5-Aminosalicylic acid inhibits colitis-associated but not sporadic colorectal neoplasia in a novel conditional Apc mouse model

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Introduction

Inflammation and carcinogenesis are pathological consequences of injury and repair at a cellular and molecular level. Several studies suggest inflammation as a risk factor for the development of cancer, including colorectal cancer (CRC) (1). Individuals suffering from either of the two main chronic forms of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn’s disease, are at increased risk of developing CRC (2,3). 5-Aminosalicylic acid (5-ASA) is a non-steroid anti-inflammatory drug, widely used in the maintenance of remission and treatment of mild inflammatory exacerbations in IBD. Most epidemiological studies have shown that the chronic use of 5-ASA in IBD has chemopreventive effects on the development of CRC, although some studies failed to show this, as described in a recent meta-analysis by Velayas et al. (4). To study prospectively whether 5-ASA has chemopreventive actions in patients is hardly feasible, due to long duration of such a study, the high patient numbers needed, and ethical considerations. Therefore, for this kind of research, animal models are used that mimic the human disease, although most models lack similarities.

The ApcMin/+ mouse model carries a germ line mutation in one allele of the adenomatous polyposis coli (Apc) tumour suppressor gene. ApcMin/+ mice develop multiple intestinal neoplasia (min), mainly in the small intestine, due to loss of function of the intact Apc+ allele (5). Apc is a crucial negative regulator of the canonical Wnt pathway, forming a complex with axins and glycogen synthase 3β kinase, targeting β-catenin for degradation. Loss of functional Apc prevents formation of the complex, resulting in accumulation of β-catenin. Upon translocation to the nucleus and association with the TCF-4 transcription factor, β-catenin induces transcriptional activation of many genes involved in cell adhesion, migration and proliferation (6). The ApcMin/+ mouse model mimics the familial adenomatous polyposis syndrome in humans, an inherited form of CRC, caused by a germ line Apc mutation and characterized by development of 100–1000 adenomas in the large intestine. Most CRCs, however, are sporadic but they share early Apc mutations in 80% of the cases.

Reports on the effects of 5-ASA on the development of intestinal polyps in ApcMin/+ mice have been conflicting. McGregor et al. (7) showed that 5-ASA reduced the number of polyps, whereas Ritland et al. (8) found no reduction in polyp numbers. The ApcMin/+ mouse model is limited for studying the chemopreventive effect of 5-ASA on the development of CRC because these mice develop only a small fraction of lesions in the colon compared with the small intestine, and intestinal tumorigenesis is most probably already initiated in the first 2 weeks of life in these mice.

Recently, we generated a novel tissue-specific, sporadic CRC mouse model, FabplCre;Apc15lox/+ mice, compared with ApcMin/+ mice, FabplCre;Apc15lox/+ mice develop relatively few (<50) intestinal tumours, at a more advanced age, almost exclusively located in the distal part of the large intestine, similar to the human disease counterpart (E.C.Robanus-Maandag, manuscript in preparation).

A widely used and thoroughly described colitis model is the dextran sodium sulphate (DSS) model. DSS dissolved in the drinking water of rodents induces intestinal inflammation, including crypt damage and crypt loss. When DSS is applied in a low concentration (2% wt/vol) for a few days, followed by a period of normal drinking water, the inflammatory period is followed by a healing and repair period, resembling exacerbation and remission stages in human UC (9,10). DSS-induced colitis in the ApcMin/+ mouse model was shown to accelerate the development of colorectal tumours and to be useful as a colitis-associated intestinal cancer model (11,12).

In the present study, we determined the effects of local 5-ASA on the development of colorectal tumours in (i) FabplCre;Apc15lox/+ mice, which develop distally located tumours, to mimic human sporadic CRC development and (ii) FabplCre;Apc15lox/+ mice exposed to DSS, to study colitis-associated development of neoplasia. 5-ASA was found to effectively reduce the development of colitis-associated tumours, whereas it was ineffective in inhibiting sporadic tumour development.

