BCL-2 Blocks Perforin-induced Nuclear Translocation of Granzymes Concomitant with Protection against the Nuclear Events of Apoptosis*

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David A. Jans§§, Vivien R. Sutton¶, Patricia Jans‡, Christopher J. Froelich¶, and Joseph A. Trapani§

From the §Nuclear Signaling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra City, Australia, ¶Cellular Cytotoxicity Laboratory, The Austin Research Institute, Heidelberg, Australia, and ‡Department of Medicine, Northwestern University Medical School, Evanston, Illinois 60201

Cytolytic granule-mediated target cell killing is effected in part through the synergistic action of the membrane-acting protein perforin and serine proteases such as granzymes (Gr) A and B. In this study, we examine the subcellular distribution of granzymes in the presence of perforin and the induction of apoptosis in mouse FDC-P1 myeloid and YAC-1 lymphoma cells that express the proto-oncogene bcl2. Using confocal laser scanning microscopy to visualize and quantify subcellular transport of fluoresceinated granzyme, we find that granzyme entry into the cytoplasm in the absence of perforin is not impaired in the bcl2-expressing lines. However, perforin-dependent enhancement of granzyme cellular uptake and, importantly, granzyme redistribution to the nucleus were strongly inhibited in the bcl2-expressing lines, concomitant with greatly increased resistance to granzyme/perforin-induced cell death. DNA fragmentation induced by granzyme/perforin was severely reduced in the bcl2-expressing lines, implying that prevention of granzyme nuclear translocation blocks the nuclear events of apoptosis. The kinetics of GrB nuclear uptake and induction of apoptosis were faster than for GrA, whereas YAC-1 cells showed greater resistance to granzyme nuclear uptake and apoptosis than FDC-P1 cells. In all cases, granzyme nuclear accumulation in the presence of perforin correlated precisely with ensuing apoptosis. All results supported the idea that GrA and GrB share a common, specific nuclear targeting pathway that contributes significantly to the nuclear changes of apoptosis.

One major active component released by cytotoxic natural killer and T cells (CTLs)1 during degranulation is perforin (1), which can damage cells through membrane perturbation. The induction of target cell apoptosis and accompanying DNA fragmentation and chromatin condensation additionally requires the action of other granule components and in particular, serine proteases termed granzymes (2). The most abundant granzyme in the mouse is granzyme A (GrA, fragmentin-1), a ~60-kDa homodimer that, in contrast to the monomeric granzyme B (GrB, fragmentin-2), is able to activate interleukin-1β directly (3) and can cleave various intracellular/extracellular proteins, including the thrombin receptor (4) and the nuclear/nucleolar protein nucleolin (5). GrA and GrB differ in their substrate specificities; although GrA cleaves at basic amino acid residues, GrB is an aspase, cleaving at aspartic acid residues (6).

Evidence for synergy between perforin and granzymes comes from a variety of biochemical and cellular studies. Nonlytotoxic rat basophilic leukemia cells transfected to express perforin with GrA and/or GrB become potent inducers of membrane damage and DNA fragmentation in conjugated target cells (7, 8). Purified GrA or GrB in combination with perforin are able to effect both the nuclear and the nonnuclear changes of apoptosis, although GrA/perforin-mediated cell killing exhibits slower kinetics (9, 10). Although CTLs from GrB gene knock-out mice exhibit only severely delayed apoptotic capacity (11), those from GrA knock-out mice do not, implying that GrA is not indispensable for the induction of apoptosis in target cells by natural killer cells and alloreactive CTL (12), although it is probably important in inducing target cell apoptosis via a distinct mechanism that is triggered after prolonged incubation of killer and target cells and/or in cells that are relatively resistant to GrB (Ref. 11; see also Refs. 10 and 13). GrA/GrB double knock-out mice are completely defective in the induction of the nuclear changes associated with apoptosis, whereas perforin-dependent target cell lysis is unaffected (13). Because the trypsin-like substrate specificity of GrAs is quite distinct from that of GrB, it must induce apoptosis by cleaving either substrates different from those of GrB or at cystytic sites within the natural substrates of GrB, this presumably being the basis of the documented role of GrA in chromosome degradation during apoptosis (9, 14).

