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# Perforin and granzyme B induce apoptosis in FasL-resistant colon carcinoma cells

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**Abstract** Cytotoxic lymphocytes may induce apoptosis in their target cells by the FasL (Fas ligand) pathway or the perforin/granzyme B pathway. It has been shown that Fas-expressing colon carcinoma (CC) cells are resistant to FasL-mediated apoptosis. The aims of this study were to determine whether CC cells are also resistant to perforin/granzyme B and whether the FasL resistance lies upstream of caspase-3 activation. The resistance of the Fas-expressing rat CC531s cells to the FasL pathway was confirmed by treating them with recombinant human soluble FasL, using rat hepatocytes as a positive control. The intracellular delivery of granzyme B by sublytic concentrations of perforin, on the other hand, resulted in many features of apoptosis (chromatin condensation, nucleus fragmentation, loss of microvilli and internucleosomal DNA fragmentation) within 3 h. Since both the FasL and perforin/granzyme B pathways converge at caspase-3, we measured caspase-3 activity to learn whether the FasL resistance was due to failure to activate this crucial executioner. Caspase-3 activation occurred in CC531s cells after perforin/granzyme B treatment, but not after the addition of recombinant FasL. Furthermore, we showed that caspase-3 activity is involved in the execution of perforin/granzyme-B-induced apoptosis in CC531s cells, since the cell-permeable caspase-3 inhibitor Z-DEVD-FMK abrogated DNA fragmentation. Together, these results suggest that CC cells are sensitive to perforin/granzyme-B-induced apoptosis by activating caspase-3 and FasL resistance lies upstream of this executioner caspase.

**Key words** Colon carcinoma · Apoptosis · Perforin Granzyme B · Fas ligand

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## Introduction

Colorectal cancer is the second cause of cancer death (in females after breast cancer and in males after lung cancer) in the Western world [27]. Cytotoxic lymphocytes (cytotoxic T lymphocytes and natural killer cells) are present both at the primary tumour site (colon) and at places of metastasis (liver, lung), and intratumoral cytotoxic lymphocytes have been associated with better prognosis [3, 14, 26, 27]. Cytotoxic lymphocytes use mainly two pathways to induce apoptosis in their target cells, namely the FasL (Fas ligand) pathway and the perforin/granzyme B pathway [1, 16, 19]. It has been shown, by using an agonistic anti-Fas antibody, that colon carcinoma (CC) cells are resistant to the FasL pathway despite Fas expression [15, 25]. However, it is not known whether perforin and/or granzyme B can induce apoptosis in CC cells. Perforin and granzyme B are stored in the granules of cytotoxic lymphocytes and are exocytosed upon a triggering signal [1]. Perforin is a pore-forming protein and granzymes are a group of serine proteases, of which granzyme B is the most potent one [19].

Caspases play a central role in the execution of apoptosis. Both the FasL and perforin/granzyme B pathway appear to converge at caspase-3, where granzyme B functionally replaces caspase-8, which is activated by trimerization of Fas and formation of a death-inducing signalling complex (DISC) [28]. In this study we investigated whether CC cells are susceptible to rapid perforin/granzyme-B-mediated apoptosis, by using isolated perforin and granzyme B, and looked for the involvement of caspase-3 activity.

## Materials and methods

### Cells and reagents

CC531s, a dimethylhydrazine-induced CC of Wag/Rij rats [11] was maintained in culture medium consisting of RPMI-1640 (Gibco, Life Technologies, Gent, Belgium), 10% fetal calf serum (Eurobiochem, Bierges, Belgium), penicillin (100 U/ml), streptomycin (100 U/ml), and L-glutamine (0.2 mM) (Gibco, Life Technologies, Gent, Belgium). This cell line has been used to evaluate interferon treatment on CC at different tumour sites [11], to study liver metastasis [7, 20], and to study interactions between CC cells and lymphokine-activated killer cells [10] or hepatic natural killer cells [24]. Rat hepatocytes were isolated according to De Smet et al., using a two-step collagenase perfusion of the liver [4]. Granzyme B and perforin were purified as described previously [6]. Recombinant human soluble (rhs) FasL and enhancer were purchased from Alexis (Läufelfingen, Switzerland). The enhancer is an antibody reacting with rhsFasL thereby increasing its activity.

