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Cytotoxic T Lymphocytes Induce Caspase-Dependent and -Independent Cell Death in Neuroblastomas in a MHC-Nonrestricted Fashion

Anna De Geer,* Rolf Kiessling,* Victor Levitsky,† and Jelena Levitskaya2*

The MHC class I-restricted processing and presentation pathway is frequently nonfunctional in tumor cells; therefore, the direct targeting of tumor cells by CTLs may be difficult, if at all possible, to achieve. We used neuroblastoma (NB), which represents a striking example of a tumor with an impaired MHC class I pathway, as a model to study bystander effects of activated T lymphocytes on tumor cells. We found that NB cell lines are susceptible to killing by differentiated CD8+ CTL clones in a MHC class I-nonrestricted manner that involves two programs of cell death distinguished on the basis of different kinetics, sensitivities to caspase inhibitors, and cytokine-blocking reagents. The “early” death exhibited characteristic features of apoptosis, whereas the “delayed” caspase-independent death exhibited features associated with necrosis and was partially inhibited by TNF-α-blocking and prevented by overexpression of Bcl-2 or Bcl-xL. Our data reveal a previously unappreciated complexity of death pathways induced in tumor cells by immune activation and suggest that redirecting nonspecific effector CTLs to even a small proportion of NB cells or activating CTLs in a tumor’s proximity may have therapeutic effects in patients with NB. The Journal of Immunology, 2006, 177: 7540–7550.

D own-regulation of MHC class I molecules or components of the MHC class I processing and presentation pathway is one of the most common mechanisms of tumor escape from immune surveillance (reviewed in Ref. 1). In some tumors this is combined with deficiencies in the apoptotic pathways, which are used by killer lymphocytes to eliminate tumor cells. A classical example of this paradigm in humans is provided by neuroblastoma (NB), the most common solid, extracranial tumor in children, accounting for 8–10% of cancers and 15% of cancer deaths in childhood (2, 3).

A proportion of NB cells undergo spontaneous regression. At least in same cases, immune-mediated mechanisms may be involved in this unusual behavior of the tumor (4–6). Several lines of evidence support the critical importance of CD8+ T cells in the clearance of tumors or the prevention of their development. However, a number of features make NB cells an unfavorable target for CTLs; among these features are low or undetectable expression of surface MHC class I complexes (7, 8), defective expression of essential components of apoptotic signaling pathways such as caspase-8 (9, 10), and a limited spectrum of potential antigenic targets for NB-specific immune responses that is restricted to weakly immunogenic self-Ags (reviewed in Refs. 11 and 12).

CTLs exploit two major effector pathways for cytotoxicity. Although the first requires specific recognition of targets expressing MHC class I-peptide complexes and involves exocytosis of cytotoxic granules, the second pathway involves production of different cytokines and death receptor ligands such as IFN-γ, TNF-α, FasL, and TRAIL, which initiate a cascade of events in target cells in a HLA-nonrestricted manner (13).

It has been shown in experimental mouse models that activated T cells can efficiently eliminate tumors by acting on the malignant cells in a bystander fashion (14, 15). However, the bystander effects of T cell activation on human tumors are not well studied. We sought to investigate the potential usefulness of CTLs specifically activated on third-party targets in eliminating NB cells in a bystander fashion. We took advantage of human CD8+ CTL clones and polyclonal cultures specific for EBV-encoded Ags. Worthy of note, up to 95% of the human population are EBV carriers and bear CTL precursors specific for a large number of the EBV-derived peptide Ags presented through different HLA alleles (reviewed in Ref. 16). This opens the possibility of reactivating a large number of CTLs by using autologous lymphoblastoid cell lines (LCLs) or EBV-derived Ags in other forms from practically every adult individual as well as a large proportion of children who acquire EBV very early in life. Importantly, these CTLs usually posses the phenotype of memory T cells and express proinflammatory cytokines as well as membrane-associated ligands for death receptors upon activation (17).

