Expression of Cellular FLICE Inhibitory Protein, Caspase-8, and Protease Inhibitor-9 in Ewing Sarcoma and Implications for Susceptibility to Cytotoxic Pathways

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Abstract

Purpose: Ewing sarcoma is a common pediatric bone tumor with an unfavorable prognosis for metastatic or recurrent disease. Cellular immunotherapy may provide new treatment options and depends on the cytolytic death receptor and perforin/granzyme pathways. Expression of death receptor pathway inhibitor cellular FLICE inhibitory protein (cFLIP), initiator caspase-8, and granzyme B inhibitor protease inhibitor-9 (PI-9) have been reported to determine susceptibility to cell- and chemotherapy-mediated killing in several tumor types. Here, we have studied their *in vitro* and *in vivo* expression in Ewing sarcoma and the implications for susceptibility to cytotoxicity.

Experimental Design: Ewing sarcoma cell lines (n = 8) were tested for cFLIP, PI-9, and caspase-8 expression. Functional significance was tested by anti-Fas antibody (death receptor pathway) or natural killer cell (perforin/granzyme pathway) treatment. Immunohistochemistry was done on 28 sections from 18 patients. In half of the cases, sequential material, including metastases, was available.

Results: Although all tested Ewing sarcoma cell lines expressed cFLIP, resistance to CD95/Fas–mediated apoptosis was only observed in two cell lines lacking caspase-8 expression. PI-9 was expressed at low levels in four of eight Ewing sarcoma cell lines, but positive cell lines remained susceptible to perforin/granzyme–mediated killing. In primary Ewing sarcoma, including metastases, cFLIP was abundantly expressed in 18 of 18 patients. Caspase-8 was expressed in all patients but showed more intertumoral and intratumoral variation in both intensity and heterogeneity of staining. PI-9, in contrast, was undetectable.

Conclusions: The expression patterns of cFLIP, caspase-8, and the absence of PI-9 provide a rationale to preferentially exploit the perforin/granzyme pathway in cytotoxic therapies against Ewing sarcoma.

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Ewing sarcoma is the second most common malignant bone tumor in children. It is mainly found in children in the second decade of life and is characterized by specific EWS/FLI-1 or EWS/ERG gene fusions (1). Approximately 25% of Ewing sarcoma patients have metastatic disease at diagnosis. The prognosis for patients with metastases or recurrent disease is very poor with <30% long-term relapse-free survival despite intensive chemotherapy, surgery, and radiotherapy (2). Especially for this group of patients, new therapeutic approaches are needed. Cellular immunotherapy with tumor-specific CTL or natural killer (NK) cells could be a new option for additional treatment of Ewing sarcoma.

Elimination of tumor cells by cytotoxic effector cells is mainly induced through two major cytolytic pathways: the death receptor pathway and the calcium-dependent granule exocytosis pathway (3, 4). The death receptor pathway is mediated by members of the tumor necrosis factor receptor superfamily. Death ligands present on CTLs and NK cells, such

as FasL (CD95L) and tumor necrosis factor–related apoptosis-inducing ligand, cross-link the respective death receptors Fas (CD95) and tumor necrosis factor–related apoptosis-inducing ligand receptors 1 and 2 on target cells. Subsequently, the intracellular death domains of these death receptors attract adaptor proteins that, in turn, recruit the proform of caspase-8. At this membrane-bound complex, called the death-inducing signaling complex, autolytic cleavage of pro-caspase-8 into active caspase-8 occurs (5). Active caspase-8 can cleave and activate "executioner" caspases (primarily caspase-3, caspase-6, and caspase-7) starting an amplifying cascade of caspase activation. This finally leads to cleavage of "death substrates," such as poly(ADP-ribose) polymerase and DNA fragmentation factor, and results in apoptosis.

The calcium-dependent granule exocytosis pathway depends on cytotoxic granules that are secreted by CTL and NK cells upon binding to a target cell. Perforin and granzyme B are the main effector molecules that are present in these granules; therefore, cytotoxicity by this pathway is also called perforin/granzyme–mediated killing. Inside the target cell, granzyme B cleaves several substrates, including Bid, thereby initiating the mitochondrial death pathway, and caspases including caspase-3, caspase-7, and caspase-8. Ultimately, these actions lead to target cell apoptosis (6).

Tumor cells, however, may acquire resistance to the apoptotic pathways mentioned above. One way of resistance is by expression of specific antiapoptotic proteins. The cellular FLICE inhibitory protein (cFLIP) and serine protease inhibitor-9 (PI-9) are antiapoptotic molecules that are expressed in different tumor types (7–11). The antiapoptotic protein cFLIP contains two death effector domains that enable it to bind to the death-inducing signaling complex where it functions as an inactive caspase-8 surrogate. In this way, cFLIP inhibits activation of the caspase cascade, thereby preventing progression toward apoptosis induced by the death receptor pathway (7, 12). The human intracellular serpin PI-9 irreversibly inactivates granzyme B, thereby inhibiting progression of the other major apoptotic pathway (13).

Expression of antiapoptotic molecules can lead to *in vivo* tumor escape from CTL-mediated killing as was shown in murine tumors that overexpressed either SPI-6, the murine counterpart of PI-9, or cFLIP (8, 14). A recent vaccination study in metastatic melanoma patients showed that PI-9 expression in the tumor was correlated with an adverse outcome (15). These studies indicate the relevance of expression of antiapoptotic proteins for the initial tumor escape from immunosurveillance as well as for tumor immunotherapy. Similar studies have, however, not yet been done in Ewing sarcoma patients. Furthermore, antiapoptotic proteins can contribute to resistance against chemotherapy (11, 16, 17), making studies on their expression relevant for current treatment protocols as well.

