5-ASA - colorectal cancer - cell death : an intriguing threesome

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Chapter 5

5-Aminosalicylic acid induces cell death in colorectal cancer cells *in vivo*

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Abstract

5-Aminosalicylic acid (5-ASA) has colorectal cancer (CRC)-preventive properties, although the mechanism is largely unknown. We aimed to determine the ability of 5-ASA to induce cell death in cancer cells *in vitro* and *in vivo.* HT29 colon cancer cells were treated with 5-ASA *in vitro*. Thirty-one patients with a colorectal neoplasm were treated with 5-ASA enemas for 14 consecutive days. Neoplastic and normal tissue biopsies were taken before and after 5-ASA treatment. Apoptosis was assessed by determination of caspase-3 activity, caspase-degraded cytokeratin 18 (M30 antigen) and total cytokeratin 18 (CK18) levels. 5-ASA induced caspase-3 activity *in vitro,* but blocking caspase-3 was not able to completely inhibit the cell death inducing effects of 5-ASA, indicating the contribution of non-caspase mediated mechanisms. 5-ASA induced caspase-3 activity in colorectal carcinomas and normal mucosa, but not significantly in adenomas, revealing the induction of apoptosis also *in vivo*. 5-ASA significantly decreased CK18 levels in carcinomas, indicating the loss of epithelial cancer cells. We conclude that 5-ASA induces caspase-dependent apoptosis in CRC cells, both *in vitro* and *in vivo*. Moreover, 5-ASA is also capable of inducing caspase-independent cell death *in vitro*, which might have contributed to the loss of cancer cells *in vivo*.

Introduction

The intestinal epithelium is under continuous renewal with a complete repopulation of this lineage within 4-5 days. Homeostasis of intestinal crypts depends on a tightly regulated balance between epithelial cell proliferation, migration up the crypt, differentiation and finally shedding and programmed cell death, i.e. apoptosis [1]. A disturbance in the balance between these processes, in favour of cell proliferation, leads to uncontrolled growth, i.e. cancer. Colorectal cancer (CRC) is one of the most common malignancies in the Western world. With 940.000 recorded cases worldwide each year it is the third most common malignancy in the world, after lung and breast cancer, causing nearly 500.000 deaths each year worldwide [2].

Apoptosis has been widely studied in CRC, with a large variation of detection techniques [3], some more specific for apoptosis than others [4]. The caspases, or death proteases, are cysteine-proteases that are responsible for the morphological changes during apoptosis. Caspase-3 is at the point of convergence of the two main apoptotic pathways, the extrinsic and intrinsic pathway, and cleaves most of the cellular substrates in the apoptotic process [5]. Measurement of caspase-3 activity is therefore a reliable measurement of apoptosis, and low tumour caspase-3 activity is an independent risk factor for disease recurrence in colon and rectal cancer [6, 7].

5-Aminosalicylic acid (5-ASA, mesalazine), a non-steroid-anti-inflammatory drug (NSAID) widely used in the treatment of inflammatory bowel disease (IBD), has been reported to have cancer-preventive properties, although the precise mechanisms are not known. It has been shown that 5-ASA can induce apoptosis in a wide variety of CRC cell lines *in vitro*, although it is not completely clear which apoptotic pathway is induced by 5-ASA treatment [8-11]. In the present study we revealed the capability of 5-ASA to induce apoptosis in CRC cells *in vitro* and *in vivo*.

Material&Methods

Cell culture and materials

HT29 colon and MCF-7 breast cancer cells were cultured in Dulbeco's Modified Eagle Medium DMEM:F12 (1:1) + GlutaMAX medium supplemented with 10 mM Hepes, 100 U/ml penicilin, 100 µg/ml streptomycin and 50 µg/ml gentamycin (all Invitrogen, Breda, The Netherlands) and 10% heat-inactivated fetal calf serum (FCS, Perbio Science, Erembodegem, Belgium). 5-ASA (Dr. Falk Pharma, Freiburg, Germany) was dissolved in

culture medium, pH adjusted (~7.4) and filter-sterilized. Staurosporine (Sigma Aldrich, Germany) was dissolved in dimethyl sulfoxide (DMSO) in a 1 mM stock solution and added in a final concentration of 1 μM. Q-VD-OPh (Apoptame^{TM}Q, MP Biomedicals, Irvine, CA, USA), a pan-caspase inhibitor, was added in a final concentration of 20 μM (1:1000) and the concentration of DMSO did not exceed 0.1 %. All incubations with 5-ASA (5 ml in volume) were protected from light as much as possible. Non-attached cell were collected by centrifugation at 350 x *g* and attached cells were de-attached by trypsin /ethylene diamine tetraacetic acid (EDTA) treatment. Fractions were pooled and cell viability was determined by trypan blue exclusion assays in a heamatocytometer.