We have recently shown that GrB can both enter the cell cytoplasm in the absence of perforin and translocate from the cytoplasm to the nucleus in target cells in the presence of perforin (15–17). Nuclear targeting occurs before the nuclear events of apoptosis such as DNA fragmentation (17), implying that nuclear translocation of granzymes may constitute a means by which the apoptotic signal is communicated to the nucleus; nuclear transport of GrB may bring it into contact with potential substrates such as the DNA repair enzyme poly- (ADP-ribose) polymerase (PARP) and the catalytic subunit of DNA-dependent protein kinase, both of which have been shown to be cleaved not only by caspases such as caspase-3, early in

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§ To whom correspondence should be addressed: Nuclear Signaling Laboratory, Division for Biochemistry and Molecular Biology, John Curtin School of Medical Research, Canberra City, Australia, ¶Cellular Cytotoxicity Laboratory, The Austin Research Institute, Heidelberg, Australia, and ‡Department of Medicine, Northwestern University Medical School, Evanston, Illinois 60201

1 The abbreviations used are: CTL, cytotoxic T lymphocytes; GrA, granzyme A; GrB, granzyme B; CLSM, confocal laser scanning microscopy; FITC, fluorescein isothiocyanate.
many forms of apoptosis, but also in vitro (18, 19) as well as in vivo (20) by GrB. On this basis, it has recently been proposed that GrB may bypass the requirement for active caspases in the presence of viral caspase inhibitors (20). Our recent work (21) has shown that GrA resembles GrB in terms of nuclear targeting in intact cells in the presence of perforin as well as in vitro and in vivo nuclear transport assays, this apparently conserved mechanism probably playing a role in contributing to the nuclear changes associated with apoptosis.

Many forms of apoptotic cell death can be blocked by the proto-oncogene bcl2 (B cell leukemia gene-2), a member of a family of regulatory proteins involved in either promoting or inhibiting apoptosis. bcl2 expression is required for long term survival of lymphoid cells (22), but following overexpression due to chromosomal translocation, can be oncogenic; unlike other oncoproteins, however, it confers cell viability without promoting cell proliferation (23). Although bcl2 inhibits apoptosis induced by a variety of stimuli including cytotoxic drugs, tumor necrosis factor, and ionizing radiation, it does not confer general resistance to CTL-induced apoptosis in myeloid cells (24–28). We have recently shown that although BCL-2 does not confer resistance to apoptosis induced by allogeneic CTL, natural killer cells, or whole cytolytic granules, it can protect against apoptosis induced by purified perforin and GrB (29).

In the present study, we use confocal laser scanning microscopy (CLSM) to assess the cellular uptake and intracellular distribution of granzymes in bcl2-expressing mouse myeloid FDC-P1 and YAC-1 lymphoma cells. We show that perforin-dependent redistribution and nuclear uptake of granzymes is strongly inhibited by BCL-2, concomitant with greatly increased resistance to granzyme-perforin-induced apoptosis and induction of DNA fragmentation in particular. The clear implication is that nuclear targeting of granzymes may participate directly in effecting the nuclear changes associated with apoptosis.

**MATERIALS AND METHODS**

**Chemicals and Reagents—**Fluorescein isothiocyanate (FITC) was from Molecular Probes. Other reagents were from the sources previously described (16, 17, 21, 29, 30).

**Cell Culture—**Mouse FDC-P1 myeloid cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and recombinant interleukin 3-containing culture supernatant (31) as described (17). Mouse YAC-1 lymphoma cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum as previously described (30). The bcl2-expressing FDC-P1 (FDBcl2 (23)) and YAC-1 (Ybcl2 (29)) cell lines, derived after transfection, have been characterized previously with respect to the lack of expression of functional BCL-2 protein and were cultured in identical fashion to the parental lines.

**Protein Purification and Labeling—**Human GrA, GrB, and perforin were all purified as described previously (21, 32–35). GrA and GrB were labeled with FITC as described, with less than 15% loss of proteolytic activity (21, 30). Protein concentrations were determined using the dye binding assay of Bradford (36) with bovine serum albumin as a standard or by measuring absorbance at 280 nm in the case of GrB (37).

**Cellular Uptake and Distribution of Granzymes—**Whole cell uptake and subcellular transport of labeled GrA or GrB were examined as previously (17, 21). Cells were harvested in the logarithmic phase of growth, washed, and resuspended (4 × 10⁶ cells/ml) in Hanks’ buffered saline solution containing 10 mM Hepes, pH 7.2, 2 mM CaCl₂, 0.4% bovine serum albumin, and 0.1% (v/v) interleukin 3-containing culture supernatant. Cells (3 μl) were incubated for the specified times with an equal volume of perforin (100–1000 units/ml, final concentration) and/or FITC-GrA or -GrB (0.25–10 μg/ml, final concentration) or a 20-kDa FITC-labeled dextran (Sigma). Perforin and GrA or GrB were diluted immediately before the assay in 10 mM Hepes, 150 mM NaCl, 1 mM EGTA, pH 7.2, and the whole mixture was pipetted onto a glass slide, which was then sealed before incubating at 37 °C and imaging of fluorescence using CLSM (38). Results were expressed in terms of Fc/Fmed (cellular uptake: fluorescence quantitated in the cytoplasm (Fc) relative to background fluorescence) and Fm/Fc (nuclear accumulation: fluorescence quantitated in the nucleus (Fm) relative to Fc after subtraction of background fluorescence), as previously (17, 21).

