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

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Chapter 9

Summarizing discussion

Introduction

Colorectal cancer (CRC) is a major disorder in the Western world [1]. Treatment of CRC, although improved in the last decade, is not very successful, and therefore much research effort is aimed at prevention and early detection. In the development of CRC both genetic predisposition and environmental factors are important [2]. The general goal of the studies described in this thesis was to evaluate the effect of 5-aminosalicylic acid (5-ASA), a non-steriodal anti-inflammatory drug used in the treatment of IBD with structural similarities to aspirin, on the development of CRC. In addition, the feasibility of introducing 5-ASA as a potential (neo-)adjuvant therapy for CRC patients was evaluated. Based on the observations from these studies the type and clinical significance of apoptosis in CRC was further investigated.

The major findings reported in this thesis are summarized in *Figure 1*.

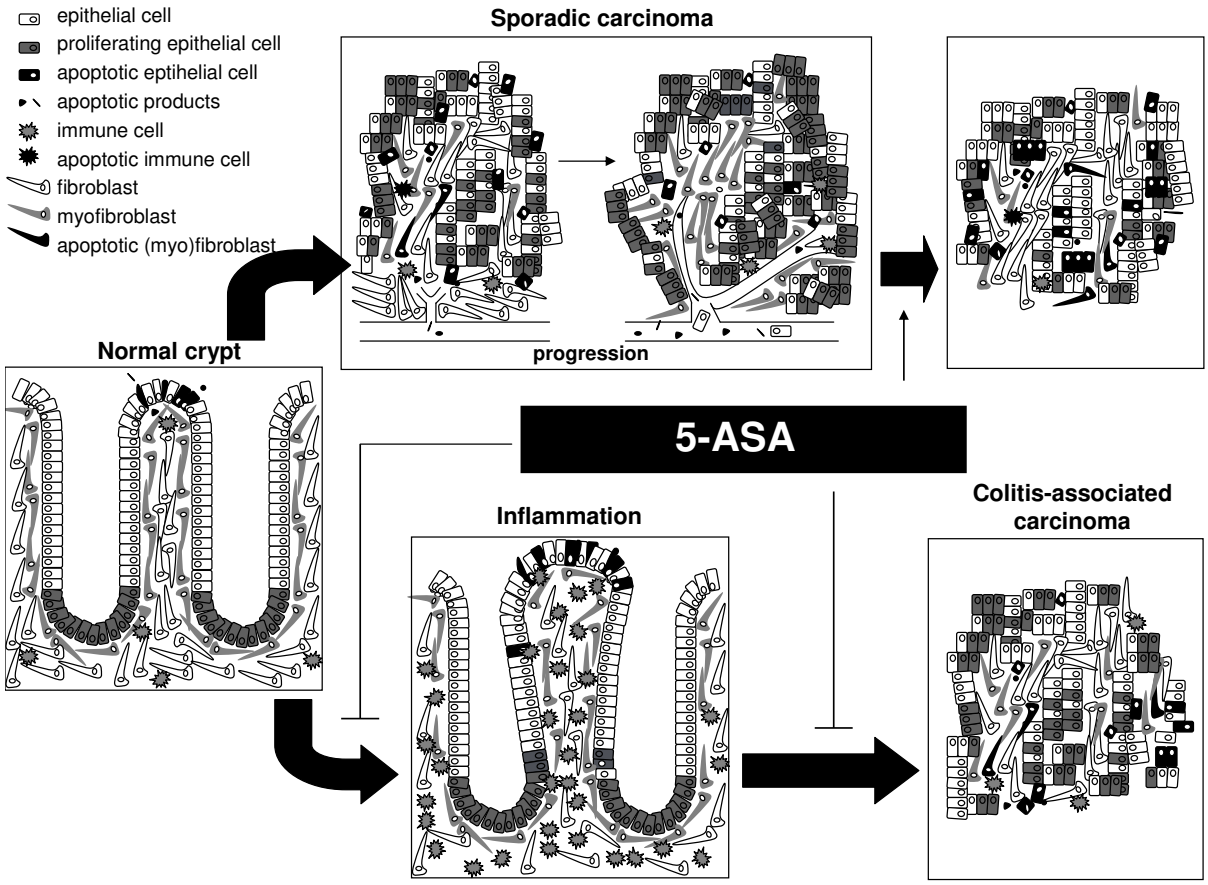

Figure 1: Summary of the results obtained in the studies as described in this thesis.

