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Original Paper

Apoptosis induction by sennoside laxatives in man; escape from a protective mechanism during chronic sennoside use?

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Abstract

Chronic sennoside use induces melanosis coli (MC) and possibly increases colorectal cancer risk. Sennosides alter colonic crypt length, proliferative activity, and bcl-2 expression 18 h after administration. To investigate possible mechanisms for carcinogenesis, the effects of acute sennoside use and the presence of MC on colorectal epithelium were studied. Colorectal biopsies from 15 subjects receiving sennosides 6 h before sigmoidoscopy (Sen), 15 controls (NSen), and 27 with MC [11 moderate (MMC) and 16 severe (SMC)]. were analysed for degree of apoptosis (H&E staining), immunohistochemical p53, p21/WAF and bcl-2 expression, and proliferative activity (labelling index, LI). Apoptosis ($p = 0.0004$), intensity of p53 staining ($p = 0.01$), and p21/WAF expression ($p = 0.008$) were increased in Sen and SMC compared with NSen and MMC. p53 expression was increased in Sen ($p = 0.004$). No difference in bcl-2 expression or LI was observed. Crypts were shorter in Sen ($p = 0.05$) and longer in SMC ($p = 0.04$) than in NSen. It is concluded that sennosides acutely induce apoptosis of colonic epithelial cells, presumably by a p53, p21/WAF-mediated pathway, resulting in shorter crypts. In severe melanosis coli, apoptosis seems to be delayed, causing longer crypts without a rise in proliferative activity or bcl-2 expression. This escape from a presumably protective mechanism may enhance the risk of carcinogenesis during chronic sennoside use. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: **sennosides; proliferation; apoptosis; p53; bcl-2; carcinogenesis; colon; laxatives**

Introduction

Abuse of laxatives is quite common; 20–30% of people over the age of 60 use laxatives more than once a week [1]. Almost 25% of the laxatives used in an English population were senna-based products [2].

Chronic sennoside use is associated with the development of melanosis coli [3–5]. Though mostly regarded as harmless, melanosis coli has recently been observed more frequently in patients with colonic adenomas and carcinomas than in controls [6]. Speculation on a carcinogenic effect of sennosides is encouraged by experiments demonstrating mutagenic [7], genotoxic [8], and carcinogenic effects [9], the latter only seen after long-term exposure to high dosages, pointing to a tumour-promoting activity. Carcinogenesis generally involves a dysbalance between cell proliferation and apoptosis [10]. A key protein in the induction of apoptosis is p53, which arrests the cell cycle in case of DNA damage [11], p21/WAF being a mediator of this mechanism [12]. When DNA can then not be repaired, the cell undergoes p53-induced apoptosis, which is regulated by members of the bcl-2 family [10,13]. The bcl-2 gene is a suppressor of p-53-mediated apoptosis [14] and is frequently abnormally activated at an early stage of colorectal carcinogenesis [15].

Sennosides probably induce apoptosis after both short- and long-term ingestion. The lipofuscin pigment in macrophages of subjects with melanosis coli is thought to be derived from apoptotic bodies of colonic epithelial cells after sennoside-induced apoptosis [16]. Epithelial changes point to apoptotic cell death after short-term senna ingestion [17,18]. Eighteen hours after a single high dose of sennosides, the crypt length was reduced by 10–20%, proliferative activity was increased, and the bcl-2 gene was up-regulated. The latter was predominantly observed in crypts with increased proliferation and both probably represent compensatory mechanisms for epithelial cell loss. Increased proliferation is generally seen as one of the first steps in the carcinogenic process [19] and is seen in subjects with a high risk of colonic cancer [20].

To unravel the mechanisms of possible carcinogenic effects after long-term senna consumption, we studied the influence of sennosides on apoptosis, on mediators of the apoptotic pathway, and on proliferative activity, both in the acute situation after a single high dose of sennosides, and in patients with melanosis coli, assuming

this to be an indicator of chronic sennoside ingestion [3,21,22].