**Apoptosis—**Apoptotic morphology was determined based on visual criteria as previously (17, 21), the validity of which has been confirmed using terminal deoxyribonucleotidyl transferase labeling of DNA strand breaks with DUTP (TUNEL) analysis (see below) and annexin V expression (17). To monitor nuclear apoptosis, cells undergoing DNA fragmentation were quantitated using a TUNEL kit purchased from Boehringer Mannheim (17). Cells (routinely 2000–5000) were analyzed immediately on a cytofluorograph (FACScan, Becton Dickinson) (17).

**RESULTS**

**Perforin-dependent Nuclear Uptake of Granzymes by Intact Cells—**We have previously shown that purified GrA and GrB can localize in the nucleus of intact rat hepatoma and FDC-P1 mouse myeloid cells in the presence of sublytic concentrations of purified perforin (16, 17, 21), this nuclear accumulation occurring before the nuclear and cell membrane changes of apoptosis. Because bcl2 expression affords protection against apoptosis in the case of purified GrB and perforin (29), we decided to investigate granzyme subcellular transport in bcl2-expressing FDC-P1 and YAC-1 cell lines using FITC-labeled granzymes and CLSM. Untransfected FDC-P1 and YAC-1 cells both showed perforin-dependent nuclear uptake of either GrA or GrB whereby two populations of cells became rapidly evident; those showing accumulation of granzymes in the nucleus (Fig. 1, left panels) and those that did not accumulate granzymes and did not undergo apoptosis ("non-apoptotic" cells).

**BCL-2 Blocks Perforin-dependent Translocation of Granzymes to the Nucleus—**Quantitative analysis of the cellular and nuclear uptake of FITC-labeled granzymes in the absence or presence of perforin indicated significant uptake of GrA and GrB into the cytoplasm, but not nuclear accumulation, in both
wild type FDC-P1 and YAC-1 cells in the absence of perforin (Figs. 2, left panel and not shown). In the presence of perforin, both granzymes were taken up by the cells to a greater extent and accumulated strongly in the nucleus, with maximum levels of accumulation in the nucleus around 2-fold those in the cytoplasm (Fig. 2, left panel). Granzyme uptake in the absence of perforin when \( bcl2 \) was expressed in the cells appeared not to be significantly different to that in the parental lines. Results are averaged from at least three separate experiments, each individual measurement representing at least five separate measurements for each of Fc, Fmed, Fn, and autofluorescence, where the S.E. was not greater than 7.2% the value of the mean.

**FIG. 2.** Uptake and nuclear accumulation of granzyme A or B or a 20-kDa dextran by FDC-P1 cells expressing (FDbl2, right panels) or not expressing (FDC-P1, left panels) \( bcl2 \) in the absence or presence of perforin. Cells were exposed to FITC-GrA, FITC-GrB, or a 20-kDa FITC-labeled dextran in the absence or presence of perforin at 37 °C as indicated and visualized at various times using CLSM. Cells with apoptotic morphology (preapoptotic) as opposed to nonapoptotic were distinguished as described under "Results" (Ref. 17; see Fig. 3 for quantitation thereof), and image analysis was performed to quantitate either cellular (Fc/Fmed, top panels) and nuclear (Fm/c, bottom panels) uptake (see "Materials and Methods") for the different cell populations. Results are averaged from at least three separate experiments, each individual measurement representing at least five separate measurements for each of Fc, Fmed, Fn, and autofluorescence, where the S.E. was not greater than 7.2% the value of the mean.

**BCL-2 Prevention of Granzyme Nuclear Uptake Correlates with Protection against DNA Fragmentation**—To determine whether the nuclear events of granzyme/perforin-induced apoptosis were specifically prevented in \( bcl2 \)-expressing cells coincident with the inhibition of granzyme nuclear translocation, \( bcl2 \)-expressing and nonexpressing FDC-P1 and YAC-1 (data not shown) cells were exposed to perforin and GrB, and DNA breakdown during apoptosis was examined by TUNEL (Fig. 5). Neither perforin nor GrB alone induced DNA fragmentation in the cell population above background levels (~3%) in any of the lines. The combination of both reagents induced a strong response in terms of DNA fragmentation in the FDC-P1 cells (maximally up to 70%) but had no significant effect on \( bcl2 \)-expressing cells (<10% TUNEL positive cells) (Fig. 5). Comparable results were obtained for the YAC-1 lines (not
shown). It was concluded that bcl2 expression prevented the nuclear events of apoptosis induced by perforin and GrB, presumably as a consequence of the inhibition of GrB nuclear translocation.