Impact of apoptosis in colorectal cancer

The study on the impact of epithelial and stromal apoptosis in the tumour tissue on the clinical outcome of CRC patients is described in **chapter 2**. The total caspase-3 activity in the tumour was found to correlate with disease progression. This was mainly due to stromal apoptosis, as represented by a reduction in stromal apototic cells in the upper panel of *Figure 1*. In addition, stromal apoptosis was the most predictive apoptotic factor in CRC, in contrast to epithelial apoptosis, and independent of other factors, like disease stage, gender or age. Many other studies already described the importance of the stromal compartent in CRC [3-6]. Our study showed that stromal cell apoptosis can be added to the important signalling pathways in the cancer-associated stroma. The importance of stromal apoptosis in CRC might also explain why not all studies investigating apoptosis in CRC report similar results [7], as detection techniques for apoptotis can differ in specificity for stromal and/or epithelial cells [8].

The significance of cell death products in the circulation of CRC patients was also investigated, as described in **chapter 3**. Cytokeratin18 (CK18) and M30 antigen levels, the caspase-generated breakdown product of CK18, were determined in the plasma of CRC patients by specific ELISAs. and were most likely elevated by the presence of the tumour. In addition, M30 antigen and CK18 levels were significantly higher in patients with progressed disease (Dukes' C and D compared with Dukes' A and B), indicated by the apoptotic products in the circulation in the upper panel of *Figure 1*. Moreover, M30 antigen levels in the circulation were of significance for the patient's disease-free survival. Also the M30/CK18 ratio in the circulation of CRC patients, indicative of the balance between apoptosis and necrosis, was found to be of prognostic significance for the patient's disease-free survival. Low ratios, indicating more necrosis over apoptosis, might be indicative of hypoxic conditions within the tumour and were associated with a worse prognosis. Since hypoxic tumours are generally more resistant to most treatments, the M30/CK18 ratio might be used to select patients for specific treatment. The post-operative levels of M30 antigen and M30/CK18 ratios in the plasma of CRC patients, i.e., after surgical resection of the tumour, were also of prognostic significance for the patient's disease-free survival, without being affected by treatment. Therefore, the detection of M30 and CK18 levels in the circulation of CRC patients might be useful for the selection of treatment and for the patient's follow-up after surgery, although larger studies are needed to confirm this hypothesis.

