Increased sensitivity to TRAIL-induced apoptosis occurs during the adenoma to carcinoma transition of colorectal carcinogenesis

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The death ligand TRAIL (ApO2L) has potential for cancer therapy, since tumour cells are thought to be more sensitive than normal cells. We investigated whether sensitivity to TRAIL increases during the adenoma to carcinoma transition of colorectal carcinogenesis. Under the same culture conditions, we compared the extent of TRAIL-induced apoptosis in four premalignant adenoma and three carcinoma cell lines. Although TRAIL induced some apoptosis in adenoma cultures, the carcinoma cell lines were significantly more sensitive (P < 0.001). This finding was recapitulated in an in vitro model of tumour progression in which conversion of the adenoma cell line AA/C1 to a tumorigenic phenotype was associated with increased TRAIL sensitivity (P < 0.001). Increased TRAIL sensitivity during colorectal carcinogenesis has been previously attributed to changes in the balance between TRAIL receptors TRAIL-R1 and -R2 and ‘decoy’ receptors TRAIL-R3 and -R4 during malignant progression. To address this, cell surface receptor expression was measured by flow cytometry. In summary, during colorectal carcinogenesis, there is a marked increase in sensitivity to TRAIL-induced apoptosis associated with progression from benign to malignant tumour that could be exploited for colon cancer therapy, but alterations in cell surface TRAIL receptor expression may not be the primary reason for this change.

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Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) mediates rapid apoptosis in cancer cell lines of varied tissue origin (Wiley et al., 1995; Petti et al., 1996) and has generated much interest as a potential therapeutic agent due to its apparent differential effects on normal and tumour cells (Wang and El-Deiry, 2003). Tumour necrosis factor-related apoptosis-inducing ligand interacts with four different membrane-bound receptors, two with cytoplasmic death domains, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), and two so-called ‘decoy receptors’ TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) (Ashkenazi and Dixit, 1998). When TRAIL binds the receptors R1 and R2, the death signal is transmitted through their intracellular protein–protein interacting death domains via cytoplasmic adaptor molecules resulting in an early activation of caspase-8 (Griffith et al., 1998; MacFarlane et al., 2000a). The activation of the caspase cascade leads, in turn, to cleavage of intracellular substrates such as poly-(ADP-ribose) polymerase (PARP) (Lazebnik et al., 1994). However, the death receptors can also transmit signals for cell survival through NF-κB, and via caspase-8 can activate JNK and p38 MAP kinase pathways (Chaudhary et al., 1997; Schneider et al., 1997; Mühlenbeck et al., 1998; MacFarlane et al., 2000b). TRAIL-R3 and TRAIL-R4 are unable to transmit the death signal, since TRAIL-R3 does not have a death domain and TRAIL-R4 has a death domain that is substantially deleted (French and Tschopp, 1999). However, in some situations, TRAIL-R4 retains the ability to activate the NF-κB pathway (Degli-Eposti et al., 1997). Initially, the differential sensitivity between normal and cancer cells was thought to be due to the presence of decoy receptors on normal cells (Ashkenazi and Dixit, 1998; Green, 1998). More recent studies have failed to find an obvious correlation between decoy receptor expression and the apoptotic response to TRAIL, with levels of TRAIL-R1 and the caspase-8 inhibitory protein c-FLIP being more likely to affect the sensitivity of some cells to TRAIL-induced apoptosis (Kim et al., 2000).

Colorectal cancer cells have been reported to be sensitive to TRAIL-induced death (Gliniak and Le, 1999; Lacour et al., 2001; Lee et al., 2001; Ravi et al., 2001). The potential of TRAIL for colorectal cancer treatment is highlighted by the ability of TRAIL to inhibit growth of xenografts of several colon carcinoma cell lines in mouse models and to cooperate with chemotherapeutic agents such as 5-fluorouracil (5-FU), cisplatin, doxorubicin and camptothecin analogue CPT-11 (Ashkenazi et al., 1999; Gliniak and Le, 1999; Lacour et al., 2001, 2003; Shimoyama et al, 2002). In the carcinoma cell line HT29, chemotherapeutic agents, as well as potentiating the mitochondrial amplification of the caspase cascade, enhance recruitment of the adapter protein FADD and caspase-8 to the death-inducing signal complex (DISC) (Lacour et al., 2003).