Protein samples were made by adding lysis buffer (10 mM Hepes, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA) to cell pellets and 3 cycles of shearing (freeze in liquid nitrogen, thaw at 37 ˚C, place tube on ice, vortex 10 s).

Patient study

Patients with a histological proven neoplastic lesion in the distal sigmoid or rectum took 4 gram 5-ASA/100 ml enemas (Yamanouchi Pharma BV, Italy) or 4 gram 5-ASA/60 gram Salofalk enemas (Trademico BV, Weesp, The Netherlands) for 14 consecutive days before bedtime. Exclusion criteria were: chronic inflammatory bowel disease, familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC, or Lynch syndrome), history of salicylate allergy, pregnancy, other NSAID use during previous 6 months and blood urea > 10 mmol/L or blood creatine > 150 μmol/L. Before starting the 5-ASA treatment a first set of endoscopical biopsies was taken from the colorectal neoplasm and normal mucosa. After the 14 day treatment a second set of biopsies was collected. Biopsies were stored at -70°C until use [12]. The clinico-pathological patient characteristics of the 31 patients with a colorectal neoplasm that were treated with 5-ASA enemas for 14 days are shown in *Table 1*. Of the 31 patients 18 patients were diagnosed with a carcinoma, 12 patients were diagnosed with an adenoma, and 1 patient with 2 adenomas. The study was performed with informed consent from the patients obtained according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre (protocols P172/94 and P219/98) and in compliance with the Helsinki Declaration.

Table 1: Patient characteristics of 5-ASA treated patients.

	Gender	Age	Dukes' stage	WHO	Diameter (cm)	Differentiation grade	Ulceration
1	M	79	B	adeno	4.7	Moderate	No
2	M	68	B	adeno	2.5	Well	No
3	F	63	B	mucinous	3.0	Moderate	Yes
4	M	64	C	adeno	4.5	Moderate	No
5	M	75	C	adeno	2.6	Well	No
6	F	61	B	adeno	4.3	Moderate	No
7	F	46	C	adeno	4.5	Moderate	No
8	M	67	B	adeno	3.0	Moderate	No
9	F	52	B	adeno	4.0	Moderate	No
10	F	51	B	adeno	5.0	Moderate	No
11	M	70	C	adeno	7.0	Moderate	Yes
12	M	44	C	adeno	3.0	Moderate	No
13	M	73	B	adeno	3.5	Well	No
14	M	44	A	adeno	3.0	Moderate	Yes
15	F	50	A	adeno	2.0	Moderate	No
16	M	81	A	adeno	4.5	Well	No
17	M	71	B	adeno	2.0	Moderate	Yes
18	F	79	B	mucinous	3.1	Moderate	Yes
			Grade of Dysplasia	Histological type			
19	F	91	LGD	Villlous	3.0		
20	F	65	LGD	Tubulovillous	1.4		
21	M	66	LGD	Villlous	< 1.0		
22	F	63	HGD	Tubulovillous	1.0		
23	F	66	HGD	Villlous	3.0		
24	M	69	LGD	Tubulovillous	< 1.0		
25	M	47					
a			LGD	Tubulovillous	< 1.0		
b			LGD	Tubulovillous	< 1.0		
26	M	64	HGD	Tubulovillous	7.5		
27	M	65	LGD	Villlous	3.9		
28	M	74	HGD	Tubulovillous	3.5		
29	F	60	LGD	Tubulovillous	5.0		
30	M	77	LGD	Tubulovillous	< 1.0		
31	M	74	LGD	Tubulovillous	< 1.0		

Clinical characteristics of patients with a colorectal carcinoma (1-18) or adenoma (19-31) that were treated with 5-ASA enemas for 14 days. Patient 25 was diagnosed with 2 adenomas. HGD: high grade dysplasia, LGD: low grade dysplasia.

Tissue homogenization and protein determination

Two frozen tissue specimens were weighed and homogenized on ice for 2 minutes in 1 ml Tris-HCl, 0.1% Tween 80, pH 7.5 per 25 mg tissue using a Potter device (B Braun, Germany), and centrifuged twice at 8000 x *g* for 2.5 min at 4°C. Protein content was measured according to Lowry *et al.* and standardized by bovine serum albumin [13].

