Apoptosis and colorectal cancer. Studies on pathogenesis and potential therapeutic targets
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Chapter 4

Expression of TRAIL (TNF-related apoptosis-inducing ligand) and its receptors in normal colon mucosa, adenomas and carcinomas.

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Abstract

Background: Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in tumour cell lines. Four membrane-bound receptors for TRAIL have been identified, two apoptosis-mediating receptors DR4 and DR5 and two apoptosis-inhibiting receptors DcR1 and DcR2. The aim of this study was to examine the role of TRAIL and its receptors in colorectal cancer development.

Methods: The immunohistochemical expression and localisation of TRAIL and its receptors were investigated in normal mucosa (n = 10), adenomas (n = 19) and carcinomas (n = 21). Correlations between expression of TRAIL and its receptors and the degree of apoptosis (assessed by M30 immunoreactivity) and histopathological characteristics were explored.

Results: TRAIL and its receptors were expressed in normal mucosal epithelium. Expression of the receptors was seen in adenomas and carcinomas. TRAIL expression was lost in a subset of colorectal tumours, more frequently in carcinomas than in adenomas (p < 0.05). DR4 and DR5 staining was stronger in neoplastic cells compared to normal cells, and was accompanied by a higher degree of apoptosis. No differences were found between tumour and normal cells regarding DcR1 and DcR2 expression. No correlations were found between TRAIL or TRAIL receptor expression and histopathological characteristics.

Conclusion: Marked changes were seen in the course of the adenoma-carcinoma sequence with respect to the expression of TRAIL and TRAIL receptors DR4 and DR5. The stronger expression of DR4 and DR5 in neoplastic cells as opposed to normal cells, together with a higher degree of apoptosis suggests a possible functional role for these receptors in apoptosis induction in colorectal neoplastic cells.

Introduction

Apoptosis plays a critical role in the normal development of multicellular organisms and in maintaining tissue homeostasis. Apoptosis also represents an effective mechanism by which cells with DNA damage can be eliminated while dysregulation of normal apoptotic mechanisms can provide a growth advantage to cancer cells. Colorectal carcinogenesis is characterised by an accumulation of molecular genetic alterations causing progressive disorders in cell growth, differentiation and apoptosis. Elucidation of the molecular mechanisms regulating these processes is therefore of primary interest.

Apoptosis is controlled through a variety of intracellular and extracellular signals. Cytokines from the tumour-necrosis factor (TNF) family have been identified as participants in the regulation of apoptosis. These cytokines are TNF-α, Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), all of which induce apoptosis by binding to their respective receptors. The role of TNF-α in the development of colorectal cancer has not been studied extensively but seems to be limited. The FasL/Fas apoptosis-induction pathway in colorectal cancer has been studied to a greater extent. Although several experiments have suggested a possible role for disturbances in this pathway in colorectal carcinogenesis, its precise impact still has to be defined. TRAIL was isolated in 1995. TRAIL induces rapid apoptosis in a wide variety of transformed cell lines, but seems to have little or no
detectable cytotoxic effect on normal cells in vitro and in vivo. It has been suggested that TRAIL may be involved in endogenous tumour surveillance. At present, five different receptors for TRAIL have been identified: two cell death-inducing receptors (DR4/TRAIL-R1 and DR5/TRAIL-R2), two non-cell-death-inducing receptors (DcR1/TRAIL-R3 and DcR2/TRAIL-R4) and osteoprotegerin. DR4 and DR5 are membrane bound receptors that contain a so-called death domain in their intracellular segment. This death domain is required for TRAIL-mediated apoptotic cell death in a manner similar to that of other death receptors in the TNF-receptor family. In contrast, DcR1 lacks the intracellular death domain and DcR2 contains a truncated death domain. Therefore, neither receptor can transduce the death signals.