Although Sheikh et al. (1999) reported overexpression of the decoy receptor TRAIL-R3 mRNA in four out of six colon cancers, with TRAIL-R3 overexpression conferring relative TRAIL
resistance, Koornstra et al (2003) found no significant alterations in expression of the decoy receptors in colonic tumours compared to normal colonic epithelium in vivo. Although loss of heterozygosity of TRAIL-R2 has been observed in approximately half of colorectal carcinomas, mutations of the two TRAIL receptor genes are rare (Arai et al, 1998). The loss of heterozygosity for TRAIL-R2 is not reflected in the level of protein in the tumours (Koornstra et al, 2001), and TRAIL-R1 and TRAIL-R2 proteins are overexpressed in colonic tumours relative to normal colonic mucosa (Koornstra et al, 2003). However, in colorectal carcinomas, expression of TRAIL-R1, but not TRAIL-R2, correlates with disease-free survival (Strätter et al, 2002a).

Normal colonocytes cultured as intact crypts embedded in collagen have previously been reported to be relatively resistant to TRAIL-induced apoptosis compared to colorectal carcinoma cells (Strätter et al, 2002b), but it is not known whether the sensitivity to TRAIL is acquired early or late in colorectal carcinogenesis, since the response of premalignant adenoma cells to TRAIL has never been examined. Koornstra et al (2003) showed that, in vivo, cytoplasmic TRAIL-R1 and -R2 expression was higher in both adenomas and carcinomas compared to normal colonic epithelium; however, there were no further changes in expression of either TRAIL receptors or decoy receptors between adenomas and carcinomas. From these results, it might be assumed that the sensitivity to TRAIL is acquired early in colorectal tumorigenesis during the formation of the adenoma, and that this is due to the changes in TRAIL receptor expression. However, the cell surface expression of TRAIL receptors in adenoma cells has not been examined, and this could have an important bearing on the relative TRAIL sensitivity of colorectal epithelial cells during tumour progression. In this study, we addressed whether TRAIL sensitivity increases in association with the transformation of an adenoma to carcinoma transition by comparing the response of four adenoma cell lines and three carcinoma cell lines to TRAIL under the same culture conditions. This is important because Larribere et al (2004) have shown that the response to TRAIL is dependent on culture conditions and that growth factor signalling through phosphatidylinositol-3 kinase and its downstream target, Akt, attenuates TRAIL-induced apoptosis of normal human melanocytes. In addition, we used a model of tumour progression in which an adenoma cell line had been transformed to a malignant phenotype in vitro (Williams et al, 1990); this allowed us to measure directly whether TRAIL sensitivity increased with acquisition of malignancy, again under the same culture conditions. To assess whether the relative abundance of TRAIL receptors on the cell surface accounted for differences in sensitivity to TRAIL-induced apoptosis, we measured cell surface expression of TRAIL-R1, -R2, -R3 and -R4 by flow cytometry.

MATERIALS AND METHODS

Cell lines and culture conditions

The adenoma cell lines used were AA/C1, RG/C2, AN/C1 and RR/C1. These cells were capable of growth after trypsinisation to single cells and are derivatives of the cell lines PC/AA (Paraskeva et al, 1984), S/RG (Paraskeva et al, 1989), S/AN (Paraskeva et al, 1989) and S/RR (Williams et al, 1992). The carcinoma cell lines used were HT29 (Fogh and Trempe, 1975), SW620, derived from a lymph node metastasis (Leibovitz et al, 1976), and K5 (Hague et al, 1997), which was derived in our laboratory using the same techniques as for the adenoma cultures. Whereas the four adenoma cell lines were anchorage-dependent and did not form tumours in nude mice (Williams et al, 1990; Hague et al, 1997), the carcinoma cell lines were anchorage-independent and tumorigenic after subcutaneous injection in nude mice. The AA/C1/SB/10 cell line is an in vitro-transformed variant of the AA/C1 adenoma cell line that is anchorage-independent and forms tumours in nude mice (Williams et al, 1990).