Caspase-3 activity measurement

For measurement of caspase-3 enzymatic activity, samples were incubated with 2.5 nmol of the enzyme substrate Ac-Aps-Glu-Val-Asp-AMC (Ac-DEVD-AMC, Bachem, Heidelberg, Germany) in a 100-mM HEPES buffer, pH 7.25, containing 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40, and 10 mM dithiothreitol (7). During incubation at 37°C, fluorescent AMC was cleaved off by active caspase-3, corresponding with the level of caspase-3 activity in the sample. The fluorescent AMC was monitored at an excitation of 360 nm and emission of 460 nm using a Fluostar Optima plate reader (BMG Labtech gmbh, Offenburg, Germany). Calibration curves were constructed using free AMC. Caspase-3 activity was indicated in pmol AMC/min/mg protein.

Flow cytometric analysis

After pooling adherent and non-adherent fractions in phosphate buffered saline (PBS) HT29 cells were centrifuged at 500 x g and fixated in 3 % formaldehyde pH 7.2 for 10 minutes at room temperature (RT). Afterwards cells were permeabilized in ice-cold 90 % ethanol on ice for 15 minutes, washed with 0.05 % Tween 20/PBS (PBST), blocked with 1 % bovine serum albumin (BSA) in PBST for 10 minutes at RT, and incubated with mouse-anti-M30 Cytodeath (1:100, Roche Applied Science, Penzberg, Germany) and rabbit-anti-active-caspase-3 (1:200, Cell Signaling technology, Danvers, MA, USA) in 1 % BSA/PBST for 1 hour at RT. After washings with PBST cells were incubated with goat-anti-mouse-Alexa594 and goat-anti-rabbit-Alexa488 (both 1:500, Invitrogen) in 1 % BSA/PBST for 30 minutes at RT. After washings with PBST cells expression was analyzed by flow cytometry on a FACScalibur (BD Biosciences, Franklin Lakes, NJ, USA). 10.000 events were stored for each sample and analyzed with Flojo software (Tree Star, Inc. Ashland, OR, USA).

Western blotting for total caspase-3

Protein samples (40 µg) were subjected to polyacrylamide SDS gel electrophoresis and blotted to a nitrocellulose membrane (Whatman, Dassel, Germany). After washings with PBST the membrane was blocked with 2.5 % milkpowder (Bio-Rad laboratories, Hercules, CA, USA) in PBST on a rocking platform for 2 hours at RT and subsequently incubated over night at 4˚C with a total-caspase-3 antibody (1:500, Cell Signaling technology) in 0.5 % BSA/PBST. After 3 washes with PBST and secondary horseradish-peroxidase (HRP)-labelled goat-anti-rabbit (1:1000, Dako, Glostrup, Denmark) incubation for 2 hours at RT the signal was developed with Supersignal West Pico Chemiluminescent Substrate (Pierce,

Rockford, IL, USA), and detected by a LCD camera (Bio-Rad). Blots were afterwards stripped with restore stripping buffer (Pierce) and probed with a β-actin antibody (1:10.000, Sigma) to check equal loading.

M30 and CK18 antigen level detection

M30 and total CK18 antigen levels were detected by M30 and M65 ELISAs, respectively, as described previously [14]. ELISAs were preformed according to protocol by manufacturer (Peviva BV, Bromma, Sweden) in 0.025-25 μg of total protein sample and expressed as antigen units per mg protein (U/mg).

Statistical analysis

Statistical analysis was performed with Statistical Package for Social Sciences (SPSS) statistical software (version 12.0 for Windows, SPSS Inc, Chicago, IL). To determine the effect of 5-ASA on caspase-3 activity *in vitro*, Student's t-tests were used. To determine effects of 5-ASA treatment on caspase-3 activity, caspase-3 activity corrected for epithelial cells (Caspase-3/CK18), M30 antigen levels, CK18 antigen levels and percentage of epithelial cells (M30/CK18 x 100) Mann-Whitney U or Wilcoxon signed-rank tests were used. For the correlation of M30 and caspase-3 activity levels the Spearman's correlation coefficient was calculated.