With regard to Ewing sarcoma, reports on the expression of these antiapoptotic proteins are limited. Until now, no studies have been reported on the expression of PI-9 in Ewing sarcoma. Expression of cFLIP has been shown in several Ewing sarcoma cell lines (18–20); however, no expression studies of cFLIP in primary Ewing sarcoma tumors have been reported yet. In the present study, we have examined the expression patterns of cFLIP, caspase-8, and PI-9 in Ewing sarcoma cell lines as well as in primary Ewing sarcoma tumors (n = 18 patients). Furthermore, we show their functional significance in Ewing sarcoma cell lines when apoptosis is induced by either of the two main cytolytic pathways.

Materials and Methods

Cell lines. In this study, the Ewing sarcoma cell lines A673, L1062, RD-ES, SK-N-MC, SK-ES-1, EW3, EW7, and COH were used. L1062 was previously established at the Department of Pathology of the Leiden University Medical Center (LUMC; Leiden, the Netherlands; ref. 21). COH and EW7 were kindly provided by Dr. A. Prieur. A673, RD-ES, SK-N-MC, and SK-ES-1 and the human erythroleukemia cell line K562 were obtained from the American Type Culture Collection (Manassas, VA). The Fas-sensitive Jurkat T-cell line E6-1 was kindly provided by Dr. J.P. Medema. All cell lines were maintained in RPMI 1640 supplemented with glutaMAX, 25 mmol/L HEPES, 10% FCS, 100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Invitrogen Life Technologies, Paisley, Scotland), at 37°C and 5% CO_2.

Patient material. Archival tumor tissue specimens, obtained from 18 Ewing sarcoma patients, were used. Tumor material obtained at diagnosis, as well as from resected, metastasized, or relapsed tumors was included. In half of the cases (n = 9), sequential material was available for analysis. All cases were retrieved from the surgical pathology files of the LUMC. Tonsils were obtained from the Department of Otorhinolaryngology of the LUMC. The tonsils were either embedded for immunohistochemistry or mechanically disrupted for RNA and protein extraction, according to the procedures mentioned below. All samples were handled in a coded fashion, according to national ethical guidelines (Code for Proper Secondary Use of Human Tissue in the Netherlands, Dutch Federation of Medical Scientific Societies).

Primary and secondary antibodies. Primary antibodies used for Western blotting or immunohistochemistry were as follows: mouse anti–PI-9-17 monoclonal antibody (Western blot 1:100, immunohistochemistry 1:800; ref. 22), rat anti-cFLIP monoclonal antibody Dave-2 (Western blot 1:1,000, Apotech, Lausanne, Switzerland), mouse anti–β-actin monoclonal antibody (Western blot 1:5,000, Sigma, St. Louis, MO), rabbit anti-cFLIP (immunohistochemistry 1:50, Sigma), mouse anti–caspase-8 monoclonal antibody (Western blot 1:50, Abcam, Cambridge, United Kingdom), rabbit anti-caspase-8 (immunohistochemistry 1:50, Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies for immunohistochemistry were tested and titrated on paraffin-embedded normal and decalcified tonsils. Secondary antibodies for Western blotting were from Santa Cruz Biotechnology: goat anti-rat IgG-horseradish peroxidase (1:5,000) and goat anti-mouse IgG-horseradish peroxidase (1:10,000). Biotinylated rabbit anti-mouse (1:200) and swine anti-rabbit (1:400) antibodies, normal rabbit and mouse IgG (used as primary antibody in comparable dilutions), and biotinylated horseradish peroxidase/streptavidin complex for immunohistochemistry were from DAKOCytomation A/S (Glostrup, Denmark).

RNA isolation, cDNA synthesis, and PCR. RNA was isolated from the cell lines by using TRIzol (Invitrogen Life Technologies, Breda, the Netherlands) according to the manufacturer's protocol. Synthesis of cDNA was done by using 1 μg of total RNA, $oligo_p(dT)_{15}$ as a primer, and avian myeloblastosis virus reverse transcriptase (Roche Diagnostics GmbH, Mannheim, Germany). PCR for PI-9 was done with the primers 5′-TCTGCCCTGGCCATGGTTCTCCTA-3′ and 5′-CTGGCCTTTGCTCCTCCTGGTTTA-3′ (61°C, 33 cycles). cFLIP was amplified with 5′-TAGGATGTCTGCTGAAGTCATCC-3′ and 5′-TTGAGCAGGTCAAATCGCCTCAC-3′ as primers (59°C, 33 cycles). PCR for glyceraldehyde-3-phosphate dehydrogenase was done with the primers 5′-GGTCGGAGTCAACGGATTTG-3′ and 5′-ATGAGCCCCAGCCTTCTCCAT-3′ (61°C, 26 cycles). PCR products were run on a 1% agarose gel containing ethidium bromide and visualized by UV illumination.

Western blotting. Cells were collected, centrifuged briefly, and lysed on ice in ice-cold radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 8.0), 0.1% SDS, 1.0% Triton X-100, 0.5%

deoxycholate, 5 mmol/L EDTA (pH 8.0), 1% NP40] supplemented with the Complete protease inhibitor cocktail (Roche Diagnostics) and 1 mmol/L phenylmethylsulfonyl fluoride (Sigma). The samples were cleared by centrifugation and assessed for protein concentration with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). SDS-PAGE gel electrophoresis (7.5% polyacrylamide gel) was done and the proteins (20 μg/lane) were electroblotted overnight onto nitrocellulose membranes (Hybond-P, Amersham Biosciences, Buckinghamshire, United Kingdom). After blocking with 5% milk in 0.5 mol/L NaCl/0.01 mol/L Tris (pH 8.0)/0.2% Tween (TBST), the nitrocellulose membranes were exposed overnight to the primary antibodies at 4°C, followed by 1 h (anti-cFLIP) or 2 h (anti-PI-9) at room temperature. After washing in TBST, the horseradish peroxidase–labeled secondary antibodies were added in 3% milk/TBST for 1 h at room temperature. The proteins were visualized with the enhanced chemiluminescence technique (Amersham Biosciences). Blots were stripped and stained overnight at 4°C with anti–β-actin monoclonal antibody to control for protein loading.