In this study we demonstrate that human CTLs activated on their relevant targets are capable of inducing death of human NB cell lines in a MHC-nonrestricted bystander mode. We show that NB cells are sensitive to both membrane-bound and soluble factors released by CTLs, which are able to trigger not only caspase-dependent, but also caspase-independent cell death. Our data suggest that the retargeting of activated CTLs to a small proportion of tumor cells or the induction of unspecific immune activation in a tumor’s proximity may have a therapeutic effect in patients with...
FIGURE 1. NB cell lines are killed by activated CTLs bypassing MHC class I-restricted recognition. A–D, NB lines were used as targets for Zan13 (A) and CAR13 (B) CTL clones as well as polyclonal EBV-specific CTL cultures (C and D) at a 5:1 E:T ratio in 18-h 51Cr-release assays. Where indicated, Jurkat cells were used as controls for FasL and TRAIL sensitivity and K562 as a control for lymphokine-activated killer activity of the effectors. The percentage of lysis from a representative experiment of two or three performed is shown for each cell line in B and D. Mean ± SD of at least three experiments is shown in A and C. E, Bystander killing of NB cells by CTLs activated on LCL third-party targets. Zan13 CTLs were incubated with either 51Cr-labeled NB cells only (open bars) at a 5:1 E:T ratio or in the presence of WEI-B1 LCL (filled bars) at a 2.5:1 effector-to-LCL ratio. The percentage of lysis from one representative experiment of two or three performed for each cell line is shown. F, Supernatant from activated CTLs induces cell death in NB cells. 51Cr-labeled NB cell lines were incubated in the presence of 10% (v/v) of control (open bars) or activated (filled bars) supernatant for 18 h. Mean ± SD of the percent 51Cr-release obtained from 5–7 performed experiments is shown.

NB deficient in MHC class I expression and/or caspase-dependent death signaling.

Materials and Methods

Cell lines

The human NB cell lines MC-IXC, CHP-212, SK-N-SH, SH-SY5Y, SK-N-Be(2), SK-N-DZ, and SK-N-AS, the acute T leukemia cell line Jurkat, and the human chronic myeloid leukemia cell line K562 were purchased from the American Type Culture Collection. The human NB cell line LAN-5 was provided by Dr. M. A. Henrikkson (Department of Microbiology, Tumor, and Cell Biology, Karolinska Institute, Stockholm, Sweden). The LCL WEI-B1 was obtained by in vitro transformation of B cells from a healthy donor by the B95.8 EBV strain. C1R-A11 is an HLA-A11-transfected subline of the MHC class I-deficient mutant LCL transformed by an A-type EBV strain that carries mutations in the HLA-A11-restricted EBV nuclear Ag-4-derived peptide epitope IVT (18). All cell lines were maintained in IMDM medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS (Invitrogen Life Technologies), 100 IU/ml penicillin, and 100 μg/ml streptomycin (complete medium).

CTL cultures

All CTL clones and polyclonal cultures were maintained in complete medium supplemented with 10 IU/ml human rIL-2 from Cetus. The HLA-A11-restricted EBV-specific CTL clones Zan13, CAR13, and BK289 and the long-term polyclonal EBV-specific CTL cultures (BK bulk) were obtained by the stimulation of lymphocytes from EBV-seropositive donors with autologous B95.8 virus-transformed LCLs as described previously (17). For the generation of short-term polyclonal EBV-specific cultures, PBMCs from healthy donors were stimulated with gamma-irradiated (25 Gy) autologous LCLs at a 40:1 ratio, and after two consecutive restimulations at day 6 and 11 the cultures were expanded in IL-2-containing complete medium.

Generation of CTL supernatants

C1R/A11 cells pre pulsed with the HLA-A11-restricted EBV peptide IVT DFSVVIK at a final concentration of 10−6 M for 1 h at 37°C were cocultured with either CAR13 or BK289 CTLs at a 5:1 E:T ratio. Culture supernatants were collected 24 h after triggering (activated supernatant (AS)). Supernatants of CTLs cocultured with APCs nonpulsed with the peptide were prepared in parallel and used as controls (control supernatant (CS)).