Results

5-ASA induces caspase-3 activity in vitro

HT29 cells were treated with increasing concentrations of 5-ASA for 24, 48 and 72 hours, which induced caspase-3 activity up to 4-fold in a concentration and time-dependent manner (*Figure 1A*). 5-ASA was not very potent in inducing caspase-3 activity when compared with staurosporine, which induced an 11-fold caspase-3 activity much quicker (24 hours) in a much lower concentration (1 μM). Flow cytometry analysis showed more cells expressing active caspase-3 due to 5-ASA treatment (48 hours, 40 mM), but indeed not as much as in staurosporine treated cells (*Figure 1B*, left) confirming the caspase-3 activity data. This was further strengthened by a similar expression pattern of caspase-degraded cytokeratin 18, i.e., the M30 antigen (*Figure 1B*, right).

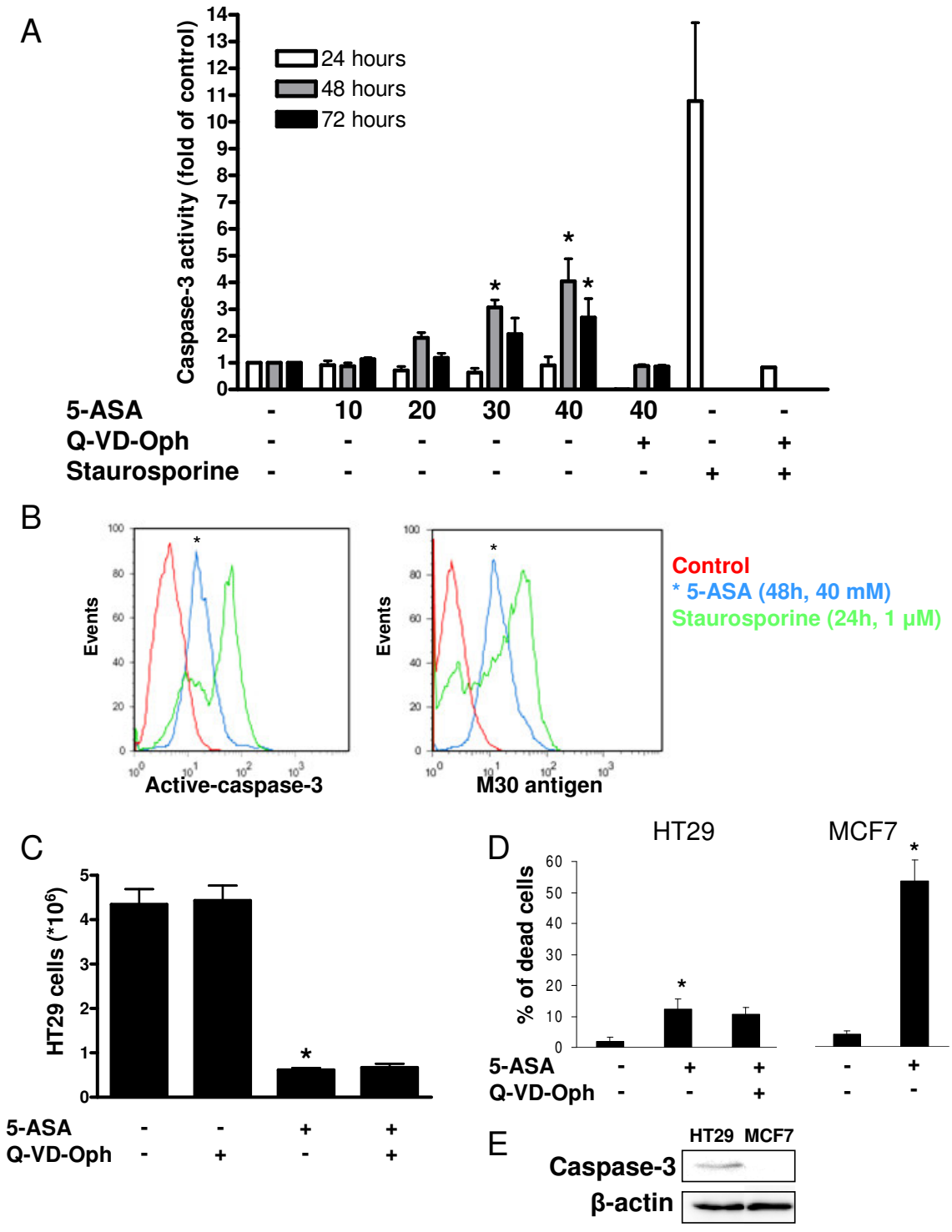

Figure 1: 5-ASA induces caspase-dependent and independent cell death.