Material and methods

Mice

All animal studies were approved by the ethical committee for animal studies of the Leiden University Medical Center and complied with national laws relating to the conduct of animal experiments. FabplCre;Apc15lox/+ C57Bl/6 mice were generated by mating FabplCre C57Bl/6 mice (line FabplCre –152Cre (13), kind gift of J.Gordon) with Apc15lox/+C57Bl/6 mice and genotyped by polymerase chain reaction (E.C.Robanus-Maandag, manuscript in preparation).
Colonic inflammation was induced in 5-week-old mice, with a median body weight of 17.5 g (range 13.0–22.0), by administration of 2% (wt/vol) DSS (MP Biomedicals, Aurora, OH; MW = 36,000–50,000) in tap water to the mice ad libitum. The solution was refreshed every other day, and the consumption was monitored daily by weighing the drinking bottle. In order to induce inflammation followed by a recovery period, the 2% DSS solution was given for 5 days followed by 16 days of tap water (Figure 1A). Mice were killed at the end of the experiment at 8 weeks of age or before the end of the experiment if they exhibited >20% weight loss, rectal bleeding, anaemia and lethargy. These latter mice were excluded from the results of the neoplasia scoring.

5-ASA medication
Salofalk 4g 5-ASA/60 gr enemas (Tramedico BV, Weesp, The Netherlands) were given daily by rectal injection of ~200 µl of enema solution in animals that were anesthetized with isoflurane/O2. The control animals were treated with 0.9% NaCl ‘placebo enema’. In the first experiment, the effect of 5-ASA was tested on DSS-induced colitis-associated neoplasia development in Fabpl-Cre; Apc^15lox^+/+ mice at 5 weeks of age, starting 2 days before DSS administration, in order to be effective before the colitis induction in the prevention protocol and to adjust the animals to the enema treatment protocol, up to the end of the 2 week recovery period (DSS/5-ASA and DSS/Placebo groups, experiment 1, Figure 1A). Similarly, Fabpl-Cre; Apc^15lox^+/+ mice were treated at 5 weeks of age with either 5-ASA or placebo enemas for 3 weeks without DSS administration, to determine the effect of 5-ASA on sporadic colorectal tumour development (5-ASA and Placebo groups, experiment 1, Figure 1A). In the second experiment, the Fabpl-Cre; Apc^15lox^+/+ mice were treated simultaneously with DSS and 5-ASA or placebo medication during the DSS-induced colitis (DSS+5-ASA and DSS+Placebo groups) or with 5-ASA only in the subsequent 2 week recovery period (5-ASA-post-DSS group, experiment 2, Figure 1B).

Assessment of colitis
The presence of blood in faeces, stool consistency and general appearance were recorded daily for each animal. Together, these factors constitute the disease activity index (DAI, range 0–6, fig 1C), as adapted from Cooper et al. (9). The daily-recorded body weight was not included in the DAI.

Histopathology
The large intestine (from caecum to anus) of killed mice was isolated and the intestinal tissues were embedded in paraffin (Swiss roll technique), sectioned at 5 µm and stained with haematoxylin and eosin, according to standard procedures. The sections were graded, in a blinded fashion, for the residual inflammatory infiltrate, with a range from 0 to 4 as to the amount and depth of infiltration: 0 = no infiltrate, 1 = focal infiltrate crypt base, 2 = infiltrate in lamina propria, 3 = infiltrate throughout the lamina propria and mucosal thickening and 4 = infiltrate extending into the submucosa. In addition, the lymphoid activity based on the number of lymph follicles was scored from 0 = none to 4 = more than three follicles. In combination a score of 0–8 could be obtained, a modification from the DSS-colitis microscopic activity index as reported before (14).