Abs and reagents

The following Abs were used in this study: rabbit polyclonal anti-human IFN-γ-specific neutralizing Ab from Nordic Biosite; the FasL-specific neutralizing mouse mAb NOK-2 from BD Pharmingen; and the anti-human neutralizing TNF-α Ab and sodium azide-free IgG1 and IgG2a isotype control Abs from R&D Systems. Enbrel (Etanercept), a soluble TNF-α receptor (TNF-R2)/Fc fusion protein, was obtained from Wyeth. Human recombinant soluble TNF-R1, the human recombinant soluble TRAIL-R2/ human IgG1-Fc fusion protein, and the control Fc fusion protein were purchased from Alexis Biochemicals. The pan-caspase inhibitor benzyloxycarbonyl (Z)-VAD-fluoromethyl ketone (fmk) was obtained from Alexis Biochemicals and the caspase-2 inhibitor Z-VDVAD-fmk was from MP Biomedicals. The kit for annexin V/propridium iodide (PI) staining was obtained from BD Pharmingen and tetrathydemethylamine ethyl ester perchlorate (TMRE) was from Molecular Probes. The FITC-conjugated monoclonal active caspase-3 Ab was from BD Pharmingen. The mouse monoclonal anti-β-tubulin and anti-β-actin Abs were from Sigma-Aldrich. The mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP) Ab was from BD Biosciences, the mouse monoclonal anti-Bcl-2 Ab was obtained from Zymed Laboratories, the rabbit polyclonal anti-Bcl-xL Ab, and the polyclonal rabbit anti-high mobility group B1 (HMGB1) Ab were from BD Pharmingen. The HRP-conjugated rabbit anti-mouse and mouse anti-rabbit Abs were from Dakopatts.

Treatment with supernatants

Neuroblastoma (NB) cell lines were cultured in complete medium alone or in the presence of 10% v/v of the CS or AS for the indicated periods of time. Because the CS failed to induce the death of NB cell lines as compared with the complete medium samples kept in complete medium alone, they are omitted from the figures. To inhibit caspase activities in long-term kinetics assays, NB cells were pretreated with 50 μM pan-caspase inhibitor Z-VAD-fmk or 20 μM caspase-2 inhibitor Z-VDVAD-fmk for 1 h at 37°C.
and subsequently exposed to supernatants containing the inhibitor, the latter being refreshed every 24 h. In TNF-α-blocking experiments, 5 g/ml Enbrel or 2 g/ml soluble TNF-R1 was added.

51Cr release assays and blocking experiments
Neuroblastoma cells labeled with Na51CrO4 (0.1 Ci/106 cells) for 1 h at 37°C were extensively washed and incubated with either effector cells at a 5:1 E:T ratio or with the CS or AS (10%; v/v) in triplicate for 18 h at 37°C. Where indicated, WEI-B1 LCLs were added at a 2.5:1 effector-to-LCL ratio. The release of 51Cr into supernatants was monitored by a gamma counter (Wallac). The percentage of lysis was determined by the following equation: percentage of 51Cr release = \[
\frac{\text{experimental} - \text{spontaneous}}{\text{maximum} - \text{spontaneous}} \times 100
\]
where significant inhibitory effects (\(p \leq 0.05\); **\(p \leq 0.01\); and ***\(p \leq 0.001\)) are indicated for each cell line and were determined using Student’s paired t test.

Assessment of cell death
Trypan blue staining. Cell viability was determined by light microscopy using trypan blue staining.
Annexin V/PI staining. Cell death was assessed using staining with PI and FITC-conjugated annexin V according to the manufacturer’s protocol and analyzed on a FACScan flow cytometer (BD Biosciences).
TMRE staining. To detect changes in mitochondrial membrane permeability (Δψm), NBs were incubated with 25 nM TMRE for 30 min at 37°C and, where indicated, followed by annexin V-FITC staining (as described above) and flow cytometry.

Active caspase-3 staining. Detection of active caspase-3 was performed using the active caspase-3 Ab apoptosis kit (BD Pharmingen) following the manufacturer’s instructions, and analyzed by flow cytometry.

DNA fragmentation assay. Cells were kept on ice for 10 min in lysis buffer (10 nM Tris (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100), followed by incubation with proteinase K (1 mg/ml) and SDS (1%) for 2 h at 50°C. Next, samples were resuspended

FIGURE 2. Different effector molecules contribute to bystander CTL killing of NB cells. A, NB lines were used as targets for either Zan13 or BK bulk CTLs in 18-h 51Cr-release assays at a 5:1 E:T ratio. B, 51Cr-labeled NB cell lines were incubated in the presence of CS or AS for 18 h; the neutralization of TNF-α, IFN-γ, FasL, and TRAIL was performed as described in Materials and Methods. The percentage of lysis in control samples (c) and samples containing blocking reagent (b) is shown for each experiment. Each line represents the data of an independent experiment performed with the indicated cell line. Statistically significant inhibitory effects (\(p \leq 0.05\); **\(p \leq 0.01\); and ***\(p \leq 0.001\)) are indicated for each cell line and were determined using Student’s paired t test.