Fas-induced apoptosis assay. Ewing sarcoma cells were seeded in 25-cm^2 flasks and grown until 60% to 70% confluence. The medium was replaced and the Fas-stimulating antibody anti-CD95 IgM clone 7C11 (Beckman Coulter, Fullerton, CA) or control mouse IgM (DAKOCytomation) was added at 0.5 μg/mL. Both adherent and detached cells were harvested after 12 h and analyzed in duplicate with propidium iodide staining as described by Nicoletti et al. (23). To measure DNA content, the fluorescence intensity of propidium iodide–stained nuclei was determined by flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany) and analyzed with Cell Quest software. Segmented apoptotic nuclei were recognized by subdiploid (sub-G_1) DNA content. Jurkat cells were concomitantly tested as positive controls. Specific anti-Fas–induced apoptosis was calculated as percentage subdiploid DNA after anti-Fas treatment minus percentage subdiploid DNA after control IgM treatment. Cells with a subdiploid DNA content of 20% above control treatment were considered anti-Fas sensitive.

^{51}Chromium release assay. Ewing sarcoma cell lines were labeled for 1 h with 100 μCi chromium-51 (^{51}Cr), washed, counted, and added to the effector cells in a 96-well round-bottomed plate at an effector to target ratio of 10:1. Interleukin-15–stimulated polyclonal NK cells of healthy blood bank donors were used as effector cells. NK cells were preincubated for 2 h at 37°C with or without 1,000 nmol/L concanamycin A (CMA; Sigma) to disrupt formation of perforin/granzyme–containing vesicles. After mixing of CMA-treated NK cells with target cells, the final CMA concentration was 500 nmol/L. The medium was isolated after 4 h and counted for ^{51}Cr release. Experiments were done in triplicate. Spontaneous and maximum releases were determined and specific lysis was calculated using the following formula:

$$(\text{experimental release} - \text{spontaneous release}/\text{maximum release} - \text{spontaneous release}) \times 100\%.$$

DNA fragmentation assay. Ewing sarcoma cell lines were labeled overnight with [^{3}H]thymidine (10 μCi/mL). Cells were washed, counted, and incubated for 18 h with NK cells as described above. Cells were lysed, harvested on fiberglass filters, and counted for ^{3}H. Specific killing was calculated using the formula [(cpm without NK cells − cpm with NK cells) / cpm without NK cells] × 100%. Fragmented DNA from apoptotic cells will be washed away from the filter, leading to lower ^{3}H counts. Experiments were done in triplicate.

Immunohistochemistry. Sections of 4 μm were cut from formalin-fixed, paraffin-embedded tumor tissue and mounted on APES-coated glass slides. Decalcified tonsils (2 weeks of formic acid treatment) served as positive controls and were used to determine optimal antibody dilutions. Immunohistochemistry was done as described previously (24), including antigen retrieval in citrate buffer. Sections were stained with diaminobenzidine/0.002% H_2O_2 and counterstained with hematoxylin. The staining was scored independently by two expert pathologists (P.C.W.H. and S.R.) as described before (25). Score for staining intensity was graded as follows: 0, absent; 1, weak; 2, moderate; and 3, intense. Score for the percentage of positive tumor cells was graded as follows: 0, absent; 1, 1% to 10%; 2, 11% to 25%; 3, 26% to 50%; 4, 51% to 75%; 5, 76% to 100% positive tumor cells. Sections with a combined score for intensity and percentage positive cells higher than three were considered positive.

Results

Expression of cFLIP and PI-9 in Ewing sarcoma cell lines. The expression of cFLIP and PI-9 was analyzed in a panel of eight Ewing sarcoma cell lines. As shown in Fig. 1A, all eight Ewing sarcoma cell lines tested expressed cFLIP mRNA. Four of these eight cell lines also expressed PI-9 mRNA. In all cases, positivity for cFLIP and PI-9 mRNA was associated with protein expression (Fig. 1B and C). A long form and a short form of cFLIP exist owing to alternative splicing. Both these forms can prevent caspase-8 cleavage and inhibit apoptosis, albeit in different ways (26). A band of 55 kDa, corresponding to