Immunohistochemistry
Paraffin sections were deparaffinized, endogenous peroxidase activity was blocked in 0.3% H2O2 (Merck, Darmstadt, Germany) in methanol for 30 min RT and rehydrated in a graded ethanol series to PBS. For antigen retrieval, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min in a microwave oven. The slides were cooled down to RT, rinsed with PBS (3 ×) and blocked with 5% goat serum (Dakocytomation, Glostrup, Denmark) in 1% bovine serum albumin PBS for 20 min at RT. Sections were incubated with the primary antibody in 1% bovine serum albumin/PBS (PBS, pH 7.2) for 24 h at room temperature (RT) and stored in 70% ethanol at 4°C. The neoplastic lesions were macroscopically counted under a dissection microscope (with an up to ×40 magnification) in a blinded fashion. The testicular tissues were embedded in paraffin (Swiss roll technique), sectioned at 5 µm and stained with haematoxylin and eosin, according to standard procedures. The sections were graded, in a blinded fashion, for the residual inflammatory infiltrate, with a range from 0 to 4 as to the amount and depth of infiltration: 0 = no infiltrate, 1 = focal infiltrate crypt base, 2 = infiltrate in lamina propria, 3 = infiltrate throughout the lamina propria and mucosal thickening and 4 = infiltrate extending into the submucosa. In addition, the lymphoid activity based on the number of lymph follicles was scored from 0 = none to 4 = more than three follicles. In combination a score of 0–8 could be obtained, a modification from the DSS-colitis microscopic activity index as reported before (14).

Statistical analysis
Statistical analysis of the changes in body weight, DAI, microscopic activity index, length of the large intestine, the number of neoplastic lesions and the immunohistochemistry scores were performed using the Student’s t-test. Differences were considered significant when P < 0.05.

Results
Increased DAI and loss of body weight due to DSS
The typical colitis symptoms, recorded as the DAI, increased dramatically in Fabpl-Cre; Apc^15lox^+/+ mice during and shortly after the 5 day DSS period in both DSS/5-ASA and DSS/Placebo groups (Figure 2A). In addition, both DSS-treated groups displayed a gradual loss of body weight at the end and shortly after the DSS period in contrast to the two non-DSS-treated groups (Figure 2B). The DSS-treated animals recovered from this severe colitis during the next two weeks, as indicated by a decrease in DAI and gain of body weight. In general, 5-ASA treatment by enema tended to a faster/better recovery of the DSS-treated mice (Figure 2A and B). This difference could not be
attributed to a difference in consumption of DSS-supplemented drinking water (Figure 2C).

**DSS-induced colitis accelerates large intestinal tumourigenesis**

Due to the DSS-induced colitis, the length of the large intestine of the DSS/Placebo group was significantly decreased compared with that of non-treated mice on average from 9.3 to 7.5 cm ($P < 0.0001$, Figure 3A and B). The DSS-induced colitis strongly accelerated intestinal tumourigenesis as indicated by the increased average number of large intestinal tumours from 5.2 tumours in non-treated mice to 28.8 tumours in DSS/Placebo mice ($P < 0.0001$, Figure 3C).

**5-ASA suppresses colitis-accelerated large intestinal tumourigenesis**

5-ASA treatment tended to increase the length of the large intestine of FabplCre;Apc$^{16lox/}$ mice treated with DSS (on average from 7.5 cm to 7.9 cm, $P = 0.17$, Figure 3B). More importantly, however, 5-ASA treatment significantly reduced the average number of colitis-accelerated large intestinal tumours by 37.4% from 28.8 tumours in the DSS/Placebo group to 18.0 tumours in the DSS/5-ASA group ($P = 0.03$, Figure 3C).

In contrast, 5-ASA treatment was unable to reduce the average number of sporadic tumours in the large intestine (5.2 and 4.8 tumours in the 5-ASA group and Placebo group, respectively, $P = 0.93$, Figure 3C).