FIGURE 3. Killing of NB cells by AS is caspase dependent at early time-points (“early” cell death). 51Cr-labeled NB cell lines were incubated with AS for 18 h in the presence or absence of Z-VAD-fmk. The percentage of lysis in control samples and samples containing the pan-caspase inhibitor is shown for each experiment. Each line represents the data of an independent experiment performed with the indicated cell line. Statistical significant inhibitory effects was determined using Student’s paired t test (**\(p \leq 0.01\); and ***\(p \leq 0.001\)).
in a mixture of phenol and chloroform (1:1; v/v) and centrifuged at 14,000 × g for 15 min at 4°C. The DNA-containing fraction was incubated with 2.5 vol of 99.5% ethanol containing 0.15 M NaCl at 70°C overnight and centrifuged at 14,000 × g for 25 min at 4°C, and pellets were lyophilized. DNA fragments were resolved in 1.5% agarose gels and images were captured using a Gel Doc digital camera system equipped with Quantity One software (Bio-Rad).

Western blot analysis

Western blot was performed as previously described (19). Briefly, total cell lysates corresponding to 1 × 10⁵ cells were separated on precast polyacrylamide gels using the Pharmacia Multiphor II electrophoresis system (Amersham Biosciences). After protein transfer onto a polyvinylidene difluoride membrane (Millipore) and probing with the relevant Abs, specific bands were visualized by ECL (Amersham Biosciences) and digitally captured by a Fujifilm LAS-1000 image reader system (Science Imaging Scandinavia).

Generation of transfectants

Neuroblastoma cells were transfected with either pcDNA3 vector alone or the pcDNA3 vector expressing Bcl-2 or Bcl-xL using LipofectAMINE 2000 (Invitrogen Life Technologies) according to the manufacturer’s instructions. The transfected cells were selected and maintained in G-418-containing medium.
(0.5–0.8 mg/ml). Expression of Bcl-2 and Bcl-xL in selected lines was analyzed by Western blotting using specific Abs.

Detection of HMGB1 release
NB cells (2 × 10^6) were treated with either CS or AS (10%; v/v) in the presence or absence of the pan-caspase inhibitor Z-VAD-fmk and/or Enbrel for the indicated periods of time. Samples were collected and centrifuged at 20,000 × g for 5 min. Resulting supernatants were then mixed at a 2:1 ratio with 3 × concentrated sample buffer [3 × (65 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 5% mercaptoethanol, and 1% bromphenol blue)], resolved by SDS-PAGE, and subsequently analyzed by Western blotting with an anti-HMGB1 Ab. Samples of total cell lysates were prepared as a positive control for HMGB1 expression. To control for the presence of HMGB1 in the CTL-derived supernatants, CS or AS (10%; v/v) was added to complete medium and samples were processed in the same way as the NB-derived supernatants. All samples were controlled for the presence of β-tubulin by Western blotting to exclude the unwanted leakage of HMGB1 from cell debris that could have been generated during our experimental procedures.

Statistical analysis
All statistical analyses were done using Analyze-It software for Excel (version 7.2). All reported p values were two-sided and calculated using the Student’s paired t test. The following values were considered statistically significant: *, p ≤ 0.05; **, p ≤ 0.01; and ***, p ≤ 0.001.

Results
Neuroblastoma cells are killed by CTLs in a bystander manner
To investigate whether CTLs can lyse NB cells independently of Ag recognition, i.e., in a bystander manner, we used an 18-h ^51^Cr-release assay using human CD8^+^ EBV-specific CTLs as effectors and a panel of NB cell lines as targets (Fig. 1). The time frame of
this assay allows the detection of cell damage induced by cytokines and death receptor ligands such as FasL and TRAIL (20). Four of eight NB lines tested (further referred to as “sensitive”) were lysed by in vitro expanded CTLs after 18 h of coincubation (Fig. 1A). In contrast, 51Cr-release was not observed after 4–6 h (data not shown), consistent with the absence of MHC class I-restricted CTL recognition. Bystander CTL lysis of NB cells was also observed with a panel of other effectors including another clone of different epitope-specificity (Fig. 1B) and long-term (Fig. 1C) or short-term (Fig. 1D) polyclonal EBV-specific CTL cultures, indicating that the capacity of CTLs to induce death in NBs is not clone or donor dependent.