### Perforin/Granzyme B treatment

CC531s cells were treated with granzyme B and perforin as described by Froelich et al. [6]. After the indicated incubation times, different methods of apoptosis detection were performed. Sublytic concentrations of perforin (defined as less than 10% specific propidium iodide positive cells in perforin-treated cells) were used in all experiments [6].

### Hoechst 33342/propidium iodide staining

CC531s cells were stained, after experimental treatment, with Hoechst 33342 (HO 342) and propidium iodide (PI), as described previously [24]. HO 342 freely passes the plasma membrane and stains DNA blue, whereas PI, a highly polar dye that is impermeable to cells with intact membranes, stains DNA red. Viable or necrotic cells were identified by intact nuclei with blue (HO 342) or pink (HO 342 plus PI) fluorescence, respectively. Apoptotic cells were detected by their fragmented nuclei, which exhibited either a blue or pink fluorescence depending on the stage in the process. During apoptosis, the plasma membrane stays intact and the apoptotic cells show blue fragmented nuclei. In vitro, however, the apoptotic cells can eventually undergo secondary necrosis and by consequence show pink fragmented nuclei [5]. For counting, recordings were made with a 40× objective lens, and ten fields per treatment were assessed. The percentage of fragmented nuclei was calculated according to following formula:

$$\%\ \text{specific fragmented nuclei} = \left[\left(\frac{\text{fragmented nuclei}}{\text{total nuclei}}\right)_{\text{EXP}} - \left(\frac{\text{fragmented nuclei}}{\text{total nuclei}}\right)_{\text{CON}}\right] \times 100$$

with: *EXP*, cells treated with perforin and/or granzyme B; *CON*, cells in medium only (no perforin or granzyme B).

### Transmission Electron Microscopy (TEM)

The preparation of the cells for electron microscopy was performed according to standard protocols, as described [2].

### Quantitative DNA fragmentation assay

DNA fragmentation was quantified as described [24]. In brief, after labelling with [methyl-$^3$H]thymidine, CC531s cells were exposed to the experimental treatment. After the indicated incubation time, medium was collected and the cells were exposed to lysis buffer (5 mM Tris pH 7.4, 2 mM EDTA, 1% Triton X-100). After ultracentrifugation (10,000 ×*g*), fragmented DNA was separated from intact DNA. Specific DNA fragmentation was calculated according to following formula:

$$\%\ \text{specific DNA fragmentation} = \left(\frac{\text{cpm}_{\text{fr. exp}} - \text{cpm}_{\text{fr.spont}}}{\text{cpm}_{\text{total}} - \text{cpm}_{\text{fr.spont}}}\right) \times 100$$

in which: $cpm_{fr}$ = the counts per minute (cpm) radioactivity in the incubation medium *plus* the cpm in the 10,000 ×*g* supernatant; $cpm_{total} = \text{cpm}_{\text{fr}}$ *plus* the cpm in the 10,000 ×*g* pellet; *exp* = experimental; *spont* = spontaneous.

### DNA extraction and gel electrophoresis

The 10,000 ×*g* supernatant (see Quantitative DNA Fragmentation Assay) was extracted with phenol, phenol:chloroform (1:1), and chloroform. The DNA in the supernatant was precipitated in 2 volumes of 100% ethanol, 0.3 M NaCl and 10 μg/ml glycogen, overnight at −70 °C. After the DNA pellet was washed with 70% ethanol and air-dried, it was submitted to an RNase treatment (100 μg/ml) for 2 h at 37 °C. Gel electrophoresis was performed for 1 h at 180 V in a 2% agarose gel. The gel was stained with SYBR Green (Molecular Probes, Leiden, The Netherlands) according to the guidelines of the manufacturer. The number of cells per sample was $2 \times 10^5$.