*A) Caspase-3 activity of HT29 treated with different concentrations of 5-ASA (mM) for different time points, or 1 uM staurosporine, and Q-VD-Oph, expressed as fold of control (mean + SEM). B) Flow cytometric analysis of active caspase-3 and M30 on HT29 cells treated with 40 mM 5-ASA (72 hours) or 1 uM stauropsorine (24 hours). C) HT29 viable cell numbers after 72 hours of 40 mM 5-ASA treatment with or without Q-VD-Oph, and controls (mean + SEM). D) Percentage of dead HT29 and MCF-7 cells after 48 hours of 40 mM 5-ASA treatment (mean + SEM). E) Total caspase-3 westernblot of HT29 and MCF-7 cells. All data of at least 3 independent experiments. * P<0.05 versus control.*

5-ASA induces caspase-independent cell death in vitro

Seventy-two hour treatment with 5-ASA reduced the number of viable HT29 cells with 86 % (*Figure 1C*). Adding Q-VD-OPh, a pan-caspase inhibitor [15], efficiently blocked the induction of caspase-3 activity (*Figure 1A*), but was nevertheless not able to decrease the effect of 5-ASA treatment on viable HT29 cell numbers (*Figure 1C*). To determine if 5-ASA induces cell death, and not apoptois only, we determined the percentage of death cells. 5-ASA induced cell death (12 %), even in the presence of Q-VD-OPh in HT29 cells (11 %, *Figure 1D*). Differences between these assays are due to the anti-proliferative effects of 5-ASA. 5-ASA was also able to induce massive cell death in MCF-7 cells (49 %, *Figure 1D*), which lack caspase-3 (*Figure 1E*). These results indicated that 5-ASA induced cell death accompanied by an induction of caspase-3 activity (classical apoptosis), and that 5-ASA is also able to induce cell death by a mechanism that does not require the activity of caspases.

5-ASA induces apoptosis in colorectal carcinomas in vivo

Treatment for 14 days with 5-ASA enemas increased the caspase-3 activity of carcinoma tissue in 14 of the 18 patients (Wilcoxon signed rank test, P=0.03), indicating the induction of apoptosis (*Figure 2*). There was no such an effect in adenomas (P=0.81), although in 7 of the 13 patients the caspase-3 activity did go up, while in the normal mucosa of all the patients there also was a significant increase in caspase-3 activity (an increase in 25 of the 30 patients, P=0.006). There were no statistical differences between the caspase-3 activity in carcinomas, adenomas and normal mucosa either before or after 5-ASA treatment.

5-ASA decreases epithelial cells in colorectal carcinomas in vivo

Because 5-ASA can alter the make up of the cells in the biopsy, we determined the amount of epithelial cells by a cytokeratin 18 (CK18) ELISA in the same protein homogenates. The 5-ASA treatment caused the loss of CK18 in carcinomas (*Figure 3A*, P=0.04), indicating the loss of epithelial cells. There was no such effect in adenomas or normal mucosa. Interestingly, adenoma biopsies tended to have higher levels of CK18 when compared with carcinoma biopsies (Mann-Whitney U test, P=0.07) and normal mucosa (P=0.005) before 5-ASA treatment, indicating a different make-up with more epithelial cells. After 5-ASA treatment carcinomas had a significant lower level of CK18 compared with adenomas or normal mucosa (both P=0.004).