We next investigated the effect of additional specific CTL activation by third-party targets on the bystander lysis of NB cells. Following activation of CTLs by a LCL expressing the relevant MHC-peptide complexes, the bystander killing of sensitive NB cell lines was further enhanced whereas some of the resistant cell lines were rendered susceptible to the activity of CTLs (Fig. 1E).

The CTL-mediated death of NB cells could be attributed to membrane-bound and/or soluble factors. To assess the contribution of soluble factors we incubated the 51Cr-labeled NB lines in the presence of supernatants collected from either resting or activated CTLs (further designated as CS or AS, respectively) and found that a number of NB cell lines were highly sensitive to the activated but not control supernatant (Fig. 1F). This finding demonstrates that soluble effector molecules released by activated CTLs can cause the death of NBs independently of direct T cell-tumor cell contact.

**Multiple CTL effector molecules contribute to cell contact-dependent bystander CTL killing of NB cells**

Upon activation, CTLs express elevated levels of death ligands such as FasL, TRAIL, and TNF-α in both membrane-bound and soluble forms (reviewed in Refs. 13, 21, and 22). Moreover, other cytokines such as IFN-γ, released from activated CTLs, were reported to enhance the death ligand-mediated cell damage of NB cells (23). To identify CTL effector molecules contributing to the bystander killing of NB cells upon their coculture with activated CTLs, we performed blocking experiments using recombinant soluble TNF-R2 and TRAIL-R2 and neutralizing Abs specific for FasL or IFN-γ. To control for blocking efficiency, targets known to be sensitive to the effector molecules were used as controls as follows: Jurkat cells for TRAIL and FasL and WEHI cells for TNF-α. Suppression of IFN-γ-induced up-regulation of surface MHC class I served as a control for IFN-γ blocking Abs (data not shown).

As shown in Fig. 2A, NB lines exhibited various patterns of responsiveness to the blocking reagents upon coincubation with CTLs. Although TNF-α, IFN-γ, and FasL appeared to contribute to cell death to different extents depending on the cell line tested, neither of the individual death inducers could explain the entire
phenomenon. However, combining TNF-α blockade with blocking Abs to IFN-γ, TRAIL, and FasL caused no further significant reduction of killing (data not shown).

**TNF-α is the major mediator of death in the supernatant from activated CTLs**

We next asked the question of which effector molecules contribute to the NB cell lysis induced by soluble factors of activated CTLs. As shown in Fig. 2B, either TNF-α-neutralizing Ab or soluble rTNF-R2 significantly inhibited AS-induced 51Cr release from all four NB cell lines tested. No significant inhibition of cell death was obtained with IFN-γ, TRAIL- and FasL-blocking reagents in any of the lines tested. In addition, the combination of blocking reagents for TNF-α, IFN-γ, TRAIL, and FasL did not result in further reduction of NB killing by AS as compared with the selective blocking of TNF-α (data not shown). We concluded that, of the molecules tested in this study, TNF-α is the most important mediator of NB cell death triggered by AS.

**Killing of NB cells by AS is caspase dependent at early time points**

TNF-α can induce cell death in both a caspase-dependent and a caspase-independent manner (24–26). We examined the effect of the pan-caspase inhibitor Z-VAD-fmk on the killing of NB cells by AS in 18-h 51Cr-release assays. Inhibition of caspases led to a significant reduction of tumor cell lysis in all four cell lines tested (Fig. 3). In some experiments, the addition of Z-VAD-fmk completely prevented the lysis of tumor cells. This led us to conclude that the killing of NB cells occurring within 18 h of exposure to AS is caspase-dependent.