Materials and methods

Acute effects of sennosides were studied in 15 healthy volunteers, the Sen group (mean age 31 years, range 18–51 years, seven male and eight female), who received 2 mg/kg (maximal 150 mg) sennosides A and B (X-praep®, ASTA, Rijswijk, The Netherlands) 6 h before sigmoidoscopy, followed 3 h later by ingestion of 2 l of lavage solution containing polyethylene glycol and electrolytes (Klean prep®, Norgine, Amsterdam, The Netherlands).

The control group (NSen) included 15 consecutive patients (mean age 53 years, range 40–72 years, seven male and eight female), who had a colonoscopy for clinical reasons, including anaemia, abdominal pain or discomfort, a history of adenomatous polyps or a family history for colon cancer. Patients with (suspicion of) inflammatory bowel disease, with diarrhoea, or at risk of hereditary non-polyposis colorectal cancer were excluded. Patients received a standard bowel preparation of 2 l of lavage solution (Klean prep®) 15 h before colonoscopy and another 1–2 l on the morning of the colonoscopy. In both the Sen and the NSen group, three biopsies were obtained from the sigmoid colon at 30 cm from the anus.

For investigation of the chronic effects of sennoside laxatives, archival biopsy material from patients with melanosis coli (MC) was obtained by selection from all colorectal specimens with the histological diagnosis of MC collected between 1990 and 1997 at the University Hospital Groningen. Only operatively removed left bowel segments or sigmoid and rectal biopsies collected during endoscopy, after bowel preparation with either Klean prep® or phosphate enemas (Colex Klysma®, Sofar, Milan, Italy), were included.

The study was approved by the Medical Ethical Committee of the University Hospital Groningen. Informed consent was obtained from all subjects in the Sen and NSen groups.

Histological evaluation of biopsies

Specimens were fixed in formalin, paraffin-embedded, and cut into 3 μm thick sections, which were applied to 2-aminopropyltriethoxysilane-coated slides and stretched on a heated plate (30 min at 60°C). Slides were dried overnight in an oven at 37°C. Slides were stained with haematoxylin and eosin (H&E) and scored under blinded conditions by an experienced pathologist. All specimens were analysed for architectural changes, which were scored as either absent (–) or present (+). The degree of MC was scored as moderate (sporadic lipofuscin pigment present in the lamina propria) (MMC) or severe (easy to find, much lipofuscin pigment per high-power field) (SMC). The presence of apoptosis was determined and scored as normal (hard to find any apoptotic bodies) (0), moderately increased (sporadic apoptotic bodies, but easy to find) (1+) or strongly increased (several apoptotic bodies per high-power field) (2+).

Immunohistochemical staining

After deparaffinization of the slides, 200 μl of blocking solution [2% blocking reagent (Boehringer, Mannheim, Germany) in maleate buffer 0.15 M NaCl, pH 6.0] was added to each slide for antigen retrieval. Slides were heated twice for 10 min at 115°C with 5 min cooling in between and subsequently washed with phosphate-buffered saline [8.750 g of NaCl, 1.370 g of Na_2HPO_4, 0.215 g of KH_2PO_4 in 1 l of H_2O, pH 7.3 (PBS)]. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in PBS for 30 min.

Monoclonal antibodies were diluted in 1% bovine serum albumin (BSA) in PBS. For p53 staining, slides were incubated for 1 h at room temperature with a 1 : 400 dilution of BP53-12-1 (Biogenex, San Ramon, CA, USA), detecting both wild- and mutant-type p53. For p21 staining, a 1 : 50 dilution of p21-WAF (Ab-1) (Calbiochem, Oncogene Research Products, Cambridge, UK) and for Ki-67-staining, a 1 : 400 dilution of MIB-1 (Immunotech, Marseille, France) were used with 1 h incubation at room temperature. For bcl-2 staining, slides were incubated overnight at 4°C with a 1 : 400 dilution of anti-bcl-2 antibody (Dako, Glostrup, Denmark). After washing with PBS, the slides were successively incubated with a 1 : 50 dilution of peroxidase-conjugated rabbit anti-mouse antibody (RaM^{per}, Dako) and a 1 : 50 dilution of peroxidase-conjugated goat anti-rabbit antibody (GaR^{per}, Dako) in 1% BSA/PBS and 1% human serum for 30 min each. Peroxidase activity was visualized by incubation with 25 mg of diaminobenzidine dissolved in 50 mg of imidazole in 50 ml of PBS and 50 μl of 30% H_2O_2. Counterstaining of the nuclei was performed using Mayer's haematoxylin (Sigma, St. Louis, MO, USA) for 2 min. For p53, a breast carcinoma specimen was taken as a positive control (2+) and for p21, a normal colon specimen was used as a positive control (1+). For bcl-2 staining, incubation with an IgG1 antibody (Dako) and subsequently RaM^{per} and GaR^{per} served as a negative control (0+) and bcl-2 staining of infiltrating lymphocytes [15] was used as a positive internal control (3+).