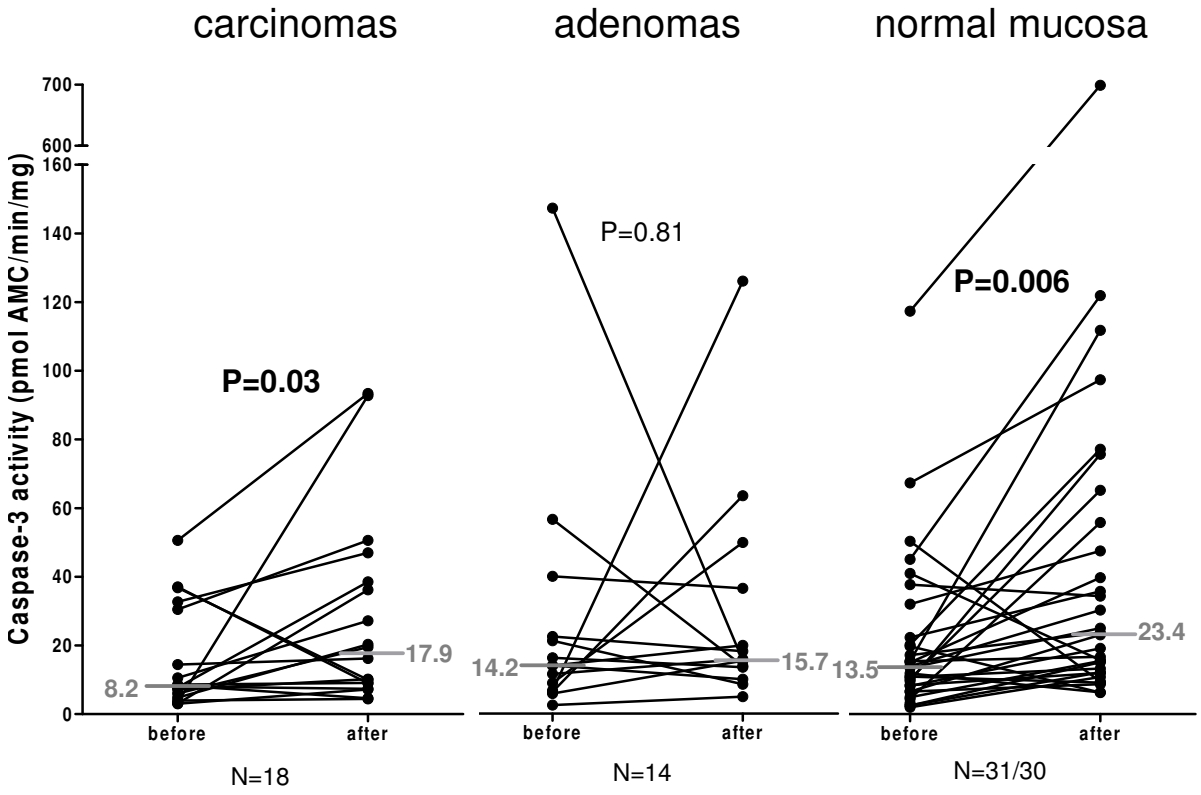

Figure 2: 5-ASA induces apoptosis in colorectal carcinomas in vivo.

Caspase-3 activity in the carcinoma, adenoma or normal mucosa of patients before and after 14 days of 5-ASA enema treatment. The differences before or after 5-ASA treatment were determined with Wilcoxon signed rank test (with P<0.05 considered significant in bold). Grey lines indicate median values. From one of the adenoma patients no qualitative normal tissue biopsies were obtained after 5-ASA treatment.

There was a significant correlation between CK18 levels and caspase-3 activity in the carcinomas, both before (Spearman's Rho= 0.476, P=0.05) and after 5-ASA treatment (Spearman's Rho=0.567, P=0.01), suggesting mainly epithelial apoptosis. When we corrected caspase-3 activity for CK18 levels (caspase-3/CK18), the increase in carcinomas and normal mucosa was also seen (data not shown, P=0.02 and P=0.05).

5-ASA does not specifically increase epithelial apoptosis

Determination of M30 antigen levels in the same protein homogenates, by an ELISA, allowed us to directly investigate epithelial apoptosis. M30 antigen levels were not significantly increased by 5-ASA treatment in carcinomas (11 up, 7 down, P=0.70, *Figure 3B*) or normal mucosa (17 up, 13 down, P=0.35), which suggests that there is no specifical epithelial apoptosis induction, but an induction of total apoptosis in carcinomas and normal mucosa. There were no differences in M30 antigen levels between carcinomas vs adenomas

vs normal mucosa either before or after 5-ASA treatment. Correction of the M30 antigen levels for CK18 levels also did not show any statistical differences between the tissues before or after 5-ASA treatment (data not shown).