Antibodies against TRAIL and TRAIL-receptors were not available until recently. Therefore, TRAIL and TRAIL-receptor expression were only reported at the level of mRNA expression. TRAIL mRNA was detected in a wide range of tissues, including the small intestine and colon. DR4, DR5 and DcR2 transcripts are expressed in normal colonic epithelium and colon adenocarcinomas. DcR1 mRNA levels were high in various tumours of the gastrointestinal tract, including colorectal cancer, in comparison to normal mucosa. Protein expression and localisation of TRAIL and its receptors in normal colonic epithelium have recently been described. There are no data available on the protein expression and localisation of TRAIL and its receptors in colorectal neoplasms.

In order to examine the possible role of TRAIL mediated apoptosis, we analysed the immunohistochemical expression and localisation of TRAIL and its receptors in different stages of colorectal cancer development. In addition, correlations between TRAIL and TRAIL receptor expression and the degree of apoptosis and histopathological characteristics were explored.

Materials and methods

Tissue collection

Paraffin-embedded tissue specimens from 10 samples of normal mucosa, 19 colorectal adenomas and 21 carcinomas were retrieved from the files of the Department of Pathology. Samples of normal mucosa were randomly selected from archival materials obtained from patients without macroscopic abnormalities at colonoscopy where random biopsies had been taken. The adenomas were the first 19 sporadic adenomas endoscopically removed at the Department of Gastroenterology in 1997. Adenomas from patients with previous or simultaneous colorectal cancer were excluded. Primary sporadic adenocarcinomas had been removed at the Department of Surgery in 1999. Mucinous carcinomas were excluded. Tumours from patients that had received chemo- or radiotherapy prior to removal of the tumour were excluded. Histologic classifications were carried out on haematoxylin and eosin (H&E) stained slides. Adenocarcinomas were staged according to the modified Dukes classification and graded into well, moderately and poorly differentiated. For statistical purposes, tumours with Dukes stages A/B were compared with Dukes stages C/D and tumours with good/moderate differentiation with tumours with poor differentiation. For adenomas, circumferential size was measured and dysplasia expressed as low- or high-
.grade. Adenomas were classified as tubular or villous when the villous component exceeded 25% of the adenoma. In all cases of adenomas and carcinomas, slides were selected in which adjacent or surrounding ‘normal’ epithelial cells were included.

**Immunohistochemistry**

**Expression of TRAIL and receptors DR4, DR5, DcR1 and DcR2**

For immunohistochemical staining, serial 3 μm-thick-sections were cut from paraffin blocks. After deparaffinisation in xylene, endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. For TRAIL, DR5 and DcR2, antigen retrieval was carried out by microwavetreatment, for DR58 min at 700 W in 0.01M citrate buffer (pH 6.0), for TRAIL and DcR2 in Tris/HCl buffer (pH 9.0). For DR4 and DcR1, staining, no antigen retrieval was required. After incubation with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA, USA) primary antibodies were applied for 1 h at room temperature. Slides were stained with a goat polyclonal IgG for TRAIL (1:25; clone C-20, Santa Cruz, CA, USA), a goat polyclonal IgG for DR4 (1:100; clone C-20, Santa Cruz), a rabbit polyclonal IgG for DR5 (1:100; Oncogene Research, Cambridge, MA, USA), a goat polyclonal IgG for DcR1 (1:50; Calbiochem, San Diego, CA, USA) and a rabbit polyclonal IgG for DcR2 (1:75; Oncogene Research). After washing with PBS, slides were incubated with a 1:300 dilution of a biotinylated rabbit-anti-goat or swine-anti-rabbit antibody (DAKO, Glostrup, Denmark) respectively, followed by addition of streptavidin-conjugated peroxidase. Peroxidase activity was visualised with diaminobenzidine. Slides were counterstained with haematoxylin.