Semi-quantitative determination of p53, p21, and bcl-2 gene protein expression

Evaluation of intensity and extent of staining was performed light microscopically by three blinded observers. Only whole-length cut crypts were evaluated and the final results were based on the evaluation of all crypts on the slide. The intensity of the staining was described as negative (0), weak (1+), strong (2+), or intense (3+). For bcl-2 and p21 staining, the percentage of the colonic crypts with $\geq 2+$ and $\geq 1+$ intensity, respectively, was determined. An extension of this staining intensity over the whole crypt from the

basal to the luminal side for bcl-2, and from the luminal to the basal side for p21, was considered 100%. For p53, the intensity of the staining was described as weak (1+), moderate (1.5+) or strong (2+). The extent of this staining from base to lumen was determined; a weighted score of the expression was calculated by multiplying the staining intensity by its extent.

Quantitative determination of proliferative activity using MIB-1 gene protein expression

Proliferative activity was determined in whole-length cut colonic crypts and expressed as the labelling index (LI). LI was defined as the percentage of labelled nuclei over the total number of nuclei; the LI of luminal, mid-, and basal compartments was determined by dividing the crypts into three equal longitudinal compartments. Crypt length was determined by counting the number of cells per crypt column. A mean number of 10.3 crypts per patient was counted, which was above 9, the minimal number of crypts sufficient to obtain a constant average value for the percentage of labelled nuclei in individual crypts, as determined by computing the running average.

Statistical analysis

The presence or absence of architectural changes and signs of inflammation were analysed with the chi-squared test. Differences between groups in the degree of apoptosis, and in the intensity and weighted score of p53 staining were determined using the Kruskal–Wallis test and the Mann–Whitney test. The extent of p53, p21, and bcl-2 staining were tested with one-way analysis of variance, using Duncan's multiple range test for determining differences between pairs of groups. Only p values less than 0.05 were considered significant.

Results

Biopsies from all 30 subjects in the Sen and the NSen group could be histologically evaluated. From 51 patients with MC, 27 (mean age 50 years, range 21–78 years, eight male and 19 female) were included for final analysis. MC was moderate in 11 (MMC group) and severe in 16 (SMC group). Architectural changes were not seen in any group. Slightly increased infiltration of the lamina propria with mononuclear cells occurred equally in all groups (Sen 2/15, NSen 4/15, MMC 2/11, SMC 6/16 patients).

More apoptosis was seen in Sen and SMC than in NSen ($p=0.001$ and 0.02, respectively) and MMC ($p=0.001$ and 0.01, respectively) (see Figures 1 and 2A–2C). Apoptotic bodies were predominantly observed in the superficial lamina propria, immediately beneath the surface epithelium. In SMC, apoptotic bodies were also observed in the inter-cryptal surface epithelium.