The effects of 5-ASA on CRC apoptosis and cell death

The effect of 5-ASA on CRC cell proliferation and growth was investigated as reported in **chapter 4**. 5-ASA was found to reduce proliferation of several CRC cell lines *in vitro* by interfering in the cell cycle progression. As a consequence CRC cells died via apoptotic processes and mitotic catastrophe at later time-points. The induction of apoptosis was determined by M30-immunohistochemistry, M30 and CK18 ELISAs and Annexin V/PI flow cytometry analysis. 5-ASA was also found to cause necrotic death of CRC cells at high concentrations. The effects of 5-ASA were found not only to be dependent on the concentration but also on the dosage, i.e., the amount, of 5-ASA per cell, which might be very relevant for the extrapolation of the data to the *in vivo* situation. Further studies with additional techniques, as described in **chapter 5**, revealed that 5-ASA can induce cell death in a both caspase and caspase-independent manner *in vitro*, indicating both apoptotic and non-apoptotic cell death. Moreover, we found that 5-ASA has the ability to induce apoptosis in CRC cells *in vivo*, as determined by an increase in caspase-3 activity on CRC biopsy material before and after 14 days of 5-ASA enema treatment. The increase of caspase-3 activity was not accompanied by an increase of M30 antigen, indicating that there was an increase of total apoptosis in the CRC biopsies but not in epithelial apoptosis, suggesting induction of stromal cell apoptosis. Immunohistochemistry with the M30 antibody on formalin-fixed paraffin imbedded tissue of the CRC biopsies of these patients to confirm absence of epithelial apoptosis, unfortunately did not give results to really quantify the level of apoptosis in these biopsies, as reported by others [9]. The M30 staining did perform well, however, on formalin-fixed paraffin imbedded resected CRC tissues (**chapter 2,** *Figure 1*). The simultaneous decrease in CK18 levels in post-5-ASA biopsies, implicating a loss of epithelial cell content due to 5-ASA treatment, could well be the result of a fast induction of epithelial cell apoptosis, or the induction of other cell death pathways, like necrosis or mitotic catastrophe, as described to be induced by 5-ASA treatment of CRC cells *in vitro***.** All these effects of 5-ASA treatment are indicated by the upper right panel of *Figure 1***.** These results are somewhat different from earlier work of our group on the apoptosis inducing effects by 5-ASA in CRC *in vivo*, showing a specific induction of tumour epithelial apoptosis by 5-ASA [10]. Actually, the biopsies of the same patients as described in the earlier study were also included in the assessments as described in **chapter 5** but showed no obvious increase in M30 antigen expression. Apoptotic stromal cells could also have been mistakenly identified as apoptotic epithelial cells in the earlier study, since we have found an induction of apoptosis most likely to be initiated in the stromal compartment. Moreover, if we extrapolate the

induction of mitotic catastrophe by 5-ASA in CRC cells *in vitro* to the *in vivo* experiments, these mitotic catastrophy cells could have been present in the biopsy material after 5-ASA treatment and easily been mistaken for apoptotic cells, thereby responsible for the discrepancies found between the studies. Nevertheless, altogether these studies show an anti-cancer effect of 5-ASA treatment *in vivo*, i.e., the induction of CRC cell death, which was the primary aim of the studies.

Other anti-CRC effects of 5-ASA

The transforming growth factor-β (TGF-β) pathway plays a dualistic role in cancer, being both tumour suppressor and tumour promotor [11]. Therefore, the regulatory effects of 5-ASA on this important TGF-β pathway in CRC was also evaluated, as reported in **chapter 6**. Hyperactivation of the TGF-β pathway, by mutations in important genes, and overexpression of TGF-β, contributes to the progression of CRC and therefore the treatment of CRC, and cancer in general, with TGF-β inhibitors is believed to hold a great promise [12]. 5-ASA was found to exert an inhibitory effect on the TGF-β pathway in CRC cell lines, as well as in normal colorectal and CRC-associated fibroblast cultures, when stimulated by the addition of exogenous TGF-β1. 5-ASA treatment had no effect on the basal TGF-β1 signalling of these cells. 5-ASA also reduced the TGF-β-induced trans-differentiation of fibroblasts into myofibroblasts, as indicated by a reduced myofibroblast content in the tumour in the upper right panel of *Figure 1*. The inhibitory effects of 5-ASA were only found on the (over)stimulated TGF-β signalling, simulating a progressing CRC, and at a relatively high 5-ASA concentration (≥ 20 mM), which is only achieved in the lumen of the colorectal area upon treatment with coated tablets and/or enemas. Moreover, the effect was specific as there was virtually no effect on the (over)stimulated and closely related bone morphogenetic protein (BMP) pathway. Treatment of CRC patients with 5-ASA could therefore be a very good strategy to inhibit TGF-β signalling in CRC, thereby inhibiting tumour progression. The effects of 5-ASA on the TGF-β pathway in the CRC patients still need to be confirmed in CRC patients *in vivo*. We tried to evaluate the activity of the TGF-β pathway in the CRC tissue of the patients described in **chapter 5**, but unfortunately it was too difficult to evaluate the immunohistochemical staining for PAI-1 or phosphorylated Smad 3 in the biopsies obtained before and after 5-ASA treatment. Both stromal and epithelial cells can express PAI-1 in CRC [13, 14], and therefore the total levels of PAI-1 are influenced by the composition of the different cell types within the biopsy. The results of that study strongly suggest that 5-

ASA altered the make-up of the cells within the tumour. Therefore, determination of PAI-1 levels in the biopsy homogenates by ELISA, could not be used to conclude anything about the effects of 5-ASA on the TGF-β pathway *in vivo*.