**Controls**

To ensure specificity of the primary antibodies several experiments were conducted. First, immunoblotting of the antibodies on several colon cancer cell lines was performed. For this study, the colon carcinoma cell lines Colo320, Caco-2 and SW948 were obtained from the ATCC (Rockville, MD). Immunoblotting of the antibodies for TRAIL, DR4 and DR5 on three cell lines and cases of colorectal cancer yielded protein products of expected size (see figure 1). The specificity of the antibodies for DcR1 and DcR2 was confirmed at the RNA and protein level on the cell lines Colo320, Caco-2 and SW948. Second, a number of tissue sections were immunostained using non-immunised goat or rabbit IgG respectively as a substitute for the primary antibodies, as described by Moller et al. In these cases, no immunostaining was detected. Third, for DR4, TRAIL and DcR1, immunohistochemical staining was performed in the presence or absence of a 10-fold excess of the corresponding blocking peptides (Santa Cruz for TRAIL and DR4, Calbiochem for DcR1), as described by Mitsiades et al. Again, in these cases no immunostaining was detected. Finally, slides were immunostained in the absence of the primary antibody and in these cases no immunostaining was detected. As positive controls, sections of normal human liver (DR4, DR5 or kidney (TRAIL, DcR1, DcR2) were included to check the reliability and reproducibility of the staining procedures.
Apoptosis

Apoptotic cells were determined by immunohistochemistry with the murine monoclonal antibody M30 (Boehringer Mannheim, Mannheim, Germany). M30 reacts with a cleavage product of cytokeratin 18, released by activated caspase-29, and the immunoreactivity is present during early apoptosis-30. M30 staining has been shown to be markedly more sensitive than the morphological identification of apoptosis by H&E staining in colorectal tissues-31. In addition, a good correlation has been found between in situ end labelling (ISEL) and expression of M30 in colorectal neoplasms-32. After deparaffinisation and blocking endogenous peroxidase activity, sections were immersed in 0.01 M citrate buffer (pH 6.0) and heated in a microwave oven for 8 min. M30 was applied to the sections for 60 min at room temperature in a 1:50 solution, followed by incubation with a secondary rabbit-anti-mouse antibody conjugated with peroxidase (DAKO, Glostrup, Denmark) and a tertiary goat-anti-rabbit peroxidase-conjugated antibody (DAKO). Peroxidase activity was visualised with diaminobenzidine. Slides were counterstained with haematoxylin. As negative controls, slides were immunostained in the absence of the primary antibody.

Evaluation of staining results

Slides were evaluated by light microscopy by at least two independent investigators without knowledge of the histopathological data. Immunostaining was evaluated in serial tissue sections. The percentage of positive cells was estimated semiquantitatively and sorted into four categories (negative, <10 %, 10-50 % and >50 %). When the observers' scores differed more than 10 %, cases were re-evaluated using a multiheaded microscope and the final grade was reached by consensus. For statistical analysis, tumours with positive staining in >10 % of tumour cells were considered positive, while tumours without staining or staining in less than 10 % of cells were considered negative. The pattern of staining was recorded as membranous, cytoplasmic or nuclear. To exclude any effects of variation in intensity of staining among different samples, the immunoreactivity of positive tumour cells was...
assessed relative to adjacent normal epithelial cells. M30 positivity was identified as brown cytoplasmic staining. M30 positive cells were determined within whole crypts in normal mucosa and adenomas and randomly in carcinomas. In all cases, at least 500 epithelial cells were counted and M30 positive cells were expressed as a percentage of the total number of cells counted.

**Statistical analysis**

For statistical assessment of differences in the expression of TRAIL, DR4, DR5, DcR1 and DcR2 between normal tissue, adenomas and carcinomas, the chi-square test was used. Differences in expression of TRAIL, DR4, and DR5 with respect to histopathological characteristics of adenomas and carcinomas were assessed using the chi-square test. Mean percentages of M30 positive cells were compared across categories of DR4, DR5 and TRAIL positive cells using the Wilcoxon test. To assess differences in the percentage of apoptotic cells between different tissue types, the Mann-Whitney test was used. P-values < 0.05 were considered significant. SPSS for Windows software (SPSS Inc., Chicago, IL) was used for all statistical analyses.
Expression of TRAIL, DR4, DR5, DcR1 and DcR2 in normal colon epithelium