**AS induces caspase-independent death of NB cells at later time points**

We observed that although Z-VAD-fmk significantly reduced tumor lysis at 18 h posttreatment, the cells still died upon prolonged incubation (Fig. 4). This “delayed” cell death was detected within...
detected only at 48 h posttreatment. A substantial portion of cells dying in the presence of the pan-caspase inhibitor were already be seen at 24 h posttreatment, considerable amounts that, although the first signs of the “delayed” type of cell death could already be seen at 24 h posttreatment, considerable amounts of cells dying in the presence of the pan-caspase inhibitor were detected only at 48 h posttreatment. A substantial (~90%) proportion of these cells had low mitochondrial potential (Fig. 4B). Notably, the first signs of the “delayed” death as monitored by annexin V, PI and TMRE stainings were detected at different time points in different cells (Fig. 5).

To evaluate the caspase blocking efficiency of Z-VAD-fmk, we monitored the expression of the active form of caspase-3 (Fig. 4B, lower panel), cleavage of PARP, a nuclear substrate for caspasas, (Fig. 4C), and DNA fragmentation (Fig. 4D) in NB cells treated with an AS. All of these molecular changes were completely prevented in the presence of Z-VAD-fmk.

Because Z-VAD-fmk is inefficient in blocking the activity of caspase-2 (27), a protease known to be involved in the transduction of death signals in different models (28), we also tested the effect of Z-VDVAD-fmk, an inhibitor of caspase-2. However, Z-VDVAD-fmk alone or in combination with Z-VAD-fmk (data not shown) failed to prevent the AS-induced death of NB cells as monitored by cell permeabilization, the externalization of phosphatidyl serine, and mitochondrial membrane depolarization (Fig. 6). Collectively, our data show that preventing the caspase activation that is induced in NB cells by the soluble factors of activated CTLs does not rescue NB cells, suggesting the initiation of another, caspase-independent type of cell death.

**Caspase-independent cell death is associated with the release of HMGB1 from NB cells**

The nuclear binding HMGB1 protein is a factor released from necrotic but not apoptotic cells (reviewed in Ref. 29). We found that AS but not CS induces HMGB1 release into the supernatant of NB cells at the time points coinciding with the “delayed” cell death. HMGB1 release was not prevented by the inhibition of AS-induced caspase activation but was substantially reduced under TNF-α-blocking conditions (Fig. 7C). These findings suggest that the molecular program of the “delayed” caspase-independent death in NB cells resembles necrosis rather than apoptosis, where TNF-α is an important contributor.

**Overexpression of Bcl-2 and Bcl-xL protects NB cells from death induced by AS**

To further characterize the molecular pathways leading to AS-induced caspase-independent death of NB cells, we generated a panel of NB lines stably expressing the anti-apoptotic Bcl-2 or Bcl-xL proteins (Fig. 8A and data not shown) known to sequester...
BH3 domain-only molecules, thereby preventing the allosteric activation of BAX, BAK, and the subsequent mitochondrial program of apoptosis (30). Overexpression of these proteins either inhibited (Bcl-2) or substantially reduced (Bcl-x<sub>L</sub>) death induced by AS in MC-IXC and CHP-212 cell lines as measured by annexin V and PI stainings (Fig. 8B). Accordingly, the overexpression of Bcl-x<sub>L</sub> almost completely inhibited the externalization of phosphatidylserine induced by AS in the SK-N-SH and SH-SY5Y cell lines as well as reduced the number of cells with decreased mitochondrial membrane potential (data not shown). Overexpression of Bcl-2 or Bcl-x<sub>L</sub> prohibited mitochondrial membrane depolarization and caspase-3 activation in AS-treated NB cells, suggesting that mitochondrial failure is required for the “delayed” cell death in NB cells and that caspase activation occurs downstream of this event. However, caspases appear to play no critical role in this form of cell death, because caspase inhibition neither rescues NB cells from the delayed death nor prevents mitochondrial dysfunction.

**Discussion**

In this study we demonstrate that human activated CD8<sup>+</sup> CTLs affect NB cells in an HLA-independent, bystander fashion. Upon coculturing NB cells with activated CTLs or their supernatants, two major programs of cell death were distinguished based on different kinetics, sensitivity to protease inhibitors, the effects of Bcl-2 or Bcl-x<sub>L</sub> overexpression, and other molecular characteristics of these programs.

Both membrane-bound and soluble factors appeared to be involved in T cell-mediated bystander lysis of NB cells, which occurred within “early” time points (18 h) postexposure to activated CTLs. The proinflammatory cytokines TNF-α and IFN-γ, as well as FasL, were identified as effector molecules contributing to the deaths of various NB cell lines under these conditions (Fig. 2A). In a given cell line, blocking either of these factors often resulted in a comparable extent of protection from death, whereas the combined blockade failed to reveal additive effects of these effector molecules in death triggering. It is possible that the effector molecules analyzed in this study synergize with each other in a complex nonlinear fashion.