5-ASA is a chemopreventive agent in colitis-associated colorectal cancer

As a proof of concept study animal experiments were performed aimed at determining the chemopreventive effect of 5-ASA, i.e., the ability to prevent the development of CRC, the results of which are described in **chapter 7**. This has been studied before by others in the *Apc*^{min} mouse model [15, 16], which is not a really good model because these animals mainly develop adenomas in the small intestine and very early in life, and almost no CRC. The generation of a novel sporadic CRC mouse model by Cre-Lox technology: Fabpl*Cre*;*Apc*$^{15lox/+}$ (Robanus-Maandag *et al.*, manuscript in preparation), allowed us to investigate 5-ASA chemoprevention in a proper animal model. Remarkably, 5-ASA was not able to prevent the development of sporadic CRC in these Fabpl*Cre*;*Apc*$^{15lox/+}$ mice. However, introduction of colonic inflammation in these Fabpl*Cre*;*Apc*$^{15lox/+}$ mice, by the administration of DSS in the drinking water, accelerated colorectal tumorigenesis creating a well suited colitis-associated CRC model. Impressively, 5-ASA was able to reduce the development of these colitis-associated colorectal tumors by 50 %, confirming epidemiological data of human studies on IBD-related CRC (**chapter 1**). The reduction of colitis-associated colorectal tumours was accompanied by a reduction of proliferation in these tumours. In contrast, no effect was seen on apoptosis or on proliferation of sporadic colorectal tumours in these Fabpl*Cre*;*Apc*$^{15lox/+}$ mice, quite unexpected, when compared with our *in vitro* and human *in vivo* experiments. The absence of effect on cell proliferation and apoptosis in these sporadic tumours correlated well with the lack of effect on the number of these tumours. Perhaps a longer 5-ASA treatment on larger/more progressed tumours in these Fabpl*Cre*;*Apc*$^{15lox/+}$ mice could have had an anti-proliferative and/or apoptosis, but that was not studied. These mouse experiments did show and confirm, however, that chronic 5-ASA treatment interferes in the colitis-induced colorectal carcinogenesis, in line with the epidemiological data in inflammatory bowel disease [17].

Potential of 5-ASA as neo-adjuvant drug

Based on the compelling evidence of inhibitory proliferation and apoptosis/cell death inducing effects, in combination with the inhibitory effects on the CRC-progression TGF-β pathway (**chapters 4, 5** and **6**), there might be a rationale for CRC treatment with 5-ASA.

The feasibility of treating CRC patients with 5-ASA medication largely depends on the interference of the 5-ASA treatment with conventional treatment strategies, with proven efficacy, that are already applied to the patients, e.g, radiation therapy (RT). Therefore the influence of 5-ASA treatment on the effect of RT on CRC cells *in vitro* was investigated. 5-ASA treatment shortly before or during RT was found to have an inhibitory effect on the ability of RT to reduce CRC cell survival (**chapter 8**). These findings indicate that 5-ASA acts as a radio-protector and should not be applied to the patient during or shortly before RT. Because of the observation that the radio-protective effect of 5-ASA treatment is lost when CRC cells are left untreated for 24 hours and then exposed to RT, 5-ASA medication could potentially be used in a neoadjuvant treatment strategy for CRC, although within a limited time-frame.

Conclusions

The animal model described in this thesis shows that chronic administration of 5-ASA has the ability to prevent colitis-associated colorectal cancer development, confirming epidemiological data on 5-ASA medication in IBD patients. The diverse studies on human cells and tissues illustrate that 5-ASA holds a great promise for the treatment of colorectal cancer, by exerting cancer cell growth inhibition, anti-tumour progression and cell death inducing effects, and thus deserves to be considered for implemention in a future treatment strategy for CRC. Furthermore, determination of cell death products in tumour tissue and the circulation of CRC patients, and the assessment of stromal apoptosis within the tumour, might constitute relevant selection criteria for additional treatment and follow-up of CRC patients.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. Int J Cancer 2001;94:153-156.