Immunohistochemical staining showed expression of TRAIL and all four TRAIL-receptors in all cases of normal epithelium, including both goblet and columnar epithelial cells, along the entire length of the crypt. There were no appreciable differences in staining intensities between individuals. For TRAIL, DR4 and DcR1, the immunoreactivity of epithelial cells increased gradually from the base of the crypt to the crypt mouth and the luminal surface. For DR5 and DcR2, there was no apparent variability in staining intensity between the basal part of the crypt and the luminal surface. The expression was cytoplasmic for TRAIL, DR4, DR5, DcR1 and DcR2. In addition, nuclear stained cells with DcR2 were seen, predominantly located in the basal part of the crypts. At the mucosal surface, TRAIL staining was seen throughout the cytoplasm whereas for DR4, DR5, DcR1 and DcR2 cytoplasmic staining was strongest at the basolateral surface of the cell.

Expression of TRAIL, DR4, DR5, DcR1 and DcR2 in colorectal adenomas and carcinomas

Staining results are summarised in Tables 1-3. Both adenomas and carcinomas exhibited cytoplasmic staining for TRAIL, DR4, DR5, DcR1 and DcR2 (figures 2, A-E: adenomas; figure 3, A-E: carcinomas).

Loss of TRAIL expression was more frequently observed in carcinomas than in adenomas (Figure 3E). Loss of TRAIL expression was seen more often in adenomas larger than 9 mm than those smaller than 9 mm (mean circumferential adenoma size, table 2). There was no relationship between loss of TRAIL expression in carcinomas and tumour stage or grade. Nuclear TRAIL staining was seen in one carcinoma. In the adenomas there were no significant correlations between the presence of TRAIL expression and growth type or degree of dysplasia.

DR4 staining was seen in all adenomas and carcinomas. Strikingly, in all adenomas and carcinomas, staining intensity was stronger in neoplastic cells relative to adjacent normal cells. The percentage of DR4 positive cells was higher in carcinomas than in adenomas.
The percentage of DR4 positive cells was not correlated to size, grade of dysplasia or growth type (tubular or villous) of adenomas and was also not correlated to stage or grade of carcinomas.

DR5 staining was detected in all adenomas and carcinomas. As for DR4, staining intensity in all adenomas and carcinomas was stronger in neoplastic cells than in adjacent normal epithelial cells. The majority of adenomas and carcinomas harboured more than 50% positive cells. The percentage of positively staining cells did not differ between adenomas or carcinomas with different histopathological characteristics.

DcR1 staining was observed in the majority of adenomas and carcinomas. Staining intensities were similar in neoplastic cells compared to adjacent normal epithelial cells. Strong staining was seen in stromal cells.

DcR2 staining was observed in all adenomas and carcinomas, with staining intensities in neoplastic cells comparable to adjacent normal cells. Nuclear staining was seen in one adenocarcinoma.

Figure 3. Representative examples of expression of TRAIL receptors and TRAIL in adenocarcinomas. A: TRAIL; B: DR4; C: DR5; D: DcR1; E: DcR2. Tumour cells express cytoplasmic immunoreactivity for DR4, DR5, DcR1 and DcR2. Loss of TRAIL expression is seen in tumour cells compared to adjacent normal cells. The intensity of staining for DR4 and DR5 is stronger in carcinoma cells as compared to normal cells. Magnification 100x. See appendix for colour pictures.
In normal mucosa, the mean percentage of M30 positive cells was 0.11 (range 0-0.26) in truly normal mucosa, 0.12 (range 0-0.27) in normal mucosa adjacent to adenomas and 0.56 (range 0.07-2.26) in normal mucosa adjacent to carcinomas. The mean percentage of M30 positive cells in adenomas was 0.51 (range 0.13-1.24). This was higher than in truly normal mucosa and normal mucosa adjacent to adenomas (p < 0.001). In carcinomas, the mean percentage of M30 positive cells was 1.66 (range 0.4-6.37). This was higher than in adenomas and normal mucosa adjacent to carcinomas (p < 0.001).