In sensitive cell lines, supernatants of activated CTLs were sufficient to trigger the early cell death which, under these circumstances, was partially accounted for by TNF-α (Fig. 2B). The commencement of “early” death correlated with increased numbers of cells expressing the active form of caspase-3 and processed PARP (data not shown). The loss of cells and the accompanying biochemical changes were significantly diminished, if not prevented, by the pan-caspase inhibitor Z-VAD-fmk (Fig. 3), pointing to a caspase-dependent mechanism of cell loss. These results appear to contradict the frequent silencing of the caspase-8 gene in NBs due to either the methylation of its promoter region or the gene deletion that renders cells resistant to death receptor-mediated apoptosis (9, 31, 32). Malignant stage IV NB cells express reduced levels of caspase-8 when compared with benign ganglioneuroma (31) and, as demonstrated recently, reconstitution of caspase-8 expression suppresses dissemination of the disease (33). Inactivation of the caspase-8 gene correlates with amplification of the oncogene MYCN and tumor severity (32) and is considered to be involved in the pathogenesis of NB (34). Two of the four sensitive cell lines investigated in this study, MC-IXC and CHP-212, exhibited high steady-state levels of caspase-8 expression, whereas in the other two lines, SK-N-SH and SH-SY5Y, caspase-8 was easily detectable after exposure to the supernatant of activated CTLs, although the protein was not detectable in those cells before the treatment (A. De Geer et al., R. Kiessling, V. Levitsky, and J. Levitskaya, manuscript in preparation). This observation seems to reconcile the contradiction between the lack of caspase-8 in NB cells and the caspase/TNF-α dependency of the early death triggered in NB cells by activated CTL supernatants.

Even though Z-VAD-fmk completely prevented the activation of caspase-3, PARP cleavage, and DNA fragmentation (Fig. 4, B–D), it failed to avert the “delayed” NB cell loss caused by AS at the later time points (48–72 h) of monitoring (Fig. 4, A and B). Thus, even in the absence of functional caspases yet another type of death can be induced in supernatant-exposed NB cells. Of note, although it has been previously suggested that Z-VAD-fmk can itself induce autophagic degeneration in tumor cells (35), the viability of the cell lines used in this study was not affected by the presence of Z-VAD-fmk in complete medium or CS-containing medium (Fig. 4C and data not shown). The caspase-independent “delayed” NB cell loss was characterized by increased numbers of annexin V/PI-double positive and TMRE-low cells and could be partially reduced by TNF-α blocking, particularly as observed in MC-IXC cells (Figs. 4B and 7A). This finding suggests that, at least in some NB cell lines, TNF-α contributes to the “delayed” cell loss; however, it may not be the sole “late death”-inducing agent in the AS. In agreement with this hypothesis, human recombinant TNF-α used alone at concentrations higher than those detected in AS failed to trigger either caspase-dependent or caspase-independent cell death in NB cell lines at both the “early” and “late” time points tested (data not shown). This indicates that either other factors present in the AS facilitate TNF-α-induced cell death or that TNF-α is an enhancer of cell death caused by another yet unidentified factor(s). In our attempts to determine such a molecule, we blocked IFN-γ, IFNα, FasL, and TRAIL; however, the neutralization of these molecules alone or in combination did not rescue NB cells from the AS-induced “delayed” death (data not shown). TNF-α can trigger caspase-dependent and caspase-independent cell death; the latter can be mediated by cathepsin B or reactive oxygen species in tumors of different origin (24, 26, 36, 37). Currently available cathepsin B inhibitors, including CA074Me, did not prevent the “delayed” death in NBs. We also failed to detect the generation of reactive oxygen species in NB upon treatment with AS (data not shown). To identify the molecular perpetrators of the “delayed” NB death, we further attempted to block a number of other proteases known to contribute to caspase-independent cell death in different cell types (reviewed in Refs. 38 and 39). The inhibition of cathepsin D and cysteine proteases by pepstatin A and E64, respectively, did not protect NB cells from death by AS, whereas the calpain inhibitor I, Z-LL-H, and chloroquine, which is used to inhibit lysosomes, were toxic when applied at the recommended concentrations in the time frame of our experimental procedures (data not shown). Therefore, the molecular executors of the “delayed” cell death in NB cells remain to be defined.