### Determination of specific caspase-3 activity

Caspase-3 activity was determined in cell extracts from CC531s cells using a kit from BIOMOL (Plymouth Meeting, Pa., USA), following the instructions of the manufacturer. In brief, after collecting and washing the cells ($2 \times 10^6$ per sample) in PBS at 4 °C, they were treated for 5 min in lysis buffer (50 mM HEPES pH 7.4, 0.1% CHAPS, 1 mM dithiothreitol, 0.1 mM EDTA) on ice. After ultracentrifugation (10,000 ×*g*, 1 min) the supernatant was collected and stored at −70 °C. Caspase-3 activity in this supernatant was determined by measuring the rate of Ac-DEVD-pNA cleavage (pmol/min). In some experiments, the caspase-3 inhibitor Ac-DEVD-CHO was added during activity measurement. From each sample the protein content was determined using the filter paper method described by Minamide et al. [12]. Specific caspase-3 activity is the activity per μg of protein.

## Results

Although CC531s cells express Fas [24], treatment of the cell line with rhsFasL failed to cause apoptosis (Fig. 1B). In comparison, rat hepatocytes treated with the same concentration of rhsFasL (100 ng/ml) underwent massive apoptosis, as shown by the presence of blue or pink fragmented nuclei (Fig. 1D), confirming a previous study [13]. It has been shown by Schneider et al. that the apoptotic inducing capacity of rhsFasL is reduced by more than 1000-fold compared with membrane-bound FasL [18]. However, restoration of the cytotoxic activity of rhsFasL was achieved with the addition of a cross-linking antibody [18]. For this reason, we included such an antibody to rhsFasL, also known as enhancer, during the treatment of the cells with rhsFasL. We preferred to use rhsFasL instead of anti-Fas antibody, since antibody agonists and natural ligand can stimulate different pathways [22].