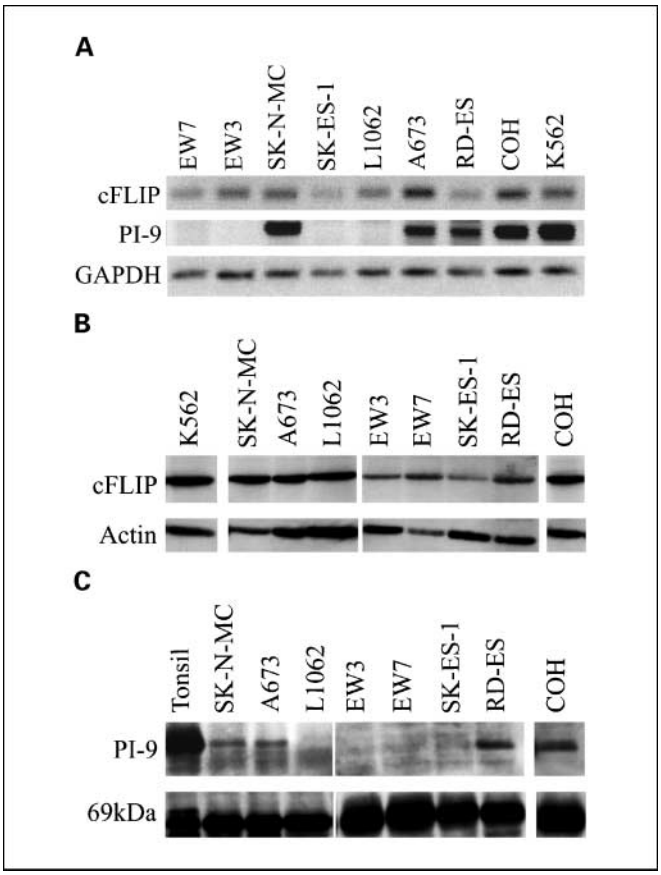

Fig. 1. mRNA and protein expression of cFLIP and PI-9 in Ewing sarcoma cell lines. *A,* reverse transcription-PCR detected cFLIP mRNA expression in all eight Ewing sarcoma cell lines as well as the K562 control cells. PI-9 mRNA was detected in half of the Ewing sarcoma cell lines and in K562. PCR for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to control for cDNA quality. *B,* Western blot showing cFLIP (55 kDa) expression in all eight Ewing sarcoma cell lines. K562 was used as a positive control, and staining for β-actin was used as a loading control. *C,* Western blot showing PI-9 protein (42 kDa) expression in half of the Ewing sarcoma cell lines. Cell lysate from a tonsil was used as a positive control. An aspecific band of 69 kDa, which was reported before with this antibody (22), was used as loading control.

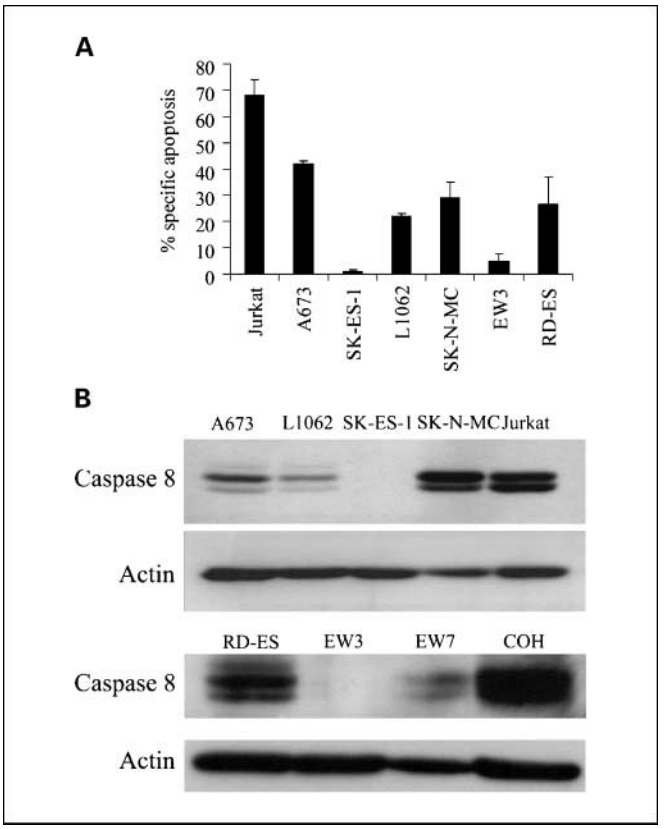

Fig. 2. Anti-Fas – induced apoptosis in Ewing sarcoma cell lines. *A,* percentage of apoptosis after 12-h treatment with 0.5 μg/mL anti-Fas monoclonal 7C11 and correction for treatment with mouse IgM control antibody (see Materials and Methods). Apoptosis was measured as the percentage of nuclei that stained as a sub-G_1 fraction with Nicoletti buffer. Experiments were done in duplicate. One of three experiments with similar results. *B,* Western blot showing absence of caspase-8 (55 and 53 kDa) expression in SK-ES-1 and EW3 cells. Jurkat cells were used as a positive control, and staining for β-actin was used as a loading control.

$cFLIP_{long}$, was detected in the Ewing sarcoma cell lines (Fig. 1B). The short form of cFLIP was not detected, although both forms can be recognized by the anti-cFLIP monoclonal Dave-2. The expression level of PI-9 in the four positive Ewing sarcoma cell lines was low when compared with the tonsil lysate that was used as a positive control in the Western blot (Fig. 1C).

CD95/Fas – induced apoptosis in Ewing sarcoma cell lines. After having shown the expression of cFLIP in our panel of Ewing sarcoma cell lines, our next aim was to determine whether cFLIP affected the susceptibility to death receptor pathway – mediated apoptotic stimuli. Therefore, the sensitivity to Fas (CD95) – mediated killing was analyzed in six cell lines that were evaluable based on similar cell surface expression of Fas (data not shown) and growth characteristics. As shown in Fig. 2A, Fas ligation induced apoptosis in four of the six cell lines. Similar results were obtained using Annexin – V staining (data not shown).

The sensitivity of these four cell lines strongly suggests that the level of cFLIP expression was insufficient to abrogate susceptibility to Fas-mediated killing. Notably, cFLIP expression was similar or even less in the resistant cell lines (SK-ES-1 and EW3) when compared with the sensitive cell lines (Fig. 1B), indicating that probably other factors determine anti-FAS resistance. The death receptor pathway starts with activation of pro-caspase-8; therefore, caspase-8 down-regulation could influence the response to the anti-Fas treatment. Caspase-8 is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b, of 55 and 53 kDa, respectively (27). Both isoforms of caspase-8 were expressed in six of eight Ewing sarcoma cell lines but not in SK-ES-1 and EW3 (Fig. 2B), thus providing a likely explanation for their resistance to Fas-mediated killing.

Perforin/granzyme – induced apoptosis in Ewing sarcoma cell lines. We next analyzed whether PI-9 expression in Ewing sarcoma cell lines affected the susceptibility to cytotoxicity via the perforin/granzyme pathway. For this purpose, interleukin-15 – activated NK cells were used as cytotoxic effector cells. Both PI-9 – positive (SK-N-MC and A673) and PI-9 – negative (SK-ES-1 and L1062) Ewing sarcoma cell lines were killed with comparable efficiency as shown by ^{51}Cr release and DNA fragmentation (Fig. 3A and B).