Possible relations were investigated between histopathological characteristics and the expression of TRAIL, DR4 and DR5, and the degree of apoptosis (table 2). No significant differences were found in the degree of apoptosis between either adenomas or carcinomas with different histopathological characteristics. Table 3 depicts the degree of positively staining cells for TRAIL, DR4 and DR5 in adenomas and carcinomas in relation to the degree of apoptotic cell death. No significant correlations were found between the degree of apoptosis and the extent of positive staining cells in adenomas and carcinomas. There was a trend towards a positive correlation between DR4 expression and the degree of apoptosis (p = 0.07). Within individual tumours, no clear patterns of immunostaining were observed with varying grades of dysplasia (adenomas) or varying degrees of differentiation (carcinomas).
Discussion

In the present study, marked changes were shown in the course of the adenoma-carcinoma sequence with respect to the expression of TRAIL and TRAIL receptors, in particular DR4 and DR5.

Our results in normal colonic epithelium are largely in accordance with the recent study by Sträter et al. They also found TRAIL, DR4, DR5 and DcR2 expression in normal colonic epithelial cells. Furthermore, as in the Sträter study, we observed TRAIL and DR4 staining predominantly in the upper third of the crypts and the surface epithelium. We found DcR1 expression in almost all samples of normal mucosa, adenomas and carcinomas whereas Sträter et al. were not able to detect DcR1 in normal colonic epithelium, neither by rt-PCR nor by immunohistochemistry. Their rt-PCR results are in contrast to a study by Sheikh et al., who demonstrated low, yet detectable, DcR1 mRNA expression in normal colonic epithelium. The contradictory results may be explained by the use of different antibodies for immunohistochemistry and the probable low level of DcR1 protein expression in normal colon.

Similar to Sträter et al., we found predominantly cytoplasmic staining of the TRAIL receptors where one would rather expect membrane staining. However, comparable results have been obtained with immunohistochemical staining of other TNF-receptor family members like Fas in the human colon and TNF-R1 and R2 in mouse colon. A possible explanation may be that TRAIL receptors, like Fas, exist in both membrane-bound and soluble forms.

In the present study, stronger immunohistochemical DR4 and DR5 expression was observed in the majority of adenomas and carcinomas compared to adjacent normal mucosa.
epithelial cells. These results suggest that both DR4 and DR5 expression are upregulated in adenomas and carcinomas. This suggestion is supported by experiments showing that both DR4 and DR5 expression are enhanced by DNA damage, induced by ionising radiation as well as by chemotherapeutic agents. The accumulation of molecular genetic alterations that characterises the development of colorectal cancer leads to increasing DNA damage and, consequently, may lead to increased expression of DR4 and DR5. Similar observations have been made in cervical and pancreatic cancer. In cervical cancer, mRNA levels of DR4 and DR5 were higher in tumour cells than in normal cells which was confirmed by stronger immunohistochemical expressions. In pancreatic cancer, mRNA levels of DR4 and DR5 but also of TRAIL were elevated in most pancreatic cancer cases, compared with normal pancreatic tissue.

The DR4 and DR5 receptors can induce apoptosis after interacting with their ligand, TRAIL, and are therefore a marker for cells that are predisposed to apoptosis. We found a higher degree of apoptosis in adenomatous and carcinomatous areas compared to adjacent normal tissue. Our results with M30 immunostaining in adenomas and carcinomas are in accordance with other studies using this method to assess apoptotic cell death. Percentages of DR4 or DR5 positively staining cells were however not correlated to the degree of apoptosis in our series.