All of the cell lines used in this study were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) containing 20% fetal bovine serum (Gibco, batch selected), glutamine (2 mM), insulin (0.2 u ml⁻¹), hydrocortisone sodium succinate (1 mg ml⁻¹), penicillin (100 u ml⁻¹) and streptomycin (100 μg ml⁻¹). Cells were grown in T25 flasks and were routinely passaged using trypsin (0.1%) (Difco, West Molesey, Surrey, UK)/EDTA (0.1%) (AnalaR, BDH).

Preparation of TRAIL and treatment of cells

The TRAIL preparation used was recombinant soluble TRAIL with N-terminal T7 and His tags obtained by nickel affinity purification as previously described (MacFarlane et al, 1997). For dose–response experiments, a stock solution of TRAIL (1 mg ml⁻¹) was prepared in EDTA (150 mM) and diluted in medium to give final concentrations of 0.25, 0.5 and 1.0 μg ml⁻¹. Duplicate or triplicate flasks were treated with each TRAIL concentration or EDTA only. Control cultures were treated with the vehicle only.

Identification of apoptosis

As previously demonstrated in these cells (Hague et al, 1993), apoptosis results in rapid detachment from the flask and the apoptotic cells float in the medium. Floating cells were collected at the end of the treatment time and adherent cells were harvested by trypsinisation. Adherent and floating cells were quantified using a counting chamber. The extent of apoptotic cell shedding was calculated as the number of floating cells per flask divided by the total cell yield (number of adherent plus floating cells). Adherent and floating cells were examined separately for apoptotic morphology by acridine orange staining as described previously (Hague et al, 1993). Unfixed cells were stained with 5 μg ml⁻¹ acridine orange in PBS for 10 min and were then viewed by fluorescence microscopy. For each experiment, the cells shed into the medium were confirmed to be morphologically apoptotic with condensed chromatin. At least 150 cells were scored per sample. To further demonstrate that TRAIL induced apoptotic death, adherent and floating cells were examined separately for DNA laddering as described previously (Hague et al, 1993). Cleavage of PARP was detected by Western blot analysis.

SDS – PAGE – Western blotting

Western blotting was carried out using standard techniques and antibody binding was detected using an enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, UK) following the manufacturer’s protocol. To detect BID, two antibodies were used: a polyclonal BID antibody (1:200) kindly provided by Dr Xiaodong Wang (Howard Hughes Medical Institute, Texas) and a polyclonal BID antibody (AF6860) (1:1000) from R&D systems. c-FLIP rabbit polyclonal antibody was a gift from Dr D Nicholson (Merck Frosst, Canada) and was used at 1:1000. Results were confirmed using a mouse monoclonal antibody, NF-6 (Alexis Corporation) at 1:1000. XIAP was detected with a mouse monoclonal antibody used at 1:500 (BD Transduction Laboratories). A mouse monoclonal α-tubulin antibody (Sigma, Poole, Dorset, UK) or a mouse monoclonal α-GAPDH antibody (Advanced Immunochemical Inc., CA, USA) was used to assess equal loading of the gels, both at 1:2000. Secondary antibodies were anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (Sigma).
Flow cytometric analysis of cell surface death receptor expression