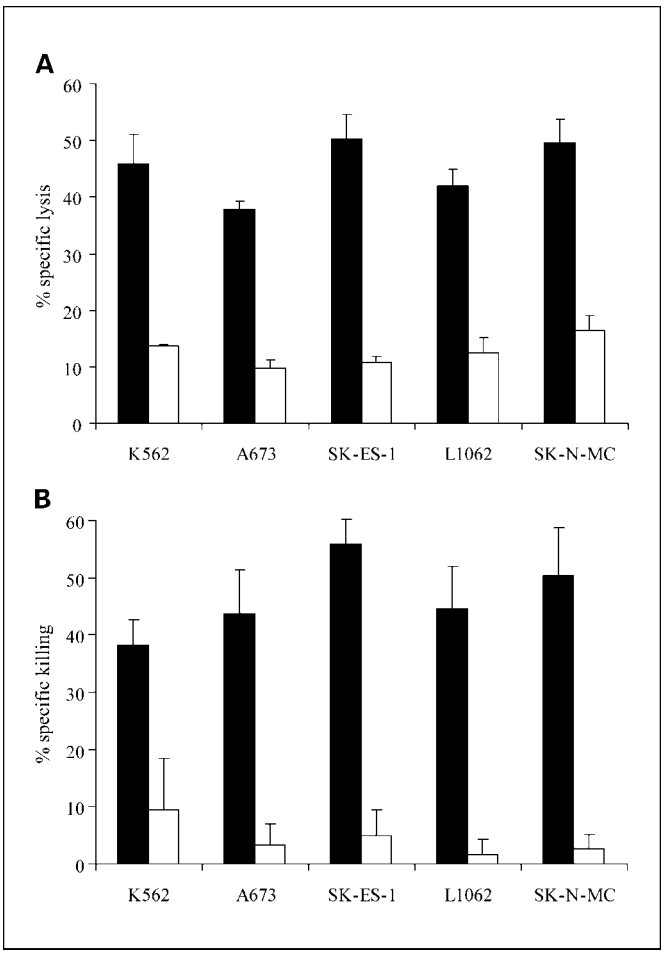

Fig. 3. Perforin/granzyme – mediated killing of Ewing sarcoma cell lines. *A,* percentage specific lysis (^{51}Cr release) of Ewing sarcoma or K562 cells by interleukin-15 – stimulated polyclonal NK cells at an effector to target ratio of 10:1. *B,* percentage specific killing (DNA fragmentation) of Ewing sarcoma or K562 cells by interleukin-15 – stimulated polyclonal NK cells at an effector to target ratio of 10:1. Effector NK cells were either preincubated for 2 h in normal medium (*black columns*) or in medium with 1 μmol/L CMA (*white columns*) before adding them to ^{51}Cr- or ^{3}H-labeled targets. K562 was included as a positive control. Experiments were done in triplicate. Incubation of Ewing sarcoma cells with CMA, but without NK cells, did not lead to higher ^{51}Cr release or lower ^{3}H counts compared with untreated cells (data not shown). One of three (*A*) or one of two (*B*) experiments with similar results. Similar results were obtained with NK cells from a different donor (data not shown).

To determine the relative contribution of the perforin/granzyme pathway, NK cells were preincubated with CMA, an inhibitor of vacuolar type H^+-ATPase that affects formation of perforin-containing granules (28). Preincubation with CMA strongly reduced killing of the Ewing sarcoma target cells (Fig. 3A and B), indicating that the NK cell–mediated apoptosis was largely perforin/granzyme pathway dependent. Similar experiments, but without CMA pretreatment, also showed efficient killing of RD-ES (PI-9^+) and EW3 (PI-9^-; data not shown). The efficient killing in the absence of CMA pretreatment shows that the expression of PI-9, as seen in some Ewing sarcoma cell lines, was not associated with a reduced susceptibility to perforin/granzyme pathway–mediated apoptosis.

Altogether, these results provide evidence that Ewing sarcoma cells can be selectively resistant to one cytolytic pathway (e.g., the death receptor pathway), while retaining their sensitivity to the other pathway used by cytotoxic effector cells. Furthermore, these results indicate that the pathway of perforin/granzyme–induced apoptosis is intact in Ewing sarcoma cells lacking caspase-8 (SK-ES-1 and EW3).

Expression of cFLIP but not PI-9 in Ewing sarcoma tumors. To determine the potential clinical relevance of these observations, the expression of cFLIP and PI-9 was evaluated in a group of 18 patients from whom 28 different tissue sections were available. The study material included diagnostic biopsies (n = 8), resection material containing vital tumor tissue after chemotherapy (n = 12), as well as metastatic (n = 7) or relapsed tumors (n = 1). Sequential material was available from nine individual patients.

Similar to our results in the cell lines, cFLIP protein was expressed in all tumor samples (Fig. 4A; Table 1). In almost every sample, the large majority of tumor cells showed positive staining (60-100% in 25 of 27 sections). The intensity of the staining varied from moderate to strong levels (Table 1). This also indicates that in cases with sequential material, cFLIP expression was relatively stable during the disease course (Table 2). In sharp contrast, PI-9 expression was not detectable in any of the tumors (Fig. 4C; Table 1). The few cells that stained positive for PI-9 and served as internal controls were infiltrating lymphocytes and dendritic cells (Fig. 4C). Decalcified tonsil that was used as control tissue also stained positive for PI-9, excluding the possibility that epitope loss occurs in Ewing sarcoma bone tissue due to the decalcification process (Fig. 4D).