In contrast to the study in pancreatic tissue, we found decreased or lost TRAIL expression in 16% of adenomas and 48% of carcinomas, while staining was intact in adjacent normal cells. The degree of apoptosis did not differ between TRAIL positive and TRAIL negative carcinomas. Expression patterns of TRAIL have recently been reported in other tumour types. In brain tumours, TRAIL was expressed in astrocytomas and glioblastomas, but not in medulloblastomas, oligodendroglomas, meningiomas, neurocytomas and schwannomas. In these brain tumours, TRAIL expression did not correlate with the degree of apoptosis. In breast cancer tissues TRAIL expression was found in 21/40 breast cancers as opposed to none of five normal breast tissue samples. The loss of TRAIL expression in a subset of colorectal adenomas and carcinomas suggest that these tumours could have evaded induction of apoptosis due to downregulation of TRAIL. The hypothesis of loss of TRAIL expression in the course of the adenoma-carcinoma sequence is supported by a recent small study. In this study, expression profiles of eight colorectal cancer tumours were compared with corresponding non-cancerous colonic cells using a DNA micro-array technique, consisting of 9216 genes. It was shown that among other genes, TRAIL was downregulated in colorectal cancer. So far, mutations in the TRAIL gene have not been studied in colorectal cancer.

No clear differences were observed in the expression of the apoptosis-inhibiting receptors DcR1 and DcR2 between colorectal neoplasms and normal mucosa. In contrast, others have shown that DcR1 mRNA was expressed at higher levels in four of six colon carcinomas than in matched control tissues. The authors suggested that colorectal tumours may gain a growth advantage by overexpressing DcR1 in order to protect themselves against TRAIL-mediated apoptosis. However, others have shown that DR4 and DR5, and not the decoy receptors, were expressed on the plasma membrane of four human colon carcinoma cell lines, and that sensitivity to TRAIL correlated with the level of expression of DR4 and DR5.
It is generally accepted that the main physiological mechanism by which colon epithelial cells die is apoptosis, yet the precise mechanisms behind the induction of apoptosis still have to be identified. Changes in the degree of apoptosis in the course of the adenoma-carcinoma sequence have been studied extensively and were recently reviewed. From these studies, it appears that the proportion of cells undergoing apoptosis increases with tumour progression, possibly as a mechanism to delete cells with sustained DNA damage. Alterations in the degree of apoptosis may be explained by changes in apoptosis-regulating death-receptor pathways. From the currently known death receptor pathways, both FasL- and TNF-α-mediated apoptosis are not thought to be involved in colorectal cancer development. In this regard, the expression of TRAIL and its receptors in the course of the adenoma-carcinoma sequence may be relevant. Our results of increased expression of the pro-apoptotic TRAIL receptors DR4 and DR5 in neoplastic cells compared to normal cells, and a high degree of apoptotic cell death in neoplastic cells, suggests a possible functional role for these receptors in the induction of apoptosis of colorectal epithelial cells with DNA damage.

The functionality of TRAIL-mediated apoptosis in normal and tumour tissues as well as the precise clinical significance and the molecular biologic regulatory mechanisms have yet to be elucidated. The cell killing properties of this cytokine have made it an exciting target for drug development. Several studies have shown that recombinant human (rh) TRAIL induces apoptosis in a variety of cancer cell lines, including colon carcinoma cell lines. Combining chemotherapy and rhTRAIL resulted in potentiation of antitumour activity in colon carcinoma cell lines as well as in mice carrying subcutaneous tumours from colorectal cell lines. Based on preclinical toxicity and activity profiling, TRAIL is considered to be of interest for clinical use. In colorectal cancer, rhTRAIL may provide a useful drug for killing tumour cells as our data suggest not only increased levels of DR4 and DR5 receptor expression but also frequent loss of TRAIL expression.

In conclusion, our study demonstrates that TRAIL and its receptors are expressed in normal colon mucosa and that several changes occur during the course of the adenoma-carcinoma sequence. Most strikingly, tumour progression is associated with an increase in expression of the pro-apoptotic receptors DR4 and DR5 and an increase in apoptotic cell death. Furthermore, the expression of TRAIL decreases in the adenoma-carcinoma sequence. More studies are needed to elucidate the precise role of TRAIL as a mediator of apoptosis in the colon, its role in the development of colorectal cancer and its potential as a therapeutic agent.
References

Chapter 4


TRAIL and its receptors in normal and neoplastic colon