**5-ASA exerts tumour-inhibiting effect during recovery period**

To determine whether 5-ASA exerted its preventive effect due to anti-inflammatory actions on the DSS-induced colitis or due to effects during the recovery period, mice were treated with 5-ASA or placebo enemas during the DSS administration period and 2 days thereafter (DSS+5-ASA group and DSS+Placebo group) and with 5-ASA enemas only during the 2 weeks of subsequent colitis recovery (5-ASA-post-DSS group, experiment 2, Figure 1B). The DAI and the body weight changes of these DSS-treated mice showed similar curves as those of the corresponding DSS-treated animals of experiment 1 (data not shown). Importantly, 5-ASA treatment during the DSS administration was not able to reduce the average number of tumours (29.5 and 29.2 tumours in the DSS+Placebo group and DSS+5-ASA group, respectively, $P = 0.96$, Figure 3C), whereas 5-ASA treatment during the recovery period tended to reduce the average tumour numbers (20.8 and 27.6 tumours in the 5-ASA-post-DSS group and Placebo group respectively, $P = 0.03$, Figure 3C).
numbers (20.8 and 28.8 tumours in the 5-ASA-post-DSS and DSS/Placebo groups, respectively, \( P = 0.07 \) and 29.2 tumours in the DSS\( + \)5-ASA group, \( P = 0.19 \) versus 5-ASA-post-DSS).

5-ASA exerts tumour-inhibiting effect in the distal large intestine
We examined the tumour-inhibiting effect of the 5-ASA treatment in more detail. First, the area covered by the 5-ASA enemas was determined in the DSS/5-ASA, 5-ASA-post-DSS and DSS\( + \)5-ASA groups. Mice were killed shortly after rectal installation of the 5-ASA enemas on the last day of the experiment and the distance covered by the 5-ASA enemas was measured. The 5-ASA enemas reached to \( \sim 3 \text{ cm} \) from the anus (Figure 4A), which was defined as the treatment area. 5-ASA significantly reduced the number of tumours in the treatment area by 51.3% in the DSS/5-ASA group as compared with the DSS/Placebo group (on average 9.4 and 19.4 tumours, \( P = 0.02 \), Figure 4B). The reduction concerned the development of both small (\( < 2 \text{ mm} \)) and larger (\( \geq 2 \text{ mm} \)) sized tumours (both, \( P = 0.02 \)), leaving 15.3% of the tumours \( \geq 2 \text{ mm} \) in the DSS/5-ASA group compared with 23.9% of the tumours \( \geq 2 \text{ mm} \) in the DSS/Placebo group. The large intestine proximal to the 5-ASA-treated area clearly showed no reduction in total tumour number, or in size of tumours (Figure 4C), and served as internal control, nicely confirming the tumour-inhibiting effect of the 5-ASA enema. Post-DSS treatment with 5-ASA also showed a consistent tendency towards a decrease in the number and size of tumours in the 5-ASA-treated area (\( P = 0.14–0.21 \), Figure 4B), leaving the more proximal large intestine unaffected (Figure 4C).

The effect of 5-ASA on histopathology, \( \beta \)-catenin expression, cellular proliferation and apoptosis
Histopathology of the distal large intestines of the DSS groups also showed many neoplastic lesions and a more severe inflammatory infiltrate (\( P < 0.001 \)), compared with that of non-treated Fabpl\( ^{-} \)Cre; Apc\( ^{-} \)lox/\( ^{-} \) mice (Figure 5A–D). Histopathological evaluation of the DSS/5-ASA group revealed less inflammatory infiltration, although not statistically significant, compared with DSS/placebo (Figure 5B, \( P = 0.19 \)) but not in the 5-ASA post-DSS group. The microscopic evaluations confirmed the reduced tumour numbers (Figure 5A) but
an exact histological quantification of the neoplastic lesions was not performed.

Immunohistochemistry showed nuclear accumulation of β-catenin in all tumours analyzed (illustrated in Figure 5D and Figure 6A,D and G), indicating that the Wnt pathway was up-regulated due to functional loss of the Apc allele in all tumours. We did not detect any obvious differences in intensity or localization, i.e. nuclear versus membranous/cytoplasmic, of β-catenin between the different groups.