Caspase-8 expression in Ewing sarcoma tumors. The experiments with the Ewing sarcoma cell lines showed that lack of

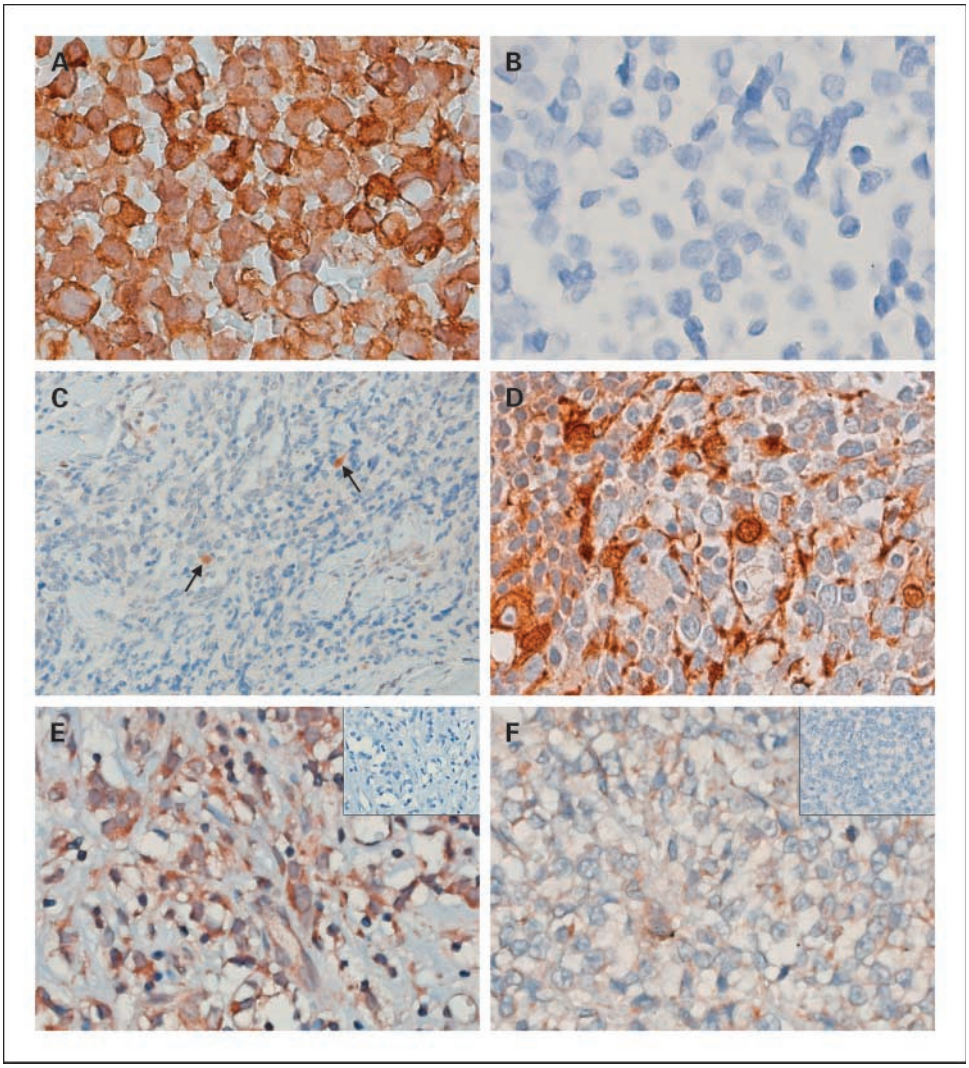

Fig. 4. cFLIP, PI-9, and caspase-8 expression in Ewing sarcoma tumors. *A,* cFLIP expression in a Ewing sarcoma tumor. *B,* negative staining of the same tumor with normal rabbit IgG as primary antibody. *C,* PI-9 was not detected in the investigated Ewing sarcoma tumors. Tumor-infiltrating lymphocytes and dendritic cells stained positive and served as internal control (*arrows*). *D,* PI-9 expression in a decalcified tonsil (positive control tissue). *E,* strong expression of caspase-8 in a Ewing sarcoma tumor. *F,* moderate expression of caspase-8 in a Ewing sarcoma tumor. *E* and *F* (*inset*), negative staining of the same tumors with normal rabbit IgG as primary antibody. Original magnification, ×100 (*A*, *B*, and *D*) and ×50 (*C*, *E*, and *F*).

Table 1. Immunohistochemical score for PI-9, cFLIP, and caspase-8 expression in Ewing sarcoma patients

	Patients	Biopsies	Resections	Metastases	Relapse
PI-9$^+$	0 of 18*	0 of 8*	0 of 12*	0 of 7*	0 of 1*
cFLIP$^+$	18 of 18	8 of 8	12 of 12	6 of 6	1 of 1
Intensity score (0-3)		3 (2-3)	3 (2-3)	3 (2-3)	3
Ewing sarcoma positivity score (0-5)		5 (3-5)	5 (4-5)	4 (1-5)	5
Caspase-8$^+$	18 of 18	7 of 8	12 of 12	5 of 6	1 of 1
Intensity score (0-3)		2 (1-3)	2 (1-3)	2 (1-3)	2
Ewing sarcoma positivity score (0-5)		4 (1-5)	4 (3-5)	4 (1-5)	5

NOTE: Sections from paraffin-embedded Ewing sarcoma tumors were stained for expression of PI-9, cFLIP, and caspase-8. A total of 28 different sections from 18 patients were available for analysis. PI-9 was not detectable in any sample, whereas cFLIP and caspase-8 were detected in all patients albeit at variable intensity or percentages. Scores for staining intensity and percentage of positive tumor cells were graded from 0 to 3 and 0 to 5, respectively, as indicated in Materials and Methods. The average scores are indicated as ranges inside parentheses.
*Number of positive patients or sections out of the total number of patients or sections.

caspase-8 expression is not a rare event and of functional relevance for cytotoxic susceptibility. Therefore, we also investigated the pattern of caspase-8 expression in primary Ewing sarcoma by immunohistochemistry. Caspase-8 was expressed in most tumor samples (Table 1). Intense and abundant expression was found, along with moderate or weak expression (Fig. 4E and F). Two cases, the biopsy of patient 6 and the metastasis of patient 4 (Table 2), were negative, as defined in Materials and Methods. The total study group showed more variability for caspase-8 than for cFLIP in both the intensity of staining (from weak to strong) as well as the percentage of positive cells per individual tumor (from <10% to 100%; Table 1). This variability was seen in all categories (diagnostic biopsies, resections, and metastases) and also when sequential samples were compared (Table 2).