Interestingly, NB cell lines defined by us as “resistant” due to their insensitivity to both, bystander killing by activated CTLs as well as CTL soluble factors at “early” time points, tolerated AS-mediated damage even after 72 h of incubation and exhibited only a minimal (3–4%) increase in the numbers of annexin V-positive and PI-positive cells, which could be prevented by the pan-caspase inhibitor and TNF-α-blocking agents (data not shown). The molecular mechanisms conferring “resistance” of NB cells to CTL-mediated bystander killing are still unclear. Our preliminary data indicate that the levels of surface death receptors involved in the bystander killing of NB cells such as FAS and TNF-R do not significantly differ between “sensitive” and “resistant” cell lines (A. De Geer, R. Kiessling, V. Levitsky, and J. Levitskaya, manuscript in preparation), suggesting that the signaling downstream of the surface death receptors may vary in these two groups of NB cells.
lines. Identification of the major factor(s) causing “delayed” NB death will allow the dissection of tumor tolerance to bystander immune activation at the molecular level.

Discrimination between “apoptotic” and “necrotic” types of cell death induced in NB cells by soluble factors released from CTLs is of special interest, because the type of cell death may define the outcome of the immune responses in the tumor milieu (reviewed in Ref. 29). The diversity of cellular death pathways described to date includes apoptosis, necrosis, autophagic degeneration, apoptosis-inducing factor (AIF)-mediated apoptosis-like death, lipoptosis, and others (Ref. 40 and reviewed in Ref. 25). Autophagy may be especially relevant for NB cells, as it has been shown that spontaneously regressing tumors have signs of autophagic degeneration (41). Our extensive efforts, including electron microscopy and autophagosome-specific staining with monodansylcadaverine, revealed no changes characteristic of autophagy (42, 43). In addition, multiple attempts to reveal AIF translocation into the nucleus of AS-treated NB cells were also unsuccessful (data not shown). This indicates that autophagic degeneration and AIF-mediated apoptosis-like cell death are unlikely to cause AS-induced, caspase-independent NB.

A number of markers for discriminating between apoptosis and necrosis have been suggested. In “healthy” cells HMGB1 is loosely associated with chromatin, and it is known that upon apoptosis HMGB1 is engaged in the nucleus and retained in the apoptotic bodies that prevent its release. However, after nonapoptotic “lytic” cellular injury HMGB1 can be released (reviewed in Ref. 44). We found an increase of HMGB1 levels in supernatants of NB cells undergoing “delayed” death, which could be diminished by blocking TNF-α (Fig. 7C). This finding points toward the occurrence of nonapoptotic cell damage that may be represented by necrosis or another, not yet identified, type of cell death characterized by the loss of structural integrity of the plasma membrane (reviewed in Ref. 45). Another indicator of the latter is an increased uptake of membrane-impermeable dyes such as trypan blue and propidium iodide by NBs exposed to AS (Figs. 4B, 7A, 8B, and data not shown). Further studies are required to characterize the molecular pathway of the “late” program of NB cell death and use it as a target for pharmacologic intervention in NB patients.

Overexpression of Bcl-2 or Bcl-xL prevented the drop in the mitochondrial membrane potential and rescued a large proportion of AS-exposed NB cells (Fig. 8). Interestingly, a negative correlation has been observed between high levels of Bcl-2 expression in NB biopsies and the frequency of spontaneous tumor regression (46), which strongly suggests that mitochondrial dysfunction is required for NB death in vitro and in vivo. This process seems to be independent of caspase activity as has been described for several forms of cell death with poorly understood molecular regulation.

The capacity of activated T cells to induce the death of NB cells independently of direct tumor cell recognition may be exploited for the development of new approaches toward immunotherapy of this malignancy. To date, a significant effort was made by others to specifically targeting of MHC-peptide complexes on the malignant cells and could involve a relatively abundant preexisting immune effectors of irrelevant specificity such as CTLs specific to EBV or any other ubiquitous human pathogen such as the CMV or the influenza virus.

Disclosures

The authors have no financial conflict of interest.

References