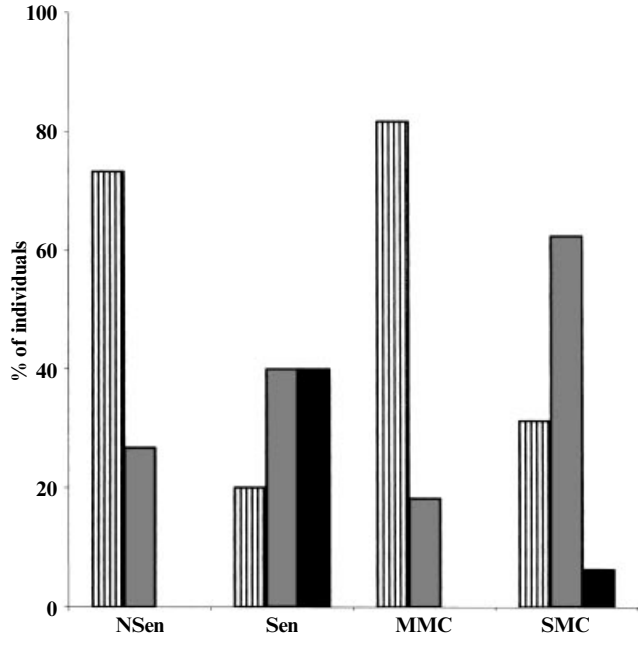

Figure 1. Degree of apoptosis in the four groups expressed as percentages of individuals with the following scores for apoptosis: ▥ 0, normal; ■ +, moderately increased apoptosis; ■ 2+, strongly increased apoptosis

p53 staining was evaluated in all Sen, Nsen, and SMC individuals, but because of limited material, in 9/11 MMC. p53 staining was observed in colonic epithelial crypt cell nuclei, with no cytoplasmic staining. Staining was predominantly basal with variable extension towards the lumen. Both the intensity and the extent of p53 staining were different in the four groups ($p=0.01$ and $p=0.004$ for intensity and extent, respectively). p53 staining was more intense in Sen ($p=0.0008$) and SMC ($p=0.02$) than in NSen (Figures 3A and 3B) and a trend for more intense staining was seen in Sen compared with MMC ($p=0.09$). Staining extended further in Sen, to $55\pm2\%$ of the crypt length, than in NSen ($42\pm3\%$, $p<0.05$), MMC ($37\pm4\%$, $p<0.05$), and SMC ($44\pm4\%$, $p<0.05$) (mean$\pm$SEM). Combining intensity and extent demonstrated an increased expression of p53 in Sen compared with NSen ($p=0.0001$) and MMC ($p=0.006$) (Figure 4), whereas in SMC it was intermediate and did not differ from the other groups. p21 expression was determined in all individuals. p21 staining was observed in the nuclei of colonic crypt epithelial cells, predominantly at the luminal crypt side, and in luminal epithelium. Extension of the p21 staining from the lumen downwards was variable and different in the four groups ($p=0.008$), extending further in Sen ($53\pm2\%$ of the crypt length), than in NSen ($40\pm3\%$, $p<0.05$) and MMC ($40\pm4\%$, $p<0.05$) (mean$\pm$SEM). p21 staining in SMC also extended further ($49\pm3\%$) than in NSen ($p<0.05$) (Figure 5). bcl-2 staining was determined in all individuals and was observed in lymphocytes of the lamina propria and in the cytoplasm of epithelial crypt cells. It was predominantly observed in the basal part of the crypts and extended towards the lumen. bcl-2