2. Heavey PM, McKenna D, Rowland IR. Colorectal cancer and the relationship between genes and the environment. Nutr Cancer 2004;48:124-141.

3. Baeten CI, Castermans K, Hillen HF, Griffioen AW. Proliferating endothelial cells and leukocyte infiltration as prognostic markers in colorectal cancer. Clin Gastroenterol Hepatol 2006;4:1351-1357.

4. Mesker WE, Junggeburt JM, Szuhai K, de Heer P, Morreau H, Tanke HJ, Tollenaar RA. The carcinoma-stromal ratio of colon carcinoma is an independent factor for survival compared to lymph node status and tumor stage. Cell Oncol 2007;29:387-398.

5. Ngan CY, Yamamoto H, Seshimo I, Tsujino T, Man-i M, Ikeda JI, Konishi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N, Monden M. Quantitative evaluation of vimentin expression in tumour stroma of colorectal cancer. Br J Cancer 2007;96:986-992.

6. Tsujino T, Seshimo I, Yamamoto H, Ngan CY, Ezumi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N, Monden M. Stromal myofibroblasts predict disease recurrence for colorectal cancer. Clin Cancer Res 2007;13:2082-2090.

7. Koornstra JJ, De Jong S, Hollema H, De Vries EG, Kleibeuker JH. Changes in apoptosis during the development of colorectal cancer: a systematic review of the literature. Crit Rev Oncol Hematol 2003;45:37-53.

8. Garrity MM, Burgart LJ, Riehle DL, Hill EM, Sebo TJ, Witzig T. Identifying and quantifying apoptosis: navigating technical pitfalls. Mod Pathol 2003;16:389-394.

9. Gosens MJ, Dresen RC, Rutten HJ, Nieuwenhuijzen GA, van der Laak JA, Martijn H, Tan-Go I, Nagtegaal ID, van den Brule AJ, Van Krieken JH. Preoperative radiochemotherapy is successful also in patients with locally advanced rectal cancer who have intrinsically high apoptotic tumours. Ann Oncol 2008;19:2026-2032.

10. Bus PJ, Nagtegaal ID, Verspaget HW, Lamers CB, Geldof H, Van Krieken JH, Griffioen G. Mesalazine-induced apoptosis of colorectal cancer: on the verge of a new chemopreventive era? Aliment Pharmacol Ther 1999;13:1397-1402.

11. Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol 2001;11:S44-S51.

12. Lahn M, Kloeker S, Berry BS. TGF-beta inhibitors for the treatment of cancer. Expert Opin Investig Drugs 2005;14:629-643.

13. Papadopoulou S, Scorilas A, Yotis J, Arnogianaki N, Plataniotis G, Agnanti N, Talieri M. Significance of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 (PAI-1) expression in human colorectal carcinomas. Tumour Biol 2002;23:170-178.

14. Illemann M, Hansen U, Nielsen HJ, Andreasen PA, Hoyer-Hansen G, Lund LR, Dano K, Nielsen BS. Leading-edge myofibroblasts in human colon cancer express plasminogen activator inhibitor-1. Am J Clin Pathol 2004;122:256-265.

15. Brown WA, Farmer KC, Skinner SA, Malcontenti-Wilson C, Misajon A, O'Brien PE. 5-aminosalicyclic acid and olsalazine inhibit tumor growth in a rodent model of colorectal cancer. Dig Dis Sci 2000;45:1578-1584.

16. Ritland SR, Leighton JA, Hirsch RE, Morrow JD, Weaver AL, Gendler SJ. Evaluation of 5-aminosalicylic acid (5-ASA) for cancer chemoprevention: lack of efficacy against nascent adenomatous polyps in the Apc(Min) mouse. Clin Cancer Res 1999;5:855-863.

17. Velayos FS, Terdiman JP, Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. Am J Gastroenterol 2005;100:1345-1353.