The extent of proliferation, as determined by immunohistochemistry for Ki67, of both neoplastic cells and normal intestinal epithelial cells was similar for the non-treated, DSS/Placebo and DSS/5-ASA groups, showing a higher percentage of proliferating neoplastic cells (all groups $P < 0.003$) than that of normal epithelial cells (Figure 6B,E,H,K and J). Quantification illustrated that 5-ASA tended to reduce the proliferation of neoplastic cells in the DSS/5-ASA group compared with the DSS/Placebo group (on average from 55.6 to 52.7%, $P = 0.09$, Figure 6J), but 5-ASA did not reduce proliferation of normal epithelial cells in these two groups ($P = 0.47$, Figure 6J). The tendency to reduce proliferation of neoplastic cells by 5-ASA was not found in the groups without DSS treatment ($P = 0.73$, Figure 6J). Overall, proliferation of neoplastic and normal epithelial cells in the DSS-treated groups was very similar to that in the non-DSS-treated groups.

Finally, we investigated the extent of apoptosis by immunohistochemical detection of active caspase-3, an apoptotic protease. Apoptotic cells were mainly found at the top of normal crypts (Figure 6L) and incidentally in the lamina propria, whereas a heterogeneous distribution of apoptotic cells was seen in tumour tissue (Figure 6C,F and I). Quantification of the number of caspase-3-positive apoptotic cells within the epithelial lining and in the submucosa, excluding crypt abscesses and extruded cells, in the distal 3 cm of the colon revealed that 5-ASA did not significantly affect overall apoptosis in the DSS/5-ASA group as compared with the DSS/Placebo group, with a mean ± SEM of 26.1 ± 6.8 versus 18.3 ± 1.7 of apoptotic cells per centimeter muscularis mucosa, respectively.

Discussion

The use of the FabplCre;Apc15lox/+ mouse model in the present study provided a unique opportunity to evaluate the chemopreventive effect of 5-ASA on both sporadic and IBD-associated neoplasia. Our results show that 5-ASA is able to reduce IBD-associated tumour development by ~50% but not that of sporadic tumours. 5-ASA medication is prescribed to IBD patients as 5-ASA prodrugs, like sulphasalazine, specially coated (pH released) tablets or enemas to circumvent 5-ASA acetylation by cells in the upper digestive tract (15,16) or 5-ASA oxidation (17). In animal studies, 5-ASA is usually given orally as supplement of the drinking water or food (7,8,18–21). However, this administration seems not to be the most ideal treatment in animal models since it does not really mimic 5-ASA medication in humans. Therefore, we have chosen to treat the mice by rectal installation of a 5-ASA enema solution that is also used to treat IBD patients and allows a good distribution and high luminal concentration of the drug (22,23). Rectal administration of 5-ASA was not effective in suppressing the acute clinical signs of DSS-colitis, i.e. the DAI and body weight loss, during the induction phase. 5-ASA was nevertheless able to significantly reduce the number and size of colorectal tumours in that part of the intestine covered by the enema, the tumourigenesis of...
which was accelerated due to the DSS-induced colitis, mimicking colitis-associated colorectal carcinogenesis. Our observation that the tumour development in the proximal part of the colon is not affected by the 5-ASA enemas also illustrate that 5-ASA does primarily act locally and not systemically. Interestingly, 5-ASA treatment during the DSS-induced inflammation period, mimicking an inflammatory exacerbation in UC, was not able to reduce the development of colorectal tumours indicating that 5-ASA does not exert its cancer-preventive effects due to anti-inflammatory actions. In contrast, it was the 5-ASA treatment during the subsequent recovery period mimicking the remission stage in UC, which showed a reduction in the number of tumours, although borderline not significantly, but not in the intestinal inflammatory infiltrate indicating tumour preventive effects of 5-ASA during the recovery period.

Fig. 5. Histopathology of sporadic tumours and of IBD-associated tumours treated with 5-ASA or otherwise. (A) Haematoxylin and eosin stained sections of the distal large intestine of a non-treated, DSS/Placebo-treated and DSS/5-ASA-treated FabplCre/Apc15lox/þ mouse (original magnification ×20). Neoplastic lesions (arrows) and infiltration of inflammatory cells (arrowhead) are indicated. The distal parts of the intestines are orientated at the right side of the picture. (B) Intestinal inflammatory infiltration of the different treatment groups, as scored by the microscopic activity index (mean ± SEM), significant P-value ≤0.05 shown in bold. (C) A higher magnification (×400) of the squared region of the DSS/Placebo haematoxylin and eosin slide in (A), illustrating neoplasia as characterized by goblet cell loss, crypt distortion and enhanced nuclear β-catenin staining in (D).