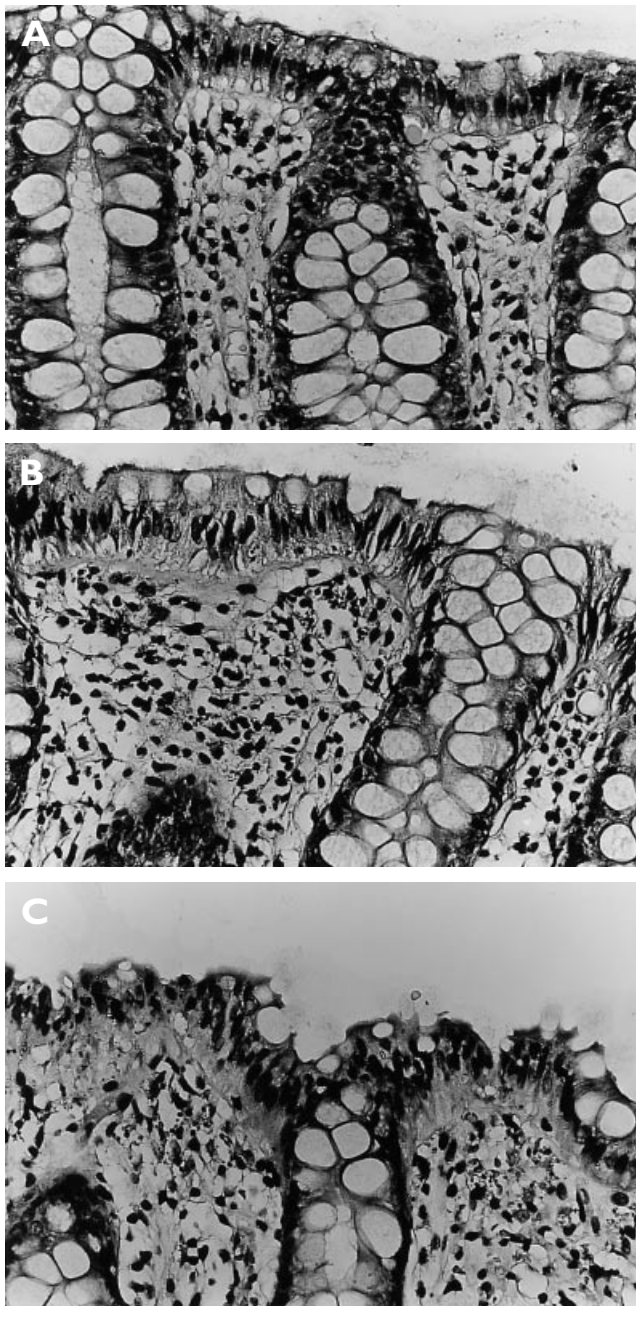

Figure 2. (A) Apoptosis in an NSen patient. Little to no apoptosis is seen. Score 0 (normal). ×220. (B) Moderately increased apoptosis in an SMC patient (score 1+). Apoptotic bodies are present in the lamina propria, and some in the epithelium. ×220. (C) Strongly increased apoptosis is seen in a Sen patient (score 2+). Apoptotic bodies are seen both in the epithelium and immediately beneath the basal membrane. ×220

staining extent did not differ between the four groups [NSen 57±6%, Sen 59±5%, MMC 41±8%, SMC 56±5, $p=0.19$ (mean±SEM)].

Proliferative activity was determined in all Sen and NSen individuals and, because of limited tissue, in 8/11 MMC and 15/16 SMC patients. LI in whole crypts was the same in all groups and no difference in luminal, mid- or basal LI could be demonstrated (Table 1). Crypt length was different in the four groups ($p=0.008$). Crypts were shorter in Sen (59.3±1.5 cells

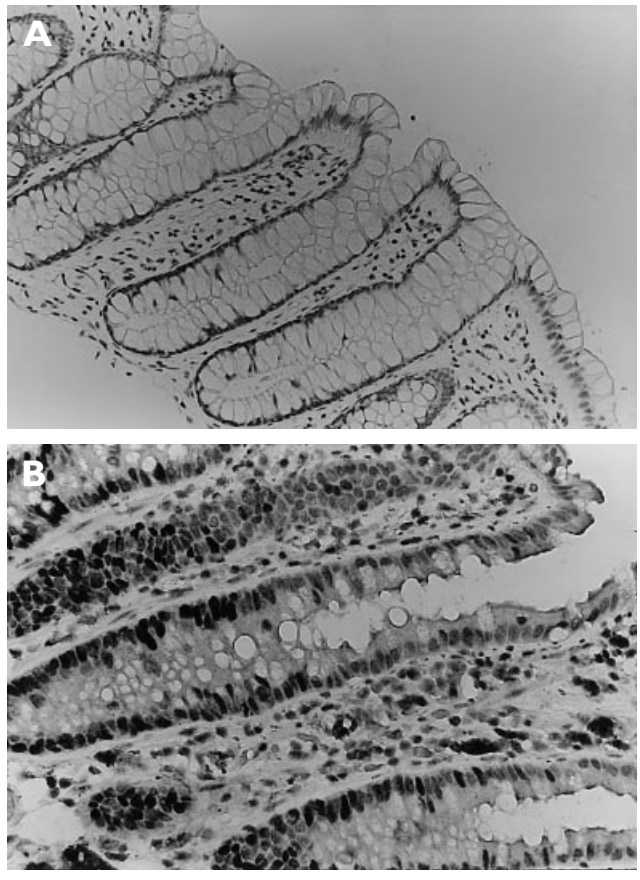

Figure 3. (A) p53 staining in an NSen patient. Intensity 1+, extent of staining 40% of crypt length from the basal part of the crypt, weighted score 40. ×110. (B) p53 staining in an SMC patient. Intensity 2+, extent of staining 50% of crypt length from the basal part of the crypt, weighted score 100. ×220