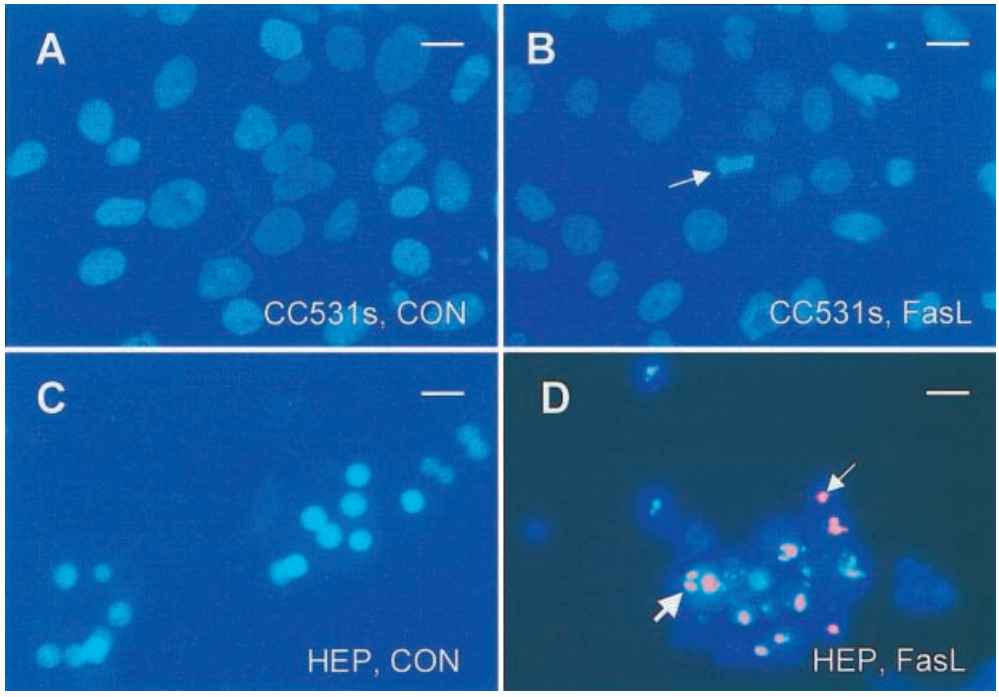


**Fig. 1** HO 342/PI staining of CC531s cells (**A, B**) and rat hepatocytes (**C, D**) showing the FasL resistance of CC531s cells. **A, C**: control; **B, D**: 100 ng/ml rhsFasL. Incubation time was 18 h. In both control and rhs-FasL-treated cells, enhancer protein was present (1 μg/ml). **B**: *arrow*, dividing cell. **D**: *thick arrow*, example of a blue fragmented nucleus; *thin arrow*, pink fragmented nucleus. *Bars* = 20 μm

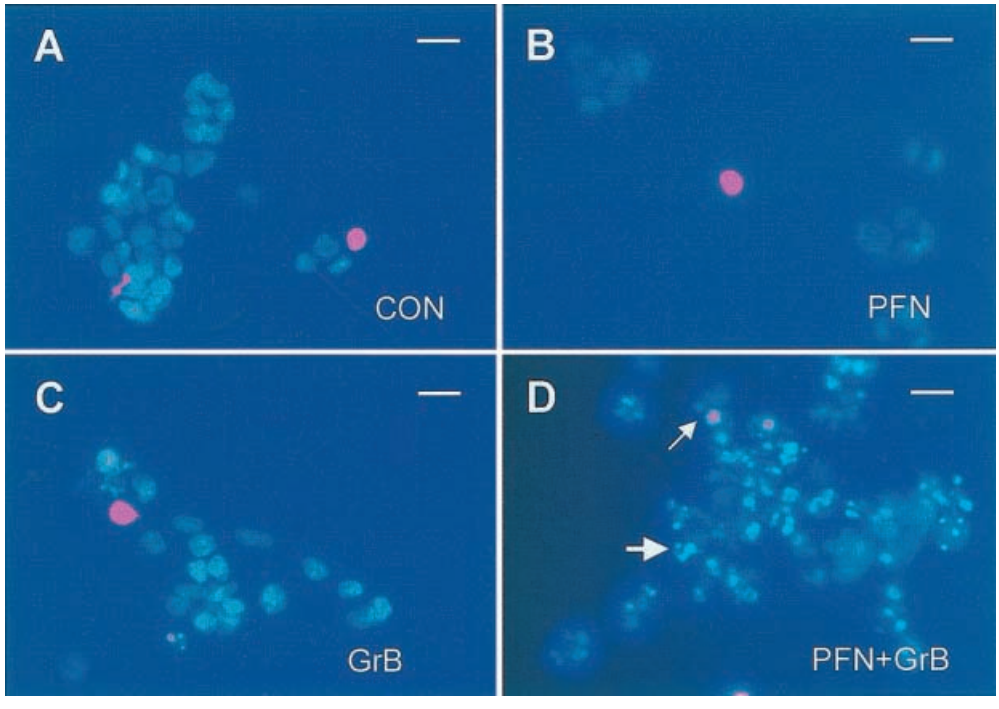


**Fig. 2** HO 342/PI staining of CC531s cells treated in medium only (**A**), 100 U/ml perforin (**B**), 5 μg/ml granzyme B (**C**) and 100 U/ml perforin + 5 μg/ml granzyme B (**D**). Incubation time was 3 h. **D**: *thick arrow*, example of a blue fragmented nucleus; *thin arrow*, pink fragmented nucleus. Only the combination of perforin and granzyme B induce an increase in apoptotic cells. *Bars* = 20 μm

In addition to the FasL pathway, cytotoxic lymphocytes may induce apoptosis by secreting perforin and granzyme B [1]. Treatment of CC531s cells with the combination of perforin and granzyme B resulted in chromatin condensation, nucleus fragmentation (Figs. 2D and 3D), cell fragmentation and condensation and loss of microvilli (Fig. 3D). These are all features of apoptosis [9]. Increasing the granzyme B concentration resulted in an increase in the number of fragmented nuclei (Fig. 4). Complementing the morphological data, we showed a marked increase in DNA fragmentation using a quantitative DNA fragmentation assay (Fig. 5 A). By gel electrophoresis, we demonstrated that the CC531s-DNA was cut in the typical ladder pattern, corresponding with internucleosomal cleavage (Fig. 5B). Perforin or granzyme B alone did not induce these signs of cell death (Figs. 2, 3, 4, 5).