TRAIL-R1, -R2, -R3 and -R4 receptor antibodies (clones M272, M413, M430 and M444, respectively) were kind gifts from Immunex Corp. (Seattle, WA, USA) and have been described previously (Griffith et al, 1999). These antibodies have been validated for flow cytometric measurement of surface TRAIL expression (Zhang et al, 2000; MacFarlane et al, 2002). A total of 10^6 trypsinised colon cells (live) were incubated in blocking buffer (PBS containing 10% normal goat serum) for 30 min on ice to block nonspecific binding. They were then incubated for 1 h with anti-TRAIL receptor antibodies (diluted 1:50) at 4°C. After two washes in PBS and centrifugation for 5 min at 400 g, the cells were resuspended in blocking buffer containing goat anti-mouse FITC-conjugated antibody (F(ab)2 fragment) (DAKO) (1:20 dilution) and incubated for 1 h at 4°C. The cells were then washed twice in cold PBS prior to analysis by flow cytometry (FACScan, Becton Dickinson). Dead cells were excluded by staining the cells with propidium iodide. The propidium iodide-positive cells could then be gated out.

Statistical analysis

The response of adenomas and carcinomas was compared by a two-way analysis of variance of TRAIL-induced apoptosis (malignant potential × TRAIL concentration). For the comparison of TRAIL sensitivity between the parental adenoma cell line AA/C1 and the transformed adenoma cell line AA/C1/SB/10, a two-way analysis of variance was conducted (cell line × TRAIL concentration). All analyses were conducted using SPSS 10 for Windows software.

RESULTS

Colorectal cancer cells are more sensitive to TRAIL-induced apoptosis than premalignant adenoma cells

In this study, we addressed whether there is differential sensitivity to TRAIL-induced apoptosis between malignant and premalignant colorectal epithelial cells. Initially, the apoptotic response of four premalignant, nonmalignant adenoma cell lines (RG/C2, RR/C1, AN/C1 and AA/C1) was compared with that of three carcinoma cell lines (HT29, SW620 and KS) by conducting dose–response experiments for each cell line. In order to determine the extent of resistance of the adenoma cells, relatively high concentrations of TRAIL were used (0.25–1 μg ml⁻¹). As in our previous studies quantifying apoptosis in colon cells, we used cell shedding as an assessment of the extent of apoptosis (Hague et al, 1993). The cells shed into the medium in response to TRAIL treatment were confirmed to be apoptotic by acridine orange staining and examination of the floating cells by fluorescent microscopy for condensed chromatin, PARP cleavage to its 85 kDa form and DNA laddering (data not shown). Treatment with TRAIL did not induce necrosis in any of the cell lines even at concentrations up to 2 μg ml⁻¹. Measurements of the extent of apoptotic cell shedding after a 16 h treatment demonstrated that, although TRAIL induced apoptosis in the adenoma cell cultures, the carcinoma cell lines were markedly more sensitive to TRAIL than the adenoma cell lines (Figure 1). When extensive shedding was obtained over the 16 h treatment period, this was reflected in reduced adherent cell yields (data not shown). The three carcinoma cell lines were markedly more sensitive than the four adenoma cell lines, a difference that was highly statistically significant (P<0.0001).

To confirm that TRAIL-mediated apoptosis was due to receptor ligation and not due to nonspecific toxicity of TRAIL, a TRAIL-R2-Fc chimeric protein, comprised of the TRAIL-R2 extracellular domain fused to human IgG, Fc, was used as a soluble receptor and blocking reagent. TRAIL-R2-Fc completely blocked TRAIL-induced apoptotic cell shedding in the carcinoma cell line HT29, confirming that death receptor-mediated apoptosis was responsible for the cell shedding induced by TRAIL treatment (Figure 2A). Consistent with this, TRAIL treatment resulted in activation of caspase-8, with cleavage of the proform of 55 kDa to 43, 41 and 18 kDa fragments (Figure 2B) together with cleavage of the 26 kDa proform of BID to yield its 15 kDa fragment (Figure 2C). Cleavage of caspase-8 and BID was accompanied by reduced levels of the full-length forms (Figure 2B and C).

