Brief Definitive Report

Granzyyme A Is an Interleukin 1β–converting Enzyme

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Summary

Apoptosis is critically dependent on the presence of the ced-3 gene in Caenorhabditis elegans, which encodes a protein homologous to the mammalian interleukin (IL)-1β–converting enzyme (ICE). Overexpression of ICE or ced-3 promotes apoptosis. Cytotoxic T lymphocyte–mediated rapid apoptosis is induced by the proteases granzyme A and B. ICE and granzyme B share the rare substrate site of aspartic acid, after which amino acid cleavage of precursor IL-1β (pIL-1β) occurs. Here we show that granzyme A, but not granzyme B, converts pIL-1β to its 17-kD mature form. Major cleavage occurs at Arg120, four amino acids downstream of the authentic processing site, Asp116. IL-1β generated by granzyme A is biologically active. When pIL-1β processing is monitored in lipopolysaccharide-activated macrophage target cells attacked by cytotoxic T lymphocytes, intracellular conversion precedes lysis. Prior granzyme inactivation blocks this processing. We conclude that the apoptosis-inducing granzyme A and ICE share at least one downstream target substrate, i.e., pIL-1β. This suggests that lymphocytes, by means of their own converting enzyme, could initiate a local inflammatory response independent of the presence of ICE.

Materials and Methods

Reagents and Substrates. Granzyme A substrate BLT (N-α-benzoxycarbonyl-α-L-lys-thiobenzyl ester) was purchased from Bachem (Bubendorf, Switzerland), and granzyme B substrate...
BAT (Boc-Ala-Ala-Asp-SBzI) was purchased from Enzyme Systems Products (Dublin, CA). The inhibitor for granzyme A (PhNHCO-NHCH2EtOIC, IGA) was a kind gift of Dr. J. Powers (Georgia Institute of Technology, Atlanta, GA), and the ICE inhibitor (Ac-Tyr-Val-Ala-Asp-CHO, YVA) was from the Glaxo Institute for Molecular Biology (Geneva, Switzerland). DCI (3,4-dichloroisocoumerin) was purchased from Boehringer Mannheim (Mannheim, Germany). The polyclonal goat antibody to mouse IL-1β was raised against purified recombinant protein. The rabbit antibody to human IL-1β was a kind gift from G. Mazzei (Glaxo Institute for Molecular Biology). The peptide corresponding to the human pIL-1β sequence Asn109 to Asp118 was purchased from Bachem.

**Purification of Proteases.** Granzymes were purified from granules of the B6.1 cytolytic T lymphocyte cell line as described (16). Active human ICE was isolated from the cytosol of the human monocytic cell line THP-1 as previously described (2). ICE was further purified by ion exchange chromatography, sequentially passing the cytosolic fraction over Q-Sepharose fast flow (Pharmacia LKB, Zürich, Switzerland) followed by chromatography on an HR 10 column (Mono-S; Pharmacia LKB). Briefly, the cytosolic fraction was dialyzed against 20 mM Hepes, pH 7.0, 5 mM EDTA, 2 mM dithiothreitol, 1 mM 4-[(2-amino-ethyl)-benzenesulfonyl fluoride, 0.1% CHAPS [3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate], and 15% ethylene glycol (buffer A), and passed over the Q-Sepharose equilibrated with buffer A. The column flow through contained ICE activity and was subsequently chromatographed on Mono-S also equilibrated with buffer A. ICE activity was eluted with a 40-column volume linear gradient from 0-0.5 M KCl in buffer A. This represents a 76-fold purification of ICE activity and sufficiently removed proteases capable of nonspecifically hydrolyzing the ICE fluorescent substrate, Ac-Tyr-Val-Ala-Asp-7-amino-4-methyl coumarin (Bachem). Using this substrate to assay for ICE activity, Mono-S-purified ICE (0.5 ng/ml) had a specific activity of 0.12 nmol AMC min⁻¹ mg⁻¹.

**Enzymatic Activity Assays.** Granzyme A activity was measured using BLT as a substrate (17). Granzyme B activity was assayed as described for granzyme A, but BLT was replaced by BAT (18). For inhibition studies, the enzymes were preincubated at 30°C for 30 min with the respective inhibitors at the following final concentrations: 170 μM DCI, 70 μM IGA, and 2 μM YVA.

**Expression of pIL-1β.** In vitro transcription and translation of human pIL-1β was carried out with the TNT Coupled Reticulocyte Lysate System (Promega Corp., Madison, WI) according to the manufacturer's instructions. The human pIL-1β DNA (Glaxo Research and Development, Greenford, UK), cloned into the pcDNA I Amp expression vector (Invitrogen, NV Leek, The Netherlands) was transcribed using the T7 promoter. For processing, 1 μl of [35S]methionine-labeled pIL-1β was incubated with enzyme (0.2 μg granzyme A, granzyme B, or Mono-S-purified ICE) in a buffer containing 50 mM Tris-HCl, pH 8.0, and 1 mM CaCl2 for 10 h at 30°C (for digestion with ICE, 0.5 mM dithiothreitol was included). Inhibition of the enzymes was performed as described above. Reaction mixtures were analyzed by SDS-PAGE and subsequent autoradiography. Recombinant bacterial human pIL-1β was kindly provided by P. Graber (Glaxo Institute for Molecular Biology).

**IL-1β Biological Assay.** Recombinant human pIL-1β (200 ng) was incubated with granzyme A (20 ng) during 4 h at 37°C, and IL-1β activity was assayed as described (19).

**Amino Acid Sequencing and Matrix-assisted Laser Desorption Mass Spectroscopy.** Granzyme