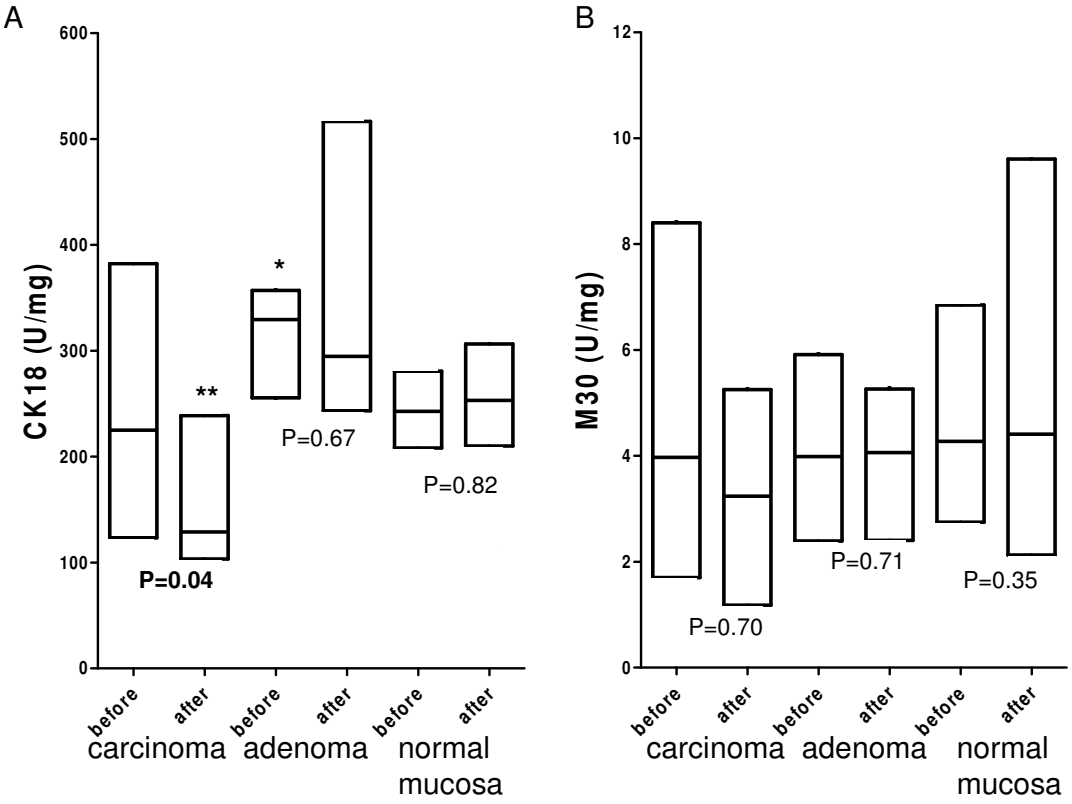

Figure 3: 5-ASA causes the loss of epithelial cells in colorectal carcinomas in vitro.

*CK18 (A) and M30 (B) antigen levels of the carcinoma, adenoma or normal mucosa of patients before and after 14 days of 5-ASA enema treatment. The differences before or after 5-ASA treatment were determined with Wilcoxon signed rank test (P-values mentioned below the boxes, with P<0.05 considered significant in bold). Lines indicate median value, box represents the inter-quartile range. Mann Whitney U tests: * P=0.07 vs carcinoma before 5-ASA and P=0.005 vs normal mucosa before 5-ASA. ** P=0.004 vs adenoma after 5-ASA and P=0.004 vs normal mucosa after 5-ASA.*

Discussion

5-ASA is an interesting candidate drug for the prevention of CRC, as IBD patients on chronic 5-ASA medication have a decreased risk of developing CRC [16]. A lot of data from *in vitro* studies has shown that it might also be an interesting agent in the treatment of CRC. The exact anti-cancer mechanism of 5-ASA has not been elucidated yet, but the reduction of proliferation and the induction of cell death has been shown in several CRC cell lines [8-11, 17, 18]. We investigated the ability of 5-ASA to induce apoptotic cell death in HT29 CRC cells by determining caspase-3 activity as a measurement of classical apoptosis. 5-ASA increased

caspase-3 activity in a concentration and time-dependent manner with a 4-fold induction after 48 hours with 40 mM 5-ASA. 5-ASA did not induce caspase-3 activity before 48 hours or at concentrations below 30 mM. In comparison with the 11-fold increase of caspase-3 activity in 24 hours with 1 μM staurosporine, 5-ASA was not capable of inducing apoptosis in such a robust way and also induced apoptosis much slower. Flow cytometric analysis of active caspase-3 and M30 confirmed these data, i.e., an induction of classical apoptosis by 5-ASA treatment but no as robust as staurosporine.

The cell death inducing and cell number reducing effects of 5-ASA could not be blocked with a very potent pan-caspase inhibitor, Q-VD-OPh, indicating 5-ASA is able to induce cell death in CRC cells via caspase-independent mechanisms. This was further illustrated by the capability of 5-ASA to induce massive cell death of MCF-7 breast cancer cells, which lack caspase-3 due to a deletion in exon of *CASP3*, and are not able to undergo classical apoptosis [19]. Furthermore, this illustrates that the cell death inducing effects of 5-ASA are not exclusive for intestinal cancer cell lines.