The in vitro-transformed adenoma cell line AA/C1/SB/10 is more sensitive to TRAIL-induced apoptosis than the parental adenoma cell line AA/C1

Since adenoma cells appeared to be more resistant to TRAIL than carcinoma cells, experiments were conducted to determine more directly whether TRAIL sensitivity increased in association with malignant progression by comparing the sensitivity of AA/C1, an anchorag-dependendent, nonmalignant, adenoma-derived cell line, and AA/C1/SB/10, an anchorage-independent, tumorigenic derivative of the AA/C1 cell line (Williams et al, 1999). In these experiments, cell shedding was confirmed to be due to apoptosis by acridine orange staining for apoptotic morphology and by detection of PARP cleavage (data not shown). Although the transformed AA/C1/SB/10 cell line had lower levels of spontaneous apoptosis than the parental AA/C1 cell line, dose–response experiments demonstrated that AA/C1/SB/10 was more sensitive to TRAIL-induced apoptosis than AA/C1 (P<0.0001) (Figure 3). The AA/C1 cells remained relatively resistant with increasing concentrations of TRAIL (0.25, 0.5 and 1 μg ml⁻¹). Even 2 μg ml⁻¹ TRAIL did not induce as much apoptotic cell shedding in AA/C1 as that induced by 0.25 μg ml⁻¹ in AA/C1/SB/10 (data not shown). Therefore, the differential sensitivity between the two cell lines was
TRAIL-induced apoptosis in colorectal tumour cells

A Hague et al

Figure 2 (A) Apoptosis induced by TRAIL is receptor-mediated and involves cleavage of caspase-8 and BID. Apoptosis induced by TRAIL in the HT29 cell line is blocked using TRAIL R2-Fc chimeric protein (R and D systems, Abingdon, UK) to compete with cellular TRAIL receptors for the death ligand. Apoptotic cell shedding induced by 0.25 \(\text{mg} \cdot \text{ml}^{-1}\) TRAIL was completely blocked by 300 \(\mu\text{g} \cdot \text{ml}^{-1}\) TRAIL-R2-Fc. (B) Caspase-8 is cleaved in HT29 cells in response to TRAIL treatment (16 h). Lane 1: control adherent cells; lane 2: adherent cells treated with 0.125 \(\text{mg} \cdot \text{ml}^{-1}\) TRAIL; lane 3: adherent cells treated with 0.25 \(\mu\text{g} \cdot \text{ml}^{-1}\) TRAIL. (C) BID is cleaved in HT29 cells in response to 16 h TRAIL treatment. Lane 1: control adherent cells; lane 2: TRAIL-treated adherent cells (0.25 \(\mu\text{g} \cdot \text{ml}^{-1}\)); lane 3: control adherent and floating cells pooled; lane 4: TRAIL-treated adherent and floating cells pooled.

Figure 3 The in vitro-transformed, tumorigenic AA/C1/SB/10 cell line is more sensitive to TRAIL-induced apoptosis than the adenoma cell line AA/C1 from which it was derived. Dose response of the adenoma cell line AA/C1 and the in vitro-transformed adenoma cell line AA/C1/SB/10 showing the extent of apoptotic cell shedding after a 16 h treatment with 0.25, 0.5 and 1 \(\mu\text{g} \cdot \text{ml}^{-1}\) TRAIL. Results shown are means \pm s.e.m. of three experiments performed in triplicate. Comparing the percentage of apoptotic cell shedding between AA/C1 and AA/C1/SB/10 by a two-way analysis of variance revealed a significantly greater extent of apoptosis in AA/C1/SB/10 (\(P<0.001\)).

Cell surface receptor expression profiles of adenoma and carcinoma cells cannot account for their differential sensitivity to TRAIL.