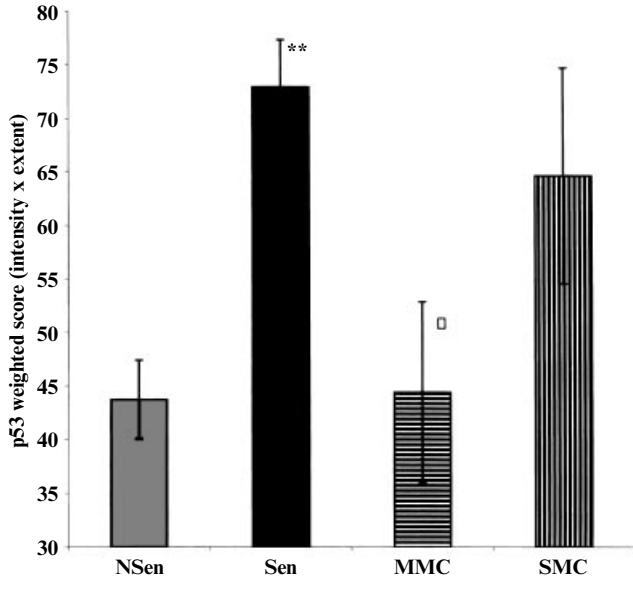

Figure 4. p53 weighted score (intensity × extent) (mean±SEM) (on the *y*-axis) in the four groups. ■ NSen, ■ Sen, ▤ MMC, ▥ SMC (on the *x*-axis). **$p=0.0001$ compared with Nsen; □ $p=0.006$ compared with Sen

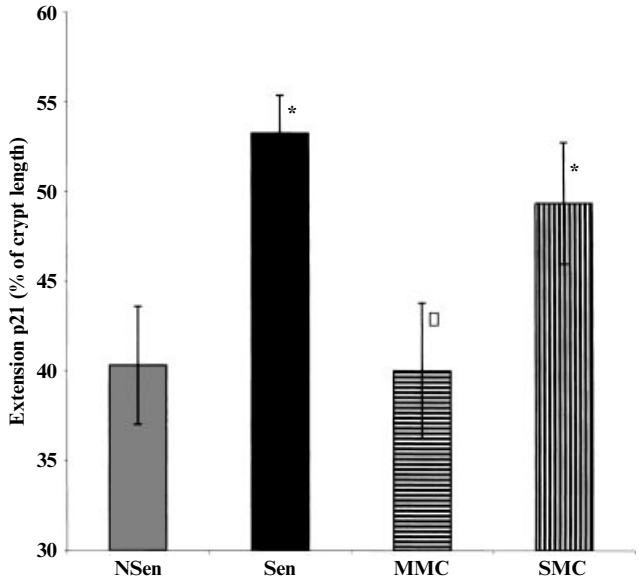

Figure 5. Extension of p21 staining (mean ± SEM) from luminal to basal side of crypt (on the *y*-axis) in the four groups; ■ NSen, ■ Sen, ▤ MMC, ▥ SMC (on the *x*-axis).* $p < 0.05$ compared with Nsen; □ $p < 0.05$ compared with Sen

per crypt) than in NSen (64.6 ± 2.0, $p = 0.05$) and SMC (72.8 ± 2.9, $p = 0.0008$). SMC also had longer crypts than the NSen group ($p = 0.04$). Crypt length in MMC (64.5 ± 4.9) did not differ from the other groups. To investigate whether this difference in crypt length could obscure an existing difference in proliferative activity, the total numbers of labelled cells per crypt were compared as well, but no difference was demonstrated (Sen 62 ± 4, NSen 60 ± 4, MMC 68 ± 9, SMC 72 ± 5, $p = 0.19$).