DISCUSSION

We have previously shown that GrB nuclear uptake precedes the onset of apoptotic DNA fragmentation (17), implying a role of nuclear granzymes in the latter. In this study we establish for the first time that bcl2 expression prevents perforin-dependent nuclear translocation of granzymes in two different cell lines, concomitant with protection against the nuclear changes associated with granzyme/perforin-mediated apoptosis and ultimately cell death. This strongly supports the idea that nuclear translocation of granzymes plays a role in the nuclear events of apoptosis, consistent with the recent demonstration that catalytic subunit of DNA-dependent protein kinase and NuMA (nuclear mitotic apparatus protein) are both
cleaved by GrB in vivo (20) as well as the fact that GrA/GrB knockout mice are completely deficient in the CTL-mediated nuclear but not cytolytic apoptotic response. Because our results here for cellular uptake (Figs. 2–4) show that GrA and GrB enter bcl2-expressing cells to an extent comparable with wild type in the absence of perforin and the sensitivity of the bcl2-expressing cells to perforin-mediated membrane damage appears to be normal (29), it can be concluded that BCL-2 action is exerted at or downstream of the point of synergy between perforin and granzymes. BCL-2 presumably targets an essential component/step in the signaling pathway, a likely candidate being caspase activation that we have recently shown to be required for both granzyme nuclear translocation and induction of the nuclear events of apoptosis (39); inhibition of caspase activation would block the downstream events of granzyme nuclear translocation and subsequent nuclear...
changes of apoptosis (17), including the cleavage of catalytic subunit of DNA-dependent protein kinase and NuMA (38). The Caenorhabditis elegans BCL-2 homolog CED-9 has been shown to exert its effect at least in part by blocking activation by CED-4 of CED-3, the homolog of the mammalian interleukin-1β-converting enzyme (ICE) (40, 41). Interestingly, the non-nuclear events in granzyme/perforin-induced cytolysis, although able to be blocked by BCL-2 (29), appear to be caspase-independent (39, 42), suggesting that BCL-2 also acts on an as yet uncharacterized caspase-independent pathway that governs nonnuclear apoptotic phenomena; that the nucleus is dispensable for apoptosis has been shown using enucleated cytoplasts (43).

Of significance in the context of this study may be the fact that, apart from being localized to the outer mitochondrial membrane and endoplasmic reticulum, BCL-2 has been shown to be present in the nuclear envelope (44–46). The complete lack of BCL-2 within the plasma membrane is consistent with...
our observations that both granzyme uptake (this study) and pore formation by perforin (this study and Ref. 29) are unaffected by BCL-2, and its absence from endocytic vesicles argues against the possibility that BCL-2 blocks perforin-induced release of granzymes from encapsulated vesicles to the cytosol (33). Structural homology between the BCL-2 family member BCL-XL and the membrane translocation domain of some bacterial toxins presumed to form a membrane pore (47) implies that BCL-2 may affect the transport of proteins or ions across membranes. That perturbation of ionic concentrations within
the nuclear envelope can impair nuclear pore complex function, and hence, nuclear protein import (48, 49) has been reported, meaning that BCL-2 within the nuclear envelope could conceivably perturb nuclear pore complex function, thereby blocking granzyme passage into the nucleus directly. Significantly, BCL-2 antagonizes the transport of p53 and cyclin-dependent kinases into the nucleus as well as that of cytochrome c from mitochondria (50, 51), consistent with this possibility.

This study demonstrates that bcl2 expression blocks perforin-dependent nuclear targeting of GrA and GrB in intact cells and concomitantly confers protection against apoptosis and its nuclear events in particular. The results here, consistent with other studies (9, 10, 13, 21), indicate that GrB nuclear uptake and induction of apoptosis were faster than for GrA, whereas YAC-1 cells showed greater resistance to granzyme nuclear uptake and apoptosis than FDC-P1 cells. Thus, in all cases, the rate of granzyme nuclear uptake correlated well with the kinetics of the induction of apoptosis, supporting the role of nuclear targeting of granzymes in apoptosis (Refs. 16, 17, 21; see also Ref. 20). That perforin-dependent induction of apoptosis by both GrA and GrB is inhibited by bcl2 expression is further evidence that granzymes utilize a common and possibly unique nuclear transport pathway (16, 21), strongly implying that nuclear targeting of granzymes plays a role in effecting the nuclear changes of apoptosis.

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bcl2 Blocks Granzyme Nuclear Targeting and Apoptosis

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