When the cell-permeable caspase-3 inhibitor Z-DEVD-FMK was added during perforin/granzyme B treatment, no increase in DNA fragmentation could be

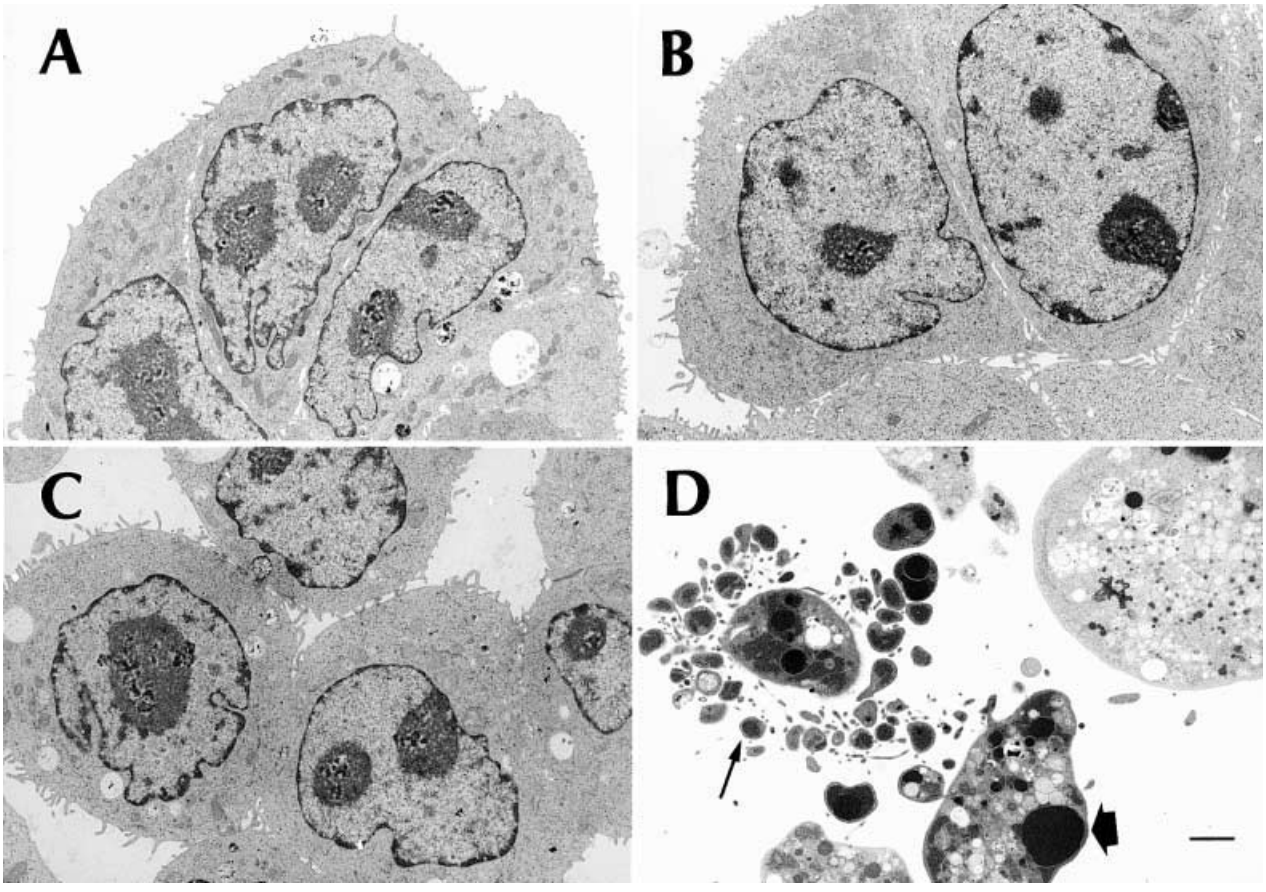


**Fig. 3** TEM of CC531s cells treated in medium only (**A**), 90 U/ml perforin (**B**), 2 μg/ml granzyme B (**C**) and 90 U/ml perforin + 2 μg/ml granzyme B (**D**) for 3 h. **D**: *thick arrow*, chromatin condensation and nucleus fragmentation; *thin arrow*, cell condensation and fragmentation; note the absence of microvilli. *Bar* = 2 μm

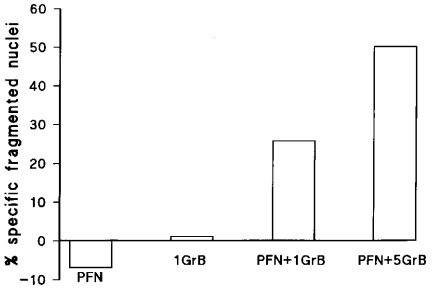


**Fig. 4** Counting of apoptotic CC531s cells, which were determined as shown in Fig. 2. An experiment representative of three independent experiments is shown. The number of pink fragmented nuclei was very low compared to the number of blue fragmented nuclei (less than 10% of the fragmented nuclei was stained pink). Incubation time was 3 h. *PFN*, 100 U/ml perforin; *1 and 5 GrB*, 1 and 5 μg/ml granzyme B

observed, showing the involvement of caspase-3 activity in the execution of apoptosis in CC531s cells (Fig. 5A, B). Recently, it has been demonstrated that intracellular delivery of granzyme B results in the initiation of the caspase pathway by proteolytic activation of caspase-3 [28]. Subsequently, caspase-3 and granzyme B work in tandem to activate the second major executioner, caspase-7. Caspase-8 is recruited by Fas functions in a similar manner to activate caspase-3. Therefore, both the FasL and perforin/granzyme B pathways of apoptosis converge at this important executioner caspase. The differential sensitivity of CC531s cells to the two death pathways suggests that FasL resistance might be due to defect(s) upstream of caspase-3 activation. Supporting this notion, an increase in caspase-3 activity was observed after CC531s cells were treated with perforin/granzyme B, but not with rhsFasL (Fig. 6). Also, at later time-points (3 h, 18 h) no caspase-3 activation could be observed in rhsFasL-treated CC531s cells (data not shown). When a caspase-3 inhibitor was present during incubation of the cells with perforin and granzyme B (Z-DEVD-FMK), or during the assay (Ac-DEVD-CHO), there was no increase in activity, demonstrating the specificity of caspase-3 activity determination (Fig. 6).

## Discussion

CC cells are resistant to FasL-induced apoptosis, as shown by ourselves (Fig. 1) and others [15, 25], despite the fact that they express Fas. In this study we investigated the sensitivity of CC cells to perforin and granzyme B. Because both the FasL and perforin/granzyme B pathways of apoptosis converge at caspase-3 [28], we