Discussion

Chronic use of sennosides has been associated with an increased risk of colonic cancer [6], implicating a possible carcinogenic potential of these laxatives. Both in the study by Siegers et al. [6] and in the present study, MC has been equated with chronic sennoside use. Although MC can probably also be caused by other laxatives [23] or factors [24], there is a strong correlation with chronic sennoside ingestion. Of patients chronically using anthranoid laxatives, 73.4% developed MC [4] and in three studies including together about 1000 patients with MC, 95% admitted habitual laxative use [3,21,22].

This study provides clues to how apoptotic colonic epithelial cells are cleared from the mucosa; shows for the first time up-regulation of p53 in grossly normal colonic mucosa; and shows structural as well as functional abnormalities of the colonic epithelium in cases of MC, which are relevant for colon carcinogenesis.

The mean age in the Sen group was lower than in the other groups. This most likely does not significantly affect the results, as in a previous study a similar reduction in crypt length was seen 18 h after sennoside ingestion in a group with a mean age of 45 years (range 15–71 years), comparable to the NSen and MC groups [18].

Six hours after ingestion of a single high dose of sennosides, increased apoptosis was demonstrated in the colonic epithelium. Apoptosis occurred almost immediately after metabolic activation, as the time required for transport to the colon is reportedly 4–5 h [25], although in our patients it was slightly shorter, due to the transit-accelerating effect of the lavage. Levels of apoptosis in our study were only measured using a semi-quantitative scale and should therefore be interpreted with a degree of caution, but the finding of enhanced apoptosis is in agreement with animal studies [16]. Also in guinea pigs, apoptotic bodies appeared almost immediately after the drug reached the caecum, 4 h after oral ingestion, whereas a peak was seen after 6 h [16]. Most apoptotic bodies were observed in the lamina propria, with smaller numbers in the intercryptal surface epithelium, as in our study. Likewise, apoptotic epithelial cells in humans are probably phagocytosed by intraepithelial macrophages and subsequently transported to the lamina propria [16], or vice versa [26]. A recent morphological study of the human colon suggested that epithelial cells undergoing apoptosis pass through fenestrations in the basement membrane to the lamina propria, where they are taken up by macrophages [27]. Although increased numbers of macrophages were not found in our study, the marked decrease of the crypt length strongly suggests an epithelial origin for the apoptotic bodies in the lamina propria.

In the acute stage, there was no sign of any compensatory reaction, such as increased proliferative

Table 1. Proliferative activity of whole crypts and in different crypt compartments (percentage of labelled nuclei of the total number of nuclei) and crypt length (number of cells) in NSen, Sen, MMC, and SMC

Crypt compartment	NSen (mean ± SEM)	Sen (mean ± SEM)	MMC (mean ± SEM)	SMC (mean ± SEM)	*p* value
Luminal	13.3 ± 3.0	14.9 ± 2.4	18.0 ± 5.1	16.9 ± 3.2	0.76
Mid	59.1 ± 4.1	63.7 ± 3.0	62.9 ± 5.7	60.5 ± 3.9	0.83
Basal	68.0 ± 3.1	66.8 ± 2.6	71.3 ± 4.9	68.7 ± 2.1	0.82
Total	48.4 ± 3.0	50.1 ± 2.6	52.4 ± 4.6	49.2 ± 2.6	0.86
Crypt length	64.6 ± 2.0	59.3 ± 1.5*†	64.5 ± 4.9	72.8 ± 2.9*	0.008

*$p \leq 0.05$ compared with NSen.
†$p = 0.0008$ compared with SMC.

activity or enhanced inhibition of apoptosis by bcl-2. The damage induced by a single dose of sennosides is a rapid effect that lasts for less than 18 h, as apoptosis was no longer present 18 [18] to 24 h [16] after ingestion.

The acute apoptotic reaction is associated with increased expression of p53 and p21, which suggests that the apoptosis is mediated through a p53-dependent pathway, inducing p21 overexpression. Members of the bcl family such as bcl-xS, bax, and bak [28] may also be involved in this process, but this was not determined. Apparently, the damage caused by sennosides is such that apoptosis ensues instead of repair.

