TRAIL Induces Apoptosis in Human Colorectal Adenoma Cell Lines and Human Colorectal Adenomas

Mathilde Jalving, Steven de Jong, Jan J. Koornstra, Wytske Boersma-van Ek, Nynke Zwart, Jelle Wesseling, Elisabeth G.E. de Vries, and Jan H. Kleibeuker

Abstract

Purpose: Recombinant human (rh) tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a potential new anticancer drug which can induce apoptosis in colorectal cancer cell lines. The aim of this study was to investigate whether it is possible to induce apoptosis in human adenoma cell lines and human adenomas using rhTRAIL.

Experimental Design: Two human adenoma cell lines were exposed to 0.1 μg/mL of rhTRAIL for 5 hours. Apoptosis and caspase activation in cell lines were evaluated using immunocytochemistry, fluorimetric caspase assays, and Western blotting. Short-term explant cultures were established from freshly removed human adenomas (n = 38) and biopsies of normal colon epithelium (n = 15), and these were incubated for 5 hours in the presence or absence of 1 μg/mL of rhTRAIL. Apoptosis was determined in paraffin-embedded tissue using morphologic criteria and cleaved caspase-3 staining.

Results: In the adenoma cell lines, rhTRAIL induced up to 55% apoptosis. This coincided with caspase-8 and caspase-3 activation and could be inhibited by a pan-caspase inhibitor. rhTRAIL induced caspase-dependent apoptosis in adenomas with high-grade dysplasia (n = 21) compared with the paired untreated counterparts (apoptotic index, 34 ± 5% versus 17 ± 2%, mean ± SE; P = 0.002), but not in adenomas with low-grade dysplasia (n = 17) or in normal colon epithelium (n = 15).

Conclusions: Colorectal adenoma cell lines and adenomas with high-grade dysplasia are sensitive to rhTRAIL-induced apoptosis, whereas normal colon epithelium is not. This suggests the potential application of rhTRAIL in the treatment of adenomas with high-grade dysplasia.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor family of death receptor ligands which have been shown to induce apoptosis in a wide variety of cancer cell lines. Unlike the other tumor necrosis factor family members, tumor necrosis factor-α and Fas-ligand, TRAIL has little or no detectable cytotoxic effect on normal cells in vitro and in vivo, and therefore has potential as an anticancer agent (1). Four membrane-bound receptors for TRAIL have been identified: two cell death–inducing receptors (DR4 and DR5) and two decoy receptors (DcR1 and DcR2; ref. 2). The intracellular segments of DR4 and DR5 contain death domains required for TRAIL-mediated apoptotic cell death (3). In contrast, DcR1 lacks the intracellular death domain and DcR2 contains a truncated death domain and therefore these receptors cannot induce apoptosis. TRAIL binds as a homotrimer to DR4 and/ or DR5, inducing the trimerization of these receptors, which leads to the formation of a death-inducing signaling complex, activation of caspase-8 and caspase-3, and eventually results in apoptosis (4). Recombinant human TRAIL (rhTRAIL) induces apoptosis in human colon cancer cell lines as well as in human colon cancer cell line xenografts in mice (5, 6). Phase I studies with rhTRAIL and phase I and II studies with agonistic monoclonal antibodies against DR4 and DR5 are currently in progress in patients with cancer. Like carcinomas, colorectal adenomas have stronger immunohistochemical expression of the proapoptotic receptors DR4 and DR5 in comparison with normal colon epithelium, suggesting that adenomas could also be sensitive to rhTRAIL-induced apoptosis (7–9). This is interesting because not only sporadic adenomas, but also adenomas from patients with hereditary non–polyposis colorectal cancer and familial adenomatous polyposis express DR4 and DR5 (9). This makes rhTRAIL a potential therapeutic agent for the treatment of colorectal adenomas as well as colorectal carcinomas. However, the TRAIL sensitivity of a premalignant lesion has never been shown. Therefore, the next step is to establish whether the...
DR4 and DR5 receptors expressed in colorectal adenomas are functional and lead to TRAIL sensitivity.

In the present study, we determined the sensitivity of human adenoma cell lines and freshly removed human colorectal adenomas and normal colon epithelium for rhTRAIL-induced apoptosis.

**Patients and Methods**

**Cell culture.** The human adenoma cell lines, VACO-235 and VACO-330, derived from a villous and tubular adenoma, respectively, were used (kindly provided by J.K. Willson M.D., Ireland Cancer Center, Cleveland, OH; ref. 10). Early passages of both cell lines are nontumorigenic in athymic mice, show anchorage-dependent growth in culture and exhibit features of well-differentiated epithelial cells, including cell polarity, microvilli and junctional complexes (10). The VACO-235 cell line is APC mutant at both alleles, has a mutant K-ras allele, and wild-type p53 (11). The mutation status of the VACO-330 cell line has not been described. Early passages of the cell lines were used in all cases. The cell lines were maintained on rat tail collagen-coated plates in MEM supplemented with 2% fetal bovine serum, insulin, transferrin, selenium, epithelial growth factor, and hydrocortisone (MEM2 + media) as previously described (10). To compare the sensitivity of adenoma cell lines for rhTRAIL to that of colon cancer cell lines, the colon cancer cell line LOVO was used (12). Continuous incubation of the LOVO cell line with 0.04 μg/mL rhTRAIL for 96 hours resulted in 50% growth inhibition (data not shown), making this cell line intermediately sensitive in comparison with other human colon cancer cell lines such as SW480, Caco-2, and Colo320 which have been used in our laboratory (13). The LOVO cell line was cultured in RPMI (Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 10% fetal calf serum.

**Flow cytometry for membrane expression of TRAIL receptors.** Analysis of TRAIL receptor membrane expression (DR4, DR5, DcR1, and DcR2) was done using a flow cytometer (Epics Elite, Coulter-Electronics, Hialeah, FL) and cells were stained as described previously (13). Membrane receptor expression is shown as mean fluorescence intensity of all analyzed cells. All experiments were done in triplicate.

**Quantification of apoptosis in cell lines.** Immunocytochemistry was used to quantify apoptosis in the adenoma and carcinoma cell lines. Cells were incubated with 0.1 μg/mL of rhTRAIL and/or 25 μg/mL of the broad-spectrum caspase inhibitor zVAD-fmk (Calbiochem, Breda, the Netherlands) for 5 hours at 37°C before cytospins were made. rhTRAIL was produced non-commercially in cooperation with IQ Biomedical (Zoetermeer, the Netherlands). The incubation time was chosen based on preliminary observations indicating that apoptosis induction by rhTRAIL in freshly removed polyps already occurs after 3 hours, whereas, after long incubation periods, the quality of the material decreases gradually in time, making evaluation more difficult. The incubation time was chosen based on preliminary observations indicating that apoptosis induction by rhTRAIL in freshly removed polyps already occurs after 3 hours, whereas, after long incubation periods, the quality of the material decreases gradually in time, making evaluation more difficult.

**Determination of apoptosis in clinical specimens.** Colorectal polyps, 0.5 to 2 cm in diameter were put in medium (RPMI supplemented with 10% fetal calf serum) at room temperature directly after endoscopic resection and processed immediately. A section of the polyp, up to 100 mm² depending on the size of the polyp, was removed by the pathologist and was transported to the laboratory in medium. The remaining section of the polyp was prepared for routine histology. Three biopsies of normal colon mucosa were obtained from the sigmoid of patients without macroscopic abnormalities at colonoscopy. One biopsy of normal colorectal mucosa was prepared for routine histology and the other two were transported directly to the laboratory in medium. In the laboratory, the removed section of polyp was cut into two equal pieces. One piece of polyp was incubated in tissue culture medium [as described by Tong et al. (15)] and the other piece was incubated in tissue culture medium with 1 μg/mL of rhTRAIL. In five cases, the polyp tissue segment was large enough to be divided into four pieces to determine whether apoptosis in freshly removed adenomas was caspase-dependent. The first two pieces were incubated as described above, the third in tissue culture medium with 50 μmol/L of the caspase inhibitor ZVAD-fmk, and the fourth in tissue culture medium with a combination of rhTRAIL and ZVAD-fmk. All cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂ for 5 hours. The incubation time was chosen based on preliminary observations indicating that apoptosis induction by rhTRAIL in freshly removed polyps already occurs after 3 hours, whereas, after long incubation periods, the quality of the material decreases gradually in time, making evaluation more difficult. The biopsies of normal colon mucosa were also incubated for 5 hours in tissue culture medium, one biopsy with, and one without 1 μg/mL of rhTRAIL. After incubation, all polyp pieces and biopsies were fixed in formalin and embedded in paraffin.

**Histologic classification.** Histologic classification of polyps and normal colorectal mucosa was carried out in H&E-stained slides by the pathologist (J. Wesseling). Adenomas and the degree of dysplasia (low or high) were defined according to WHO guidelines (16). In adenomas with high-grade dysplasia, the percentage of the adenoma in which high-grade dysplasia was present was estimated semiquantitatively (<25%, 25-50%, 50-75%). The location of the adenomas was retrieved from endoscopy or pathology reports. The biopsies of normal colorectal mucosa were examined for signs of inflammation or dysplasia and, if present, the biopsies from that patient were excluded from further analysis.

**Determination of apoptosis in clinical specimens.** All paraffin-embedded clinical specimens were H&E-stained, to determine the morphologic characteristics and the degree of apoptosis, and were stained for cleaved caspase-3 (Cell Signaling Technology) following the manufacturer’s instructions to determine the degree of apoptosis. Two
Fig. 1. A, membrane expression of the TRAIL receptors DR4, DR5, DcR1, and DcR2 in the adenoma cell lines VACO-235 and VACO-330 as determined by flow cytometry. Columns, mean fluorescence intensity of at least three independent experiments; bars, ± SE. B, degree of apoptosis (%) in VACO-235, VACO-330, and LOVO after treatment with rhTRAIL and/or zVAD-fmk for 5 hours as determined by immunocytochemistry for cleaved caspase-3. Experiments were done in triplicate. Columns, mean; bars, ± SE. * P < 0.05, percentage of apoptotic cells is higher in the rhTRAIL-treated group as compared with the other three treatments for each cell line. C, Western blot analysis of cleavage of caspase-8, caspase-3, and PARP in adenoma cell lines. rhTRAIL induces cleavage of caspase-8 into the intermediate 41 to 43 kDa product and the active p18 subunit. If cells are apoptotic, caspase-3 (32 kDa) is cleaved, resulting in two fragments (17 and 19 kDa) and full-length PARP (112 kDa) is cleaved, resulting in an 85 kDa piece. Representative of at least three different experiments. Actin (40 kDa) is shown as loading control. D, caspase activity in adenoma cell lines after 5 hours of incubation with 0.1 μg/mL of rhTRAIL. Caspase-3 and caspase-8 activity was determined using fluorimetric assays. Caspase activity is expressed as the ratio of treated to untreated cells. Experiments were done in triplicate; columns, mean bars, ± SE.

Results

**rhTRAIL induces apoptosis in adenoma cell lines.** The adenoma cell lines both expressed the receptors, DR4 and DR5 (Fig. 1A). rhTRAIL induced apoptosis in both cell lines. As shown in Fig. 1B, incubation with rhTRAIL for 5 hours induced apoptosis in both the VACO-235 and the VACO-330 cells. Apoptosis induction by rhTRAIL could be inhibited using a pan-caspase inhibitor (Fig. 1B). The amount of apoptosis was similar in the adenoma cell lines and in the intermediately TRAIL-sensitive colon cancer cell line, LOVO (Fig. 1B). Western blot analysis showed that induction of apoptosis was accompanied by activation of caspase-8 and caspase-3 and cleavage of the early apoptosis marker PARP (Fig. 1C). Caspase enzyme activity assays also showed activation of caspase-8 and caspase-3 by rhTRAIL (Fig. 1D). In summary, rhTRAIL induces caspase-dependent apoptosis in human colon adenoma cell lines.

rhTRAIL induces apoptosis in adenomas, but not in normal colon epithelium. To elucidate whether (subgroups of) freshly removed adenomas are sensitive to rhTRAIL-induced apoptosis a series of polyps (n = 48) was tested. Five polyps were excluded from further analysis because they were classified as hyperplastic polyps or juvenile polyps and five others were excluded because <50% of the crypts were evaluable. Thirty-eight adenomas were included in the final analysis. Patient characteristics are shown in Table 1.

Figure 2 shows two pieces of the same adenoma with high-grade dysplasia, one treated with rhTRAIL showing extensive apoptosis (B) and one untreated in which normal cell morphology is visible (A). In order to show that this apoptosis was caspase-dependent and not an artifact of the culture model, we incubated five of the larger adenomas in combinations of the broad-spectrum caspase inhibitor zVAD-fmk and rhTRAIL. rhTRAIL also induced apoptosis in these adenomas, as determined in H&E-stained slides and slides stained for cleaved caspase-3, and this apoptosis could be completely inhibited by zVAD-fmk (Fig. 3). This proves that the rhTRAIL-induced
apoptosis in adenomas is mediated through caspase activation. There was no difference in the degree of apoptosis between untreated and zVAD-fmk treated adenoma tissue pieces indicating that zVAD-fmk alone did not cause or prevent apoptosis.

In a large series of adenomas tested, no difference was observed in the mean size of the tissue pieces between the rhTRAIL-treated and the untreated groups (3.4 ± 0.2 mm versus 3.4 ± 0.2 mm; mean ± SE). Adenoma tissue segments treated with rhTRAIL had a higher mean degree of apoptosis than untreated segments (Table 2). Subgroup analysis revealed that apoptosis induction was higher in the rhTRAIL treated segments than in the paired untreated segments in adenomas with high-grade dysplasia (n = 21), but not in adenomas with low-grade dysplasia (n = 17, Table 2; Fig. 4A and B). In normal sigmoid epithelium (n = 15) the mean degree of apoptosis in untreated segments was lower than in the untreated adenoma segments. There was no difference in the mean degree of apoptosis between treated and untreated biopsies of normal colonic mucosa (Table 2; Fig. 2C and D).

In general, larger tissue pieces were easier to handle than smaller tissue pieces resulting in less mechanical damage. Concomitantly, larger untreated tissue pieces had lower degrees of apoptosis (R = −0.317, P = 0.05). To adjust for these variations in apoptosis in the control tissue segments, the degree of treatment-specific apoptosis was also calculated. Using this calculation, adenomas with high-grade dysplasia were again shown to be sensitive to rhTRAIL-induced apoptosis, whereas adenomas with low-grade dysplasia and normal colon epithelium were not sensitive. Furthermore, adenomas with a higher percentage of high-grade dysplasia were shown to be more sensitive to rhTRAIL-induced apoptosis (R = 0.6, P = 0.004; Fig. 4C and D).

### Discussion

The proapoptotic TRAIL receptors, DR4 and DR5, are immunohistochemically expressed in colorectal adenomas. However, immunohistochemical studies cannot predict whether these receptors induce apoptosis following the binding of rhTRAIL. In the present study, we show that two human adenoma cell lines, one derived from a villous adenoma and the other from a tubular adenoma, express DR4 and DR5, and are both sensitive to rhTRAIL-induced apoptosis. In a clinically more relevant model, freshly removed human colorectal adenomas with high-grade dysplasia, but not those with low-grade dysplasia, were also sensitive to rhTRAIL-induced apoptosis. Importantly, freshly removed normal human colonic epithelium was not sensitive to rhTRAIL.

It is well known that in contrast to normal cells, cancer cell lines as well as immortalized and transformed normal cell lines

![Table 1. Patient characteristics](image)

<table>
<thead>
<tr>
<th></th>
<th>Adenomas</th>
<th>Normal colon</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>38</td>
<td>15</td>
</tr>
<tr>
<td>Male (%)</td>
<td>61</td>
<td>47</td>
</tr>
<tr>
<td>Mean age in years (range)</td>
<td>65 (41-87)</td>
<td>51 (24-74)</td>
</tr>
<tr>
<td>Localization (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascending/transverse</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td>Descending/sigmoid</td>
<td>68</td>
<td>100</td>
</tr>
<tr>
<td>Rectum</td>
<td>16</td>
<td>—</td>
</tr>
<tr>
<td>Low-grade dysplasia (%)</td>
<td>45</td>
<td>—</td>
</tr>
<tr>
<td>High-grade dysplasia (%)</td>
<td>55</td>
<td>—</td>
</tr>
</tbody>
</table>

![Fig. 2.](image)
are sensitive to rhTRAIL-induced apoptosis. This is not only true for artificially immortalized cells (viral and telomerase immortalization) but also for spontaneously transformed cells (18–22). This had not previously been reported for colorectal adenoma cells which could, under certain conditions, be grown in culture and could therefore be described as transformed cells. The rhTRAIL sensitivity of the two adenoma cell lines used in this study is, therefore, in line with previous results in transformed cell lines. In contrast, Hague et al. reported that four adenoma cell lines, which were similar to those used in this study, were not sensitive to 1 μg/mL of TRAIL, unless transformed to a malignant phenotype in vitro (23–26). The histidine-tagged TRAIL used by Hague et al. is, however, known to be less potent than native rhTRAIL at inducing cell death in a number of cancer cell lines (27, 28). Our cell line results indicate that adenomas could be sensitive to rhTRAIL-induced apoptosis. However, the number of adenoma cell lines available is limited and their validity as a model for adenomas is often questioned. To determine whether human colorectal adenomas are truly sensitive to rhTRAIL, it is necessary to use a model that is closer to the in vivo situation, such as a short-term explant culture model.

In the present study, we show that, in this clinically more relevant model, freshly removed human colorectal adenomas with high-grade dysplasia, but not those with low-grade dysplasia, are sensitive to rhTRAIL-induced apoptosis. Importantly, in the same model, freshly removed normal human colon epithelium is not sensitive. The latter is in agreement with toxicity studies in rodents, monkeys, and chimpanzees, and a study in isolated human normal colon crypts which also showed that native soluble rhTRAIL is not toxic for normal cells (6, 28–30). The advantage of using short-term culture of adenoma explants as opposed to single-cell suspensions and adenoma cell lines is that they contain representative, unselected combinations of cell populations in an environment with stroma and intact cell-cell interactions (31–33). The amount of apoptosis in the cultured untreated control segments was relatively high as compared with untreated samples from the same patient which were directly fixed in formalin (data not shown) and also in comparison to the cultured normal colonic epithelium. This apoptosis is probably caused by detachment from surrounding connective tissue on the one hand and damage occurring between resection and incubation on the other, the latter being more prominent for the adenoma sections than for the biopsies of normal colonic epithelium (34). This is supported by the fact that this apoptosis was not inhibited by a pan-caspase inhibitor and was higher in the more difficult to handle smaller adenoma segments. The rhTRAIL sensitivity of high-grade dysplastic adenomas in this culture model is in line with the results in the cell line model suggesting that the rhTRAIL sensitivity is not an artifact of the culture model. In a small subset of cases, apoptosis was higher in the control segment compared with the treated segment. This is also most likely to be a consequence of the culture model as described above. Another possibility is that rhTRAIL-induced inhibition of apoptosis in these adenomas. rhTRAIL inhibition of apoptosis of cancer cell lines has been described, however, this is rare and mostly a consequence of mutations rarely found in colorectal adenomas or carcinomas (35, 36).

Subgroup analysis showed that only adenomas with high-grade dysplasia were sensitive to rhTRAIL-induced apoptosis. Furthermore, adenomas with high-grade dysplasia were more sensitive when a larger percentage of the adenoma was highly dysplastic. These results indicate that TRAIL sensitivity is acquired relatively late in the adenoma-to-carcinoma sequence (37). Immunohistochemical expression of the TRAIL receptors is the same in adenomas with low-grade and high-grade dysplasia, therefore, this cannot explain the observed difference in sensitivity (8). Inactivation of the TRAIL receptors by mutation or methylation is thought to be rare in colorectal cancer (36). It is, however, possible that the availability of the

### Table 2. Degree of apoptosis (%) in tissue pieces and biopsies cultured in tissue culture medium (untreated) or in tissue culture medium with 1 μg/mL of rhTRAIL

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>H&amp;E Untreated (mean ± SE)</th>
<th>rhTRAIL (mean ± SE)</th>
<th>P*</th>
<th>Cleaved caspase-3 Untreated (mean ± SE)</th>
<th>rhTRAIL (mean ± SE)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>All adenomas</td>
<td>38</td>
<td>17 ± 2</td>
<td>26 ± 3</td>
<td>0.003</td>
<td>11 ± 2</td>
<td>16 ± 3</td>
<td>0.030</td>
</tr>
<tr>
<td>High-grade dysplasia</td>
<td>21</td>
<td>17 ± 2</td>
<td>34 ± 5</td>
<td>0.002</td>
<td>14 ± 3</td>
<td>21 ± 4</td>
<td>0.048</td>
</tr>
<tr>
<td>Low-grade dysplasia</td>
<td>17</td>
<td>16 ± 3</td>
<td>17 ± 3</td>
<td>not significant</td>
<td>7 ± 2</td>
<td>9 ± 2</td>
<td>not significant</td>
</tr>
<tr>
<td>Normal colon</td>
<td>15</td>
<td>6 ± 1</td>
<td>6 ± 1</td>
<td>not significant</td>
<td>5 ± 1</td>
<td>6 ± 1</td>
<td>not significant</td>
</tr>
</tbody>
</table>

*Paired Wilcoxon signed rank test.
receptors at the membrane is different between high-grade and low-grade dysplastic adenomas. Furthermore, little is known about downstream expression levels of genes involved in the TRAIL apoptosis pathway in adenomas and whether these differ between adenomas with low-grade and high-grade dysplasia. It has been shown that mutations in the oncogene Ras, which are acquired during the adenoma-carcinoma sequence, can sensitize cells to rhTRAIL-induced apoptosis and this mechanism could be, at least in part, responsible for the sensitivity of adenomas with high-grade dysplasia (38, 39).

Early clinical trials involving rhTRAIL and agonistic TRAIL receptor antibodies are currently in progress in patients with cancer (http://www.hgsi.com/). The rhTRAIL-sensitivity of a premalignant lesion such as a colorectal adenoma is interesting and indicates that the application of rhTRAIL, agonistic TRAIL receptor antibodies or other, yet to be developed, TRAIL-receptor agonists may be possible in this setting. A potential application could be the downsizing of endoscopically irresectable adenomas with high-grade dysplasia to allow endoscopic removal. In patients with hereditary non-polyposis colorectal cancer, this could prevent a subtotal colectomy and in patients with familial adenomatous polyposis with ileorectal anastomosis, it might mean that proctectomy can be averted. It is interesting to note that nonsteroidal antiinflammatory drugs, which are frequently used in the treatment of patients with the latter condition, have been shown to potentiate TRAIL-induced apoptosis in colorectal cancer cells in vitro (36).

In conclusion, rhTRAIL induces apoptosis in colorectal adenoma cell lines as well as in freshly removed human colorectal adenomas with high-grade dysplasia, but not in those with low-grade dysplasia or in normal colon epithelium. TRAIL-based therapy could therefore be a potential adjuvant in the treatment of adenomas with high-grade dysplasia.

Acknowledgments

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References

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