Discussion

Tumors can evade the host immune system in different ways. They can, for example, down-regulate expression of MHC antigens, secrete immunosuppressive cytokines, or induce T-cell tolerance (29). Here, we investigated the expression and functional relevance of the antiapoptotic proteins cFLIP and PI-9 and the proapoptotic initiator caspase-8 in Ewing sarcoma cell lines and primary tumors.

PI-9 is normally expressed in dendritic cells, lymphocytes, and at immune privileged sites (22). Its expression has been shown in primary colon carcinoma as well as in cell lines derived from human solid tumors such as melanoma, and breast, cervical, and colon carcinomas (8, 15). Estrogen-induced expression of PI-9 protected human hepatoblastoma

Table 2. Expression of PI-9, cFLIP, and caspase-8 in patients from whom sequential material was available

Patient	Material	PI-9	cFLIP intensity	cFLIP positivity	Caspase-8 intensity	Caspase-8 positivity
1	Biopsy	—	3	5	1	5
	Resection	—	3	5	3	5
	Metastasis L*	—	2	5	2	3
2	Biopsy	—	3	5	3	5
	Metastasis L*	—	2	5	1	5
3	Metastasis L*	—	2	4	2	4
	Metastasis R*	—	3	5	2	3
4	Resection	—	2	5	3	5
	Metastasis B*	—	3	1	1	1
5	Resection	—	3	5	2	5
	Metastasis B*	—	ND	ND	ND	ND
6	Biopsy	—	3	5	2	1
	Resection	—	3	5	2	4
7	Biopsy	—	3	5	2	2
	Resection	—	3	5	3	5
8	Biopsy	—	3	3	2	2
	Resection	—	2	4	3	5
9	Biopsy	—	2	5	1	5
	Resection	—	3	4	1	3

NOTE: Sections from paraffin-embedded Ewing sarcoma tumors were stained for expression of PI-9, cFLIP, and caspase-8. Sections from sequential stages of the disease were available from 9 of 18 patients. Of these nine patients, only patient 7 went into complete remission. PI-9 was not detectable in any sample. Scores for staining intensity and percentage positive tumor cells were graded from 0 to 3 and 0 to 5, respectively, as indicated in Materials and Methods.
Abbreviations: L, lung; R, rib; B, bone; ND, not determined (because of lack of viable tumor cells).
*Metastases were located in the lung, rib, or bone.

cells against *in vitro* killing by CTLs or NK cells (30). Recently, a study using irradiated autologous tumor cells for vaccination showed that PI-9 expression, especially in patients with >50% positive tumor cells, was associated with an unfavorable outcome in metastatic melanoma (15). This suggests a role for PI-9 in immune evasion by human solid tumors. In our panel of 18 Ewing sarcoma patients, PI-9 was undetectable by immunohistochemistry. However, it cannot be excluded that low levels of PI-9 are expressed and that PI-9 expression can be induced under selective pressure. The finding that some Ewing sarcoma cell lines express PI-9, although at a low level, is compatible with this option. At present, however, our findings indicate that (over)expression of PI-9 is unlikely to be a major mechanism of immune escape in Ewing sarcoma.

In contrast to PI-9, cFLIP is expressed in all investigated Ewing sarcoma cell lines. For RD-ES, SK-N-MC, and SK-ES-1, this is in agreement with previous reports (18–20). In the present study, we show cFLIP positivity for the A673, EW3, EW7, L1062, and COH cell lines as well. Despite cFLIP expression, death receptor–induced apoptosis was not inhibited after *in vitro* anti-Fas treatment of cell lines that were cFLIP and caspase-8 positive. Conflicting results have been published with anti-Fas treatment of SK-N-MC showing either sensitivity (31) or resistance (20). Comparing the results of anti-Fas treatment of Ewing sarcoma cells can be complicated because of differences in both experimental approach and analysis by, e.g., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay, electron microscopy, or the terminal deoxyribonucleotide transferase–mediated nick-end labeling. In the study of Kontny et al. (20), apoptosis was measured by the Nicoletti assay; according to the criteria they have used (i.e., a subdiploid DNA content of 20% above the control level), SK-N-MC was positive in our assay. In this same article (20), SK-N-MC was very sensitive to tumor necrosis factor–related apoptosis-inducing ligand, which is another argument against resistance of SK-N-MC to the death receptor pathway. Together, these results do not show a relation between cFLIP expression in Ewing sarcoma cell lines and resistance to death receptor pathway–mediated apoptosis.

The role of cFLIP might be more complex *in vivo*. A previous report showed that overexpression or naturally up-regulated expression of cFLIP in two murine tumors resulted in tumor growth despite efficient CTL responses in wild-type and perforin-deficient mice (14). This result suggests an *in vivo* role for cFLIP in immune evasion and for the death receptor pathway in immunosurveillance. In our Ewing sarcoma patient group, all samples were cFLIP positive. Microarray analysis of either experimentally induced expression (32) or knockdown (33) of EWS/FLI-1 did not identify cFLIP as a direct target gene of the Ewing sarcoma–specific oncogene. This indicates that Ewing sarcoma does not necessarily associate with cFLIP (over)expression. The cFLIP expression pattern in our patient samples could thus be a more general phenomenon of transformed cells. However, a selection for cFLIP-positive cells cannot be excluded. From this perspective, it will be interesting to study possible changes in cFLIP expression in Ewing sarcoma tumors from patients that have encountered immunologic therapeutic pressure.