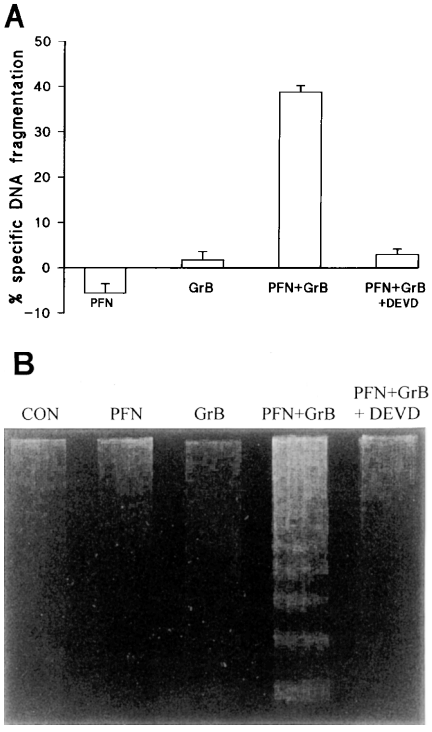


**Fig. 5** Quantitative DNA fragmentation in CC531s cells (**A**) and gel electrophoresis of extracted CC531s-DNA (**B**). *CON*, control (no perforin or granzyme B); *PFN*, 150 U/ml perforin; *GrB*, 1 μg/ml granzyme B; *DEVD*, preincubation of cells with 160 μM Z-DEVD-FMK for 30 min, 80 μM during incubation with perforin and granzyme B. Incubation time was 1 h. *Error bars*, SD of duplicate determinations

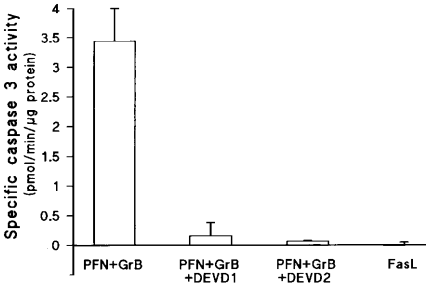


**Fig. 6** Specific caspase-3 activity in CC531s cells. *PFN*, 100 U/ml perforin; *GrB*, 2 μg/ml granzyme B; *DEVD1*, preincubation of cells with 160 μM Z-DEVD-FMK for 30 min, 80 μM during incubation with perforin and granzyme B; *DEVD2*, presence of 0.1 μM Ac-DEVD-CHO during caspase-3 activity determination; *FasL*, 100 ng/ml rhsFasL + 1 μg/ml enhancer. Controls (no PFN/GrB or FasL) have been subtracted. Incubation time was 1 h. *Error bars*, SD of duplicate determinations

asked ourselves whether the FasL resistance of CC cells is due to failure to activate this executioner caspase.

Using different methods to assess the occurrence of apoptosis, we showed that the combination of perforin and granzyme B induced apoptosis in FasL-resistant CC cells. We also demonstrated that caspase-3 is involved in the execution of perforin/granzyme-B-induced apoptosis and that FasL failed to activate this caspase. This suggests that the FasL resistance lies upstream of caspase-3 activation. The FasL resistance of CC531s cells may be due to defective Fas capping or inhibition of caspase-8 activation by endogenous factors such as FLIP (FLICE inhibitory protein) [25, 23]. Expression of FLIP in Jurkat T cells protects the cells from FasL-induced apoptosis, but not perforin/granzyme-B-mediated apoptosis [8]. It remains to be determined whether FLIP is used by CC cells to prevent FasL-mediated apoptosis. A soluble decoy receptor (DcR3) has been discovered, that binds to FasL and inhibits FasL-induced apoptosis, and it was shown that 9 of 17 colon tumours had DcR3 gene amplification [17]. Recently, another report published the discovery of a new component of the DISC, namely 'small-accelerator for death signalling' or SADS [21]. Inhibition or removal of SADS delays FasL-mediated cell death. Furthermore, it was shown that 6 of 7 colon tumours had a genetic disorder of SADS and 4 of 7 colon tumours had SADS mRNA depletion [21]. Taken together, it appears that CC cells can use several different mechanisms upstream of caspase-3 activation to escape FasL-induced apoptosis.

A possible explanation for the development of the FasL resistance can be the prevention of apoptosis induction by the co-expression of Fas and FasL, since it has been demonstrated that CC cells can express functional FasL [15]. On the other hand, expression of perforin and granzyme B is restricted to cytotoxic lymphocytes and can explain why CC cells did not develop resistance to this pathway.

In conclusion, we showed that Fas-expressing CC cells, which are resistant to FasL, are not resistant to the combination of perforin and granzyme B and that the FasL resistance has to be upstream of caspase-3 activation. The differential sensitivity of CC cells to the two death pathways has to be taken into account when developing immunotherapeutic strategies against CC.

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