Since it has been reported that there are no differences in total TRAIL receptor expression between colorectal adenomas and carcinomas (Koornstra et al, 2003), we addressed whether the differential sensitivity between nonmalignant and malignant colonic epithelial cells could be explained by relative cell surface expression of TRAIL receptors. To do this, flow cytometric analysis of antibody-labelled nonfixed cells was performed. The antibodies used to detect TRAIL receptors have been well characterised for efficient receptor binding by Griffith et al (1999) and for detection of cell surface TRAIL receptors by flow cytometry (Zhang et al, 2000; MacFarlane et al, 2002). TRAIL-R1 levels were low and similar in adenoma and carcinoma cell lines (Table 1). TRAIL-R2 was expressed in all of the eight cell lines and was the most abundant of the four receptors. AA/C1, which was relatively resistant to TRAIL, had the highest levels of TRAIL-R2, whereas KS, which was sensitive to TRAIL-induced apoptosis, had low levels. TRAIL-R3 was expressed in the adenoma cell lines AN/C1 and AA/C1, and in the transformed adenoma cell line AA/C1/SB/10, but interestingly, the other five colonic tumour cell lines did not express cell surface TRAIL-R3. Similarly, only three cell lines expressed the TRAIL-R4 receptor (at low levels): the carcinoma cell line HT29, the adenoma cell line AN/C1 and the transformed

Table 1 Flow cytometric analysis of TRAIL receptor expression

| Adenoma cell lines | Transformed adenoma | Carcinoma cell lines |
|--------------------|---------------------|---------------------|
|                    | RR/C1   | AN/C1   | AA/C1   | AA/C1/SB/10 | HT29    | SW620   | KS      |
| RG/C2              | 3.72    | 5.48    | 7.22    | 6.09       | 3.79    | 3.37    | 3.79    | 2.37      |
| NEG/C1             | 4.28    | 5.95    | 7.26    | 6.61       | 3.98    | 3.65    | 4.05    | 2.55      |
| TRAIL-R1           | 5.50    | 7.49    | 8.70    | 7.55       | 4.84    | 5.19    | 5.84    | 2.81      |
| TRAIL-R2           | 13.75   | 25.69   | 27.14   | 98.18      | 27.97   | 17.01   | 24.67   | 4.52      |
| TRAIL-R3           | 4.10    | 5.90    | 9.39    | 10.60      | 4.10    | 3.92    | 3.91    | 2.49      |
| TRAIL-R4           | 4.31    | 5.80    | 8.02    | 6.67       | 5.54    | 4.38    | 4.20    | 2.48      |

Table 1 Flow cytometric analysis of TRAIL receptor expression

|                      | RG/C2   | RR/C1   | AN/C1   | AA/C1   | AA/C1/SB/10 | HT29    | SW620   | KS      |
|----------------------|---------|---------|---------|---------|-------------|---------|---------|---------|
| CON\(^a\)            | 3.72    | 5.48    | 7.22    | 6.09    | 3.79        | 3.37    | 3.79    | 2.37    |
| NEG\(^b\)            | 4.28    | 5.95    | 7.26    | 6.61    | 3.98        | 3.65    | 4.05    | 2.55    |
| TRAIL-R1             | 5.50    | 7.49    | 8.70    | 7.55    | 4.84        | 5.19    | 5.84    | 2.81    |
| TRAIL-R2             | 13.75   | 25.69   | 27.14   | 98.18   | 27.97       | 17.01   | 24.67   | 4.52    |
| TRAIL-R3             | 4.10    | 5.90    | 9.39    | 10.60   | 4.10        | 3.92    | 3.91    | 2.49    |
| TRAIL-R4             | 4.31    | 5.80    | 8.02    | 6.67    | 5.54        | 4.38    | 4.20    | 2.48    |

Values are the mean fluorescent intensity of live (propidium iodide-excluding) cells stained with monoclonal antibodies and detected using an FITC-conjugated mouse secondary antibody. \(^a\)Cells incubated without primary or secondary antibody. \(^b\)Cells incubated with secondary antibody only.
The relative resistance of the adenoma cell lines to TRAIL-induced apoptosis cannot be accounted for by higher expression of XIAP or c-FLIP.