Antiapoptotic proteins can affect not only sensitivity to immunotherapy but also to chemotherapy. Expression of cFLIP in solid tumor cell lines protected them against chemotherapy-induced apoptosis *in vitro* (16, 17). In Burkitt's lymphoma, cFLIP expression correlated with a poor prognosis and chemoresistant disease (34). In primary nodal diffuse large B-cell lymphomas, in contrast, cFLIP expression correlated with a better response to chemotherapy (35). To establish a possible relation between cFLIP expression and chemotherapy resistance in Ewing sarcoma, a uniformly treated group should be screened. The present study group did not show a clear relation between clinical outcome and expression of cFLIP or caspase-8. Three patients obtained complete remission, whereas the others succumbed to the disease (n = 14). The outcome for one patient is unknown.

High numbers of PI-9– and BCL-2–positive neoplastic cells before treatment correlated with a poor clinical outcome in systemic anaplastic large-cell lymphoma (11). This suggests that PI-9, like cFLIP, could influence sensitivity to chemotherapy. The absence of detectable PI-9 expression in our Ewing sarcoma patient material, however, makes it unlikely that PI-9 will play an important role in chemotherapy resistance *in vivo*.

Mutation or down-regulation of Fas or caspases are other ways that can lead to tumor resistance against the death receptor pathway. Expression of caspase-8, but not of other components of this pathway, was down-regulated in several Ewing sarcoma cell lines including SK-ES-1 (36). Conflicting reports, however, have been published on the regulation of caspase-8 expression in Ewing sarcoma. Fulda et al. (36) reported that this down-regulation was caused by promoter methylation. Treatment with a demethylating agent induced caspase-8 expression and restored sensitivity of the resistant Ewing sarcoma cell line CADO to tumor necrosis factor–related apoptosis-inducing ligand, anti-Fas antibody, and chemotherapeutic agents. Others have reported that caspase-8 down-regulation was frequently found in neuroblastoma but not in Ewing sarcoma cell lines (37). In the same article of Fulda et al. (36), methylation of the caspase-8 promoter was present in 13 of 20 Ewing sarcoma patients. Immunohistochemistry of six patients showed reduced caspase-8 expression in those Ewing sarcoma patients that had caspase-8 promoter methylation. A different study, in contrast, found no methylation of the caspase-8 promoter in eight Ewing sarcoma tumors (38).

Here, we confirm the down-regulation of caspase-8 in SK-ES-1 and show similar down-regulation in EW3. The observed resistance of SK-ES-1 and EW3 to anti-Fas treatment is in agreement with the CADO cell results. Our immunohistochemistry-based results showed considerable variation in caspase-8 expression between different patients and also within individual tumors (sections with positivity scores of 2, 3, or 4). These intra-individual differences may particularly complicate the search to unravel the underlying molecular mechanisms. The variation of expression that we found in our study group supports the results of Fulda et al. (36) and might play a role in tumor escape and chemotherapy resistance.

Down-regulation of caspase-8 could play a role in metastasis formation. Loss of caspase-8 did not affect growth of primary neuroblastomas but enhanced formation of metastases in chicken embryos and mice (39). It was found that caspase-8 deficiency in neuroblastomas prevented apoptosis induced by integrins from the surrounding stroma and in this way could enhance tumor spreading. In our patient group, there was not a preference for caspase-8 deficiency in Ewing sarcoma metastases. Patient 4 (Table 2), however, was positive in the resection

and negative in the metastatic material. Furthermore, patient 2 showed a reduction in staining intensity for the metastatic material. This shows that down-regulation of caspase-8 expression can occur in Ewing sarcoma metastases. Whether this functionally contributes to metastases formation in Ewing sarcoma should be further investigated in animal models and in a larger group of patients.

Killing by the perforin/granzyme pathway can occur through caspase-dependent as well as caspase-independent mechanisms (6). This pathway can, therefore, circumvent down-regulation of caspase-8 as shown by perforin/granzyme–induced killing of the caspase-8–negative and anti-Fas–resistant cell lines SK-ES-1 (Fig. 3A and B) and EW3 (data not shown). The ability of this pathway to kill tumor cells that are resistant to caspase-mediated killing has also been described in leukemia, but not myeloid leukemia (40), and in solid tumors like renal cell carcinoma (41) and colon carcinoma (42).

Tumor-infiltrating lymphocytes that were isolated from melanoma or glioma tumors (43) mainly used the perforin/granzyme pathway for killing *in vitro*. The requirements to trigger CTLs to use the perforin/granzyme or the death receptor pathway, however, differ (44). Compared with the death receptor pathway, a stronger T-cell receptor/MHC interaction might be needed to activate the perforin/granzyme pathway (45). Although the NK cells in our *in vitro* experiments mainly used the perforin/granzyme pathway, they can, like CTLs, also use the death receptor pathway for killing target cells (4, 46). Future animal studies should investigate the possibility to enhance the perforin/granzyme pathway *in vivo*. Increased expression of perforin and/or granzymes in cytotoxic cells has been shown after treatment with the immunomodulators interleukin-27 *in vitro* (47) or with interleukin-2 or IFN-α in melanoma patients (48, 49).

The absence of PI-9 in our study group points at Ewing sarcoma tumors as a sensitive target for the perforin/granzyme pathway. Our present work provides a basis for further investigations on this pathway in Ewing sarcoma animal models. To our knowledge, these experiments have not yet been done. One vaccination study in mice showed a T cell– and NK cell–mediated antitumor response against A673 but did not investigate the involved pathway (50). Our present work shows that A673 can be killed by both the death receptor and the perforin/granzyme pathway, leaving open their involvement in this vaccination experiment. If future animal studies show the same efficacy of the perforin/granzyme pathway *in vivo* as in our *in vitro* study, then this would add further support to explore the use of this pathway clinically in Ewing sarcoma.

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Expression of Cellular FLICE Inhibitory Protein, Caspase-8, and Protease Inhibitor-9 in Ewing Sarcoma and Implications for Susceptibility to Cytotoxic Pathways

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