proliferation zone and anti-hyperplastic effects, since predominant nuclear GI-GPx is known to be confined to the proliferating cells at the crypt base. It still remains to be elucidated whether this is a direct effect on GI-GPx expression due to RS3 diet or whether the downregulation is secondary due to anti-hyperproliferative effects. Secondary effects are more likely since cytosolic GI-GPx expression in the luminal crypt compartment was not significantly affected by RS3.

There are various physiological differences between the proximal and distal rat colon that must be considered. An important observation in our study is that PKC-δ, HSP25 and GI-GPx have a generally higher expression in epithelium of the distal colon compared with the proximal colon. Moreover, RS3 influence on the epithelial expression of PKC-δ, HSP25 and GI-GPx was confined to the distal colon. The position of the stem cells also differs between segments, which leads to localization of the proliferating cells in the proximal colon to the mid-crypt region and in the distal colon to the bottom of the crypt (69). Furthermore, the distal colon has a higher proliferation rate than the proximal colon, as also showed in the present study. The application of DMH increases the proliferation zone in both colonic segments (57), but RS3 diet significantly inhibited proliferation only in the distal colon. The distal rat colon is considered to be more comparable with human colon than the proximal (58).

Although the observations suggest that proliferation has less predictive value for colon tumorigenesis compared with apoptosis (58), a decrease in proliferation may still be linked to the preventive effect of RS3 found. In contrast, enhanced apoptosis was observed in both colonic compartments in the RS3
group. Therefore, we suspect that enhanced apoptosis was a more sensitive process in tumor prevention.

Colonic microbial composition and activity is known to be regio-specific as well. The immediate environment of the intestinal bacteria is the colonic mucus layer as a part of the extracellular barrier, which is produced by the goblet cells of the crypts. The MUC protein composition, and even more the oligosaccharide side chains vary between intestinal regions showing dependence on bacterial colonization (70). As in previous studies, we observed an increase in acidic and sulfated mucins from the proximal to the distal colon regardless of diet. In our study the quantity of mucin producing goblet cells was not affected by diet in any colonic segment. However, we found a significant increase in acidic mucins and a reduction of neutral mucins with RS3 compared with standard diet in the distal colon. This might be beneficial, since acidic mucins form a better barrier against bacterial translocation than neutral mucins (39). We assume that the increase in the acidic mucins to be partly a consequence of the prebiotic effect of the RS3 by changing the microbial population in the colon in favor of butyrate-producing species. RS3 stimulates the growth of bifidobacteria in non-associated (71) and human microbiota-associated rats (72), and thus may disrupt the growth of pathogenic bacteria and therefore contribute to the homeostasis of the microbiota in the colonic lumen. Parallel to microbial-derived influence of RS3 on the mucin composition in the colon, it also remains possible for butyrate to directly affect the expression of MUC and/or glycosylation-related genes. An upregulation of MUC2, MUC3 and MUC5B gene expression by butyrate has been demonstrated in vitro in a colonic goblet cell line (34). In particular, an upregulation of MUC2 would be considered beneficial since it has been involved in suppression of colorectal cancer (73).

In summary, we demonstrated that the hydrothermally treated RS3 is preventive of tumor development in vivo. Moreover, we observed enhanced apoptosis and decreased cell proliferation under the RS3 diet. Therefore, we suspect that an enhanced removal of damaged cells and an increased repair efficiency owing to lower proliferation may be involved in the tumor prevention by RS3. This coincided with diminished GI-GPx expression. Apparently, these effects can be ascribed to the used RS3 preparation providing a stable butyrate supply for the colonic mucosa, since we could also show further known butyrate-mediated effects like upregulation of PKC-δ, HSP25 and changes in mucus composition in situ.

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References


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