5-ASA has been found to decrease levels of survivin, an inhibitor of apoptosis protein (IAP), *in vitro* [10]. Silencing survivin expression in breast cancer cells leads to apoptosis via caspase-3 independent pathways [20]. Although we could not confirm the decrease in survivin expression by 5-ASA in HT29 cells in another study we did find that 5-ASA can induce a cell cycle arrest, with an aberrant mitotic spindle formation leading to a form of cell death named mitotic catastrophe [21], which is presumed to be executed without activation of caspases and lacks the classic apoptotic features, e.g., DNA fragmentation [22]. The induction of cell death without an induction of caspase activity is in line with the results presented here. It remains to be ilucidated if other proteins, for instance apoptosis inducing factor (IAF), are implicated in the caspase-independent cell death induced by 5-ASA.

To extend our previous research on the 5-ASA anti-cancer effects *in vivo* in patients with malignant or premalignant lesions in their colorectum we studied the capability of 5-ASA to induce apoptosis in the cells of these lesions *in vivo*. 5-ASA, in a 14 days enema treatment, was very capable to induce classical apoptosis in colorectal carcinomas, as indicated by a significant increase in caspase-3 activity in the post 5-ASA biopsies. M30 antigen levels either corrected or uncorrected for total CK18 levels were not significantly increased in post 5-ASA biopsies, indicating that both stromal, which are CK18 negative, and epithelial cells are responsible for the 5-ASA induced caspase-3 activity.

The significant loss of total CK18 antigen levels in the post 5-ASA carcinoma biopsies indicates that 5-ASA caused the elimination of epithelial (cancer) cells within the 14

day treatment period. 5-ASA is capable of inducing apoptosis more rapidly in colorectal cancer cells *in vitro* [8, 11] which we have confirmed here: caspase-3 activation within 72 hours. Moreover, we have found that 5-ASA induces caspase-independent cell death in tumour cells. Both these mechanisms, i.e., a fast induction of cell death and the induction of caspase-independent cell death besides caspase-dependent cell death, could have contributed to the observed decreased epithelial tumour cell content in colorectal carcinoma biopsies. The loss of epithelial cells could have decreased epithelial barrier function, allowing a better exposure of stromal cells to the 5-ASA treatment, resulting in the induction of apoptosis of these stromal cells, including mononuclear leukocytes, which is thought to be one of the modes of action of 5-ASA[23, 24]. This is exactly what our data suggest, an increase in total apoptosis (caspase-3 activity) and not in specific epithelial apoptosis (M30 antigen, M30/CK18), indicating (also) non-epithelial apoptosis after 14 days of 5-ASA treatment.

Suprisingly, there were no such effects on adenomas, i.e. no significant increase in caspase-3 activity or loss of CK18, indicating there was no major induction of apoptosis or loss of epithelial cells. Higher amounts of CK18 in the adenomas, indicating a higher percentage of epithelial cells, might indicate a higher epithelial cell turnover and repopulation, a better epithelial barrier function at the start of the 5-ASA treatment, and a more rapid and better acetylation of 5-ASA. This would lead to a relatively lower concentration of 5-ASA in the neoplastic tissue and, as shown in the *in vitro* experiments, the 5-ASA concentration is crucial for the induction of apoptosis.

In this particular study, caspase-3 activity, M30 and CK18 antigen levels were as high in biopsies of normal and neoplastic tissue, which suprised us as we have shown in an earlier study that all are increased in the resection specimens of CRC versus normal tissue [11]. This most likely has to do with the operating procedure versus biopsy procedure.

The caspase-3 activity was also increased in the normal mucosa of the patients, indicating an induction of apoptosis in the normal mucosa. The apoptosis inducing effects of 5-ASA in the normal tissue of sporadic adenoma patients has been reported before [25], in line with the results presented here.

In conclusion, in the present report we show that 5-ASA is an inducer of apoptotic and non-apoptotic cell death in CRC cells *in vitro*. The induction of apoptosis was also found in CRC *in vivo*. Moreover, the loss of tumour cell content *in vivo* indicates that 5-ASA might be powerful in killing tumour cells, either by apoptotic or non-apoptotic cell death pathways *in vivo*. Therefore, our observations give rationale for neo-adjuvant therapy studies with 5-ASA in CRC patients.

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