Two recent reports showed that 5-ASA is also able to reduce colitis-associated neoplasia by ~50% in the DSS/azoxymethane model. Clapper et al. (20) described a suppressive effect of 5-ASA on the colitis-induced colorectal carcinogenesis when 5-ASA was administered before, during and after three DSS periods. Surprisingly, in this report only the lowest of three different dosages of 5-ASA supplemented to the drinking water, simultaneously with DSS during the DSS periods, was effective. Ikeda et al. (21) found a reduction in large intestinal polyp numbers and size when 5-ASA-supplemented to the food was administered in the remission stage, after two cycles of DSS-induced colitis. In both reports, however, it was not exactly clear which 5-ASA dosage the animals received because the animals were not housed individually
and the dosages are only based on the assumption that each animal has the same consumption of 5-ASA supplemented to the water and the food. However, due to the induced colitis each individual animal alters its eating or drinking pattern, which might influence the dosage intake considerably. In Clapper et al. (20), the mean different dosages of 5-ASA display different effects, indicating that the exact dosage per animal is very important. Moreover, both reports lacked an internal control and did not investigate the effect on sporadic tumour development without colitis induction. In the present study, we show that local administration of 5-ASA specifically represses colitis-associated neoplasia in the treatment area, when applied chronically during and after acute intestinal inflammation. This corresponds to human epidemiological studies showing a chemopreventive effect on colitis-associated CRC (4). One of the mechanisms of action of 5-ASA could be to reduce the proliferation of neoplastic cells as seen in our colitis-associated CRC model, in line with the report from Ikeda et al. (21).

5-ASA was not able to prevent the development of sporadic tumours in the FabplCre:Apcre15lox/+ mice. Apparently, 5-ASA cannot prevent colorectal tumorigenesis that is solely driven by the Apcre mutation and thereby mimicking sporadic CRC development. Moreover, 5-ASA did not affect the proliferation of neoplastic cells in these sporadic CRCs, in line with the absence of a preventive effect. These observations correspond to the data from Ritland et al. (8), describing no effect on the development of adenomas in the ApcreMin/+ mouse model. The only study performed in humans, addressing whether 5-ASA prevents sporadic CRC, examined the recurrence rate of sporadic colorectal adenomas in patients who underwent polypectomy and investigated apoptosis, which worked well but hampered the exact identification and counting of the percentage of apoptotic cells, because of additional staining of other (non-epithelial) apoptotic cells and cellular debris in the crypts and of extruded cells. Detection of the caspase-degraded product of cytokeratin 18 by M30 immunohistochemistry, widely used on human tissues and easy to quantify epithelial apoptosis, unfortunately did not work in our hands (data not shown) since it is probably not specific for mouse. 5-ASA might also induce cell death via non-caspase mechanisms (P.J.Koelink and H.W.Verspaget, unpublished results).

Fig. 6. Effect of 5-ASA on β-catenin expression, proliferation and apoptosis. Immunohistochemical analysis of neoplastic lesions in the distal large intestine from non-treated (A–C), DSS/Placebo-treated (D–F) and DSS/5-ASA-treated (G–I) FabplCre:Apcre15lox/+ mice and normal crypts from non-treated mice (K and L) for β-catenin (A,D and G), for Ki67 to determine the extent of proliferation (B,E,H and K), and for active caspase-3 to determine the extent of apoptosis (C,F,G and L). Magnification ×400. (J) Quantification of proliferation in the distal large intestines of different mouse panels. Percentages of normal (grey bars) and neoplastic cells (black bars) and P-values are shown (mean ± SEM).
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References


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