In MC patients, the degree of apoptosis varied widely, but severe forms of MC showed an increase of apoptotic bodies compared with controls. It is unclear why the MMC group does not exhibit enhanced apoptosis, but it may be due to a longer time interval between the last sennoside ingestion and biopsy, as it is known that the lipofuscin pigment gradually disappears over a period of 6–11 months after stopping sennoside use [29].

Apoptosis in SMC patients is probably also mediated by a p53-dependent pathway, as this protein was overexpressed in this group. However, only the intensity of p53 staining was increased, whereas its extent was comparable to controls.

Opinions differ as to when staining is positive for p53; some authors accept the presence of any positive cells [30], whereas others require the staining of more than a certain percentage [31], or use a weighted score including both extent and intensity [32]. Bartek *et al.* [33] agree that the intensity of the staining in itself is a measure of the amount of p53 present. Originally, immunohistochemical detection of p53 protein was interpreted as evidence of a p53 mutation, as p53 protein from a mutated gene often has an increased half-life compared with wild-type and was therefore more easily detected [34]. Increased amounts of wild-type p53 can, however, also be detected immunohistochemically and overexpression of the normal protein can occur in response to DNA damage [10]. In this study, we used the BP53-12-1 antibody, which detects both mutant and wild-type p53 protein. After acute sennoside administration, increased p53 expression was observed within hours, excluding the possibility that it involved mutated p53. In SMC patients, it is also almost certain that increased expression of wild-type p53 is involved. Expression of mutated p53 mostly marks the transition from benign to malignant neoplasia and is first observed in severe dysplasia in the colon [35], but in our patients, dysplasia was never observed. We therefore consider the sennoside-induced overexpression of p53 as that of wild-type protein.

Continued overexpression of p53 could enhance the risk of colorectal cancer. Accumulation of wild-type p53 protein was present in colonic adenomas [36] and in different types of carcinomas [37]. Again, in 30% of the colorectal carcinomas, overexpression of the p53 protein did not represent mutations of the gene [38]. Additionally, *in vitro* studies have demonstrated that overexpression of wild-type p53 can play a role in the malignant transformation of epithelial cells [39] and fibroblasts [40].

Despite increased expression of p53 and p21 protein and enhanced apoptosis, epithelial crypt length in SMC was increased compared with both the Sen and the NSen group. Previous studies demonstrated excessive proliferation [17,18] and increased bcl-2 expression [18] 18 h after a high dose of sennoside laxatives [17,18], but after chronic sennoside use, these compensatory mechanisms do not seem to occur. Other mechanisms should therefore be involved in causing the remaining epithelial cells to survive and in prolonging their life-span. Maybe these cells become to a certain degree refractory to sennoside-induced apoptosis during long-term sennoside exposure, perhaps through overexpression of multidrug resistance-associated protein 1 (MRP_1) or P-glycoprotein (P-gp). These drug efflux pumps are present in colonic epithelial cells and are involved in protection against xenobiotics [41]. An *in vitro* study has demonstrated that rhein, the active metabolite of sennosides, is indeed a substrate for the MRP_1 pump [42]. Increased survival of colonic epithelial cells and resistance to apoptosis could enhance the risk of mutation and therefore of neoplastic change.

This study demonstrates for the first time that apoptosis induced by short-term sennoside use is probably mediated by a p53/p21-dependent pathway. Six hours after administration, crypt length is decreased and the compensatory mechanisms seen after 18 h, such as increased proliferation [17,18] and inhibition of apoptosis by enhanced bcl-2 expression [18], are not yet present. Although the colon is able to react adequately to a single high dose of sennoside laxatives by repairing the induced damage, chronic use leads to persistent p53 and p21 overexpression and subsequent apoptosis. However, an increasing number of cells seem to become refractory to apoptotic cell death, thus possibly enhancing the risk of colorectal cancer. Future studies will have to determine whether the colonic epithelium of MC patients has become refractory to acute sennoside stimulation and whether drug efflux pumps, in particular MRP_1, are involved in this phenomenon. In view of these results and of other studies suggesting that sennosides have a tumourigenic potential, chronic use of these laxatives should be discouraged and alternative therapeutic approaches should be sought for constipated patients.

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