Given the lack of correlation between malignant potential and TRAIL receptor expression, we further investigated the relative resistance of adenoma cell lines to TRAIL by examining intracellular components of the death receptor signalling pathway that have previously been shown to modulate TRAIL sensitivity. c-FLIP, an inhibitor of caspase-8 activation, and XIAP, an inhibitor of active caspases-3 and -9, were expressed in all of the cell lines. c-FLIP levels were markedly higher in the transformed adenoma cell line AA/C1/SB/10 than in the parental adenoma cell line AA/C1 (Figure 5, top panel, compare lanes 4 and 5). Thus, c-FLIP levels did not appear to correlate with TRAIL sensitivity. Although c-FLIPs serve to inhibit TRAIL-induced apoptosis in some cell types (Bin et al, 2002), none of the colorectal tumour cell lines expressed c-FLIPs. XIAP levels also did not appear to correlate with TRAIL sensitivity. Although XIAP levels were slightly higher in the parental adenoma cell line AA/C1 than in AA/C1/SB/10, the other cell lines had similar expression levels of XIAP (Figure 5, middle panel).

**DISCUSSION**

Interest in TRAIL as a potential chemotherapeutic agent stems from observations suggesting that it may selectively induce apoptosis in cancer cells while sparing normal cells. Increased sensitivity to TRAIL during carcinogenesis is not a feature of all tissues; for example, comparisons of the response of normal and malignant ovarian cells to TRAIL have shown normal cells to be sensitive to TRAIL whereas cells from different ovarian carcinomas varied in their responses (Lane et al, 2004). Normal colonocytes have previously been reported to be relatively resistant to TRAIL-induced apoptosis compared to colorectal carcinoma cells: normal human colonic epithelial cells did not undergo apoptosis in response to 0.1 μg ml⁻¹ TRAIL, but COLO205 colonic carcinoma cells were sensitive to this concentration (Strätter et al, 2002b). Koornstra et al (2003) reported that colonic tumours over-expressed the TRAIL receptors TRAIL-R1 and TRAIL-R2 in vivo relative to normal mucosa, but that overall expression of TRAIL receptors was not significantly different between adenomas and carcinomas. On the basis of these observations, one might predict that the acquisition of TRAIL sensitivity would occur early in colorectal carcinogenesis and that adenoma cells would be as sensitive to TRAIL-induced apoptosis as carcinoma cells. However, it was not known whether there was a difference in TRAIL sensitivity between adenoma and carcinoma cells and whether TRAIL receptor expression on the cell surface, rather than intracellular receptor pools, would correlate with sensitivity to TRAIL-induced apoptosis. We addressed this hypothesis by comparing the extent of apoptosis induced by TRAIL in nonmalignant and malignant colorectal tumour cell lines. We initially questioned whether TRAIL was able to induce apoptosis of human colorectal adenoma cells, since this has not been reported before. The adenoma-derived cell lines did exhibit some apoptosis in response to TRAIL; however, all four adenoma cell lines, AA/C1, AN/C1, RR/C1 and RG/C2, were markedly more resistant than the three carcinoma cell lines HT29, SW620 and KS. The differential sensitivity between the adenoma and carcinoma cell lines was highly statistically significant (P<0.001). Furthermore, we used an in vitro model of colorectal carcinogenesis to demonstrate that the adenoma cell line AA/C1 became more sensitive to TRAIL after in vitro transformation to a malignant phenotype (P<0.001). Our studies provide direct evidence for increasing TRAIL sensitivity during colorectal carcinogenesis during the adenoma to carcinoma transition. They also show that the increased sensitivity to TRAIL can be recapitulated in vitro, and therefore in vivo selection pressures are not necessarily required for the acquisition of TRAIL sensitivity. The increased TRAIL sensitivity may relate to increasing growth rate or growth autonomy, or to the attainment of anchorage independence.

In normal colonic epithelium, apoptosis is primarily at the crypt lumen (Brodie et al, 2004), and for the most part at the top of the crypts (Hall et al, 1994). In early adenomas, there is apoptosis close to the basement membrane, particularly at crypt branching points (Brodie et al, 2004), whereas crypt lumen apoptosis is a feature of larger adenomas, high-grade dysplasia and adenocarcinomas. It is unknown to what extent TRAIL responsiveness contributes to the changing patterns of apoptosis in human colorectal tumours. However, the contribution of TRAIL-R2 to colorectal tumour...
growth and apoptosis sensitivity has recently been demonstrated by Wang and El-Deiry (2004) using stable human colon cancer cell lines in which the function of TRAIL-R2 was ablated using inducible RNA interference in a mouse xenograft model. Inducible silencing of TRAIL-R2 in vivo accelerated growth of bioluminescent tumour xenografts and conferred resistance to the chemotherapeutic agent 5-FU. Strätter et al (2002a) found that in normal colonic epithelium, TRAIL and TRAIL-R2 were expressed primarily in the surface epithelium, whereas TRAIL-R1 and TRAIL-R4 were detected all along the crypt axis. In adenomas, this expression pattern was mostly retained, although some adenomas also expressed abnormally high levels of TRAIL-R3. In carcinomas, the expression of TRAIL and TRAIL receptors was much more variable, but TRAIL-R1 expression was significantly associated with disease-free survival. Koonstra et al (2003) also reported overall expression of TRAIL receptors, which have a large cytoplasmic component, to be similar in adenoma and carcinoma cells; therefore, immunohistochemical studies have not revealed a mechanism for the differences in TRAIL sensitivity between the adenoma and carcinoma cells. We therefore examined the cell surface expression of the four TRAIL receptors by flow cytometry. The importance of examining cell surface expression of TRAIL receptors has been highlighted recently by Jin et al (2004), who showed that TRAIL-resistant variants of the colon carcinoma cell line SW480 had reduced cell surface expression of TRAIL-R1 compared to the parental cell line, although total protein expression remained unchanged. However, it is interesting that in our study the cell surface expression of the four TRAIL receptors did not correlate with the increased sensitivity to TRAIL that is associated with malignancy. Importantly, this indicates that the increased sensitivity to TRAIL may instead be due to alterations in intracellular signalling. In this regard, it is worth noting that we found no correlation between the expression of c-FLIP or XIAP, or of Bcl-2 family members Bcl-2, Bcl-xL, Bax or Bak to explain the differential sensitivity (data not shown). Furthermore, TRAIL-induced NF-κB activity did not correlate with malignant potential or sensitivity to TRAIL-induced apoptosis (data not shown).

In summary, using an in vitro model of colorectal tumour progression, we have demonstrated for the first time that colorectal adenoma cells are more resistant to TRAIL-induced apoptosis than carcinoma cells and that TRAIL sensitivity increases during the conversion of an adenoma cell line to a malignant phenotype. Importantly, since the adenoma cultures are grown under the same culture conditions as the carcinoma cultures, this cannot be attributed to differences in survival factors or medium composition. The results are important for the potential application of TRAIL as a treatment for colorectal cancer because of the current focus on the mechanisms of TRAIL resistance in colorectal tumours – it will be important to define the ‘window’ of TRAIL sensitivity. Although the mechanism for the differential sensitivity of benign and malignant cells to TRAIL remains elusive, our data indicate that death receptor expression alone cannot explain why cells become sensitive to TRAIL on acquisition of the malignant phenotype. Therefore, in addition to defining mechanisms by which resistant variants may subsequently arise after prolonged TRAIL treatment, further investigations are also needed to define the mechanisms by which colorectal carcinoma cells become sensitive to TRAIL and what selective advantage of tumour cells has given rise to this sensitivity. Together, these investigations will assist in the optimisation of TRAIL for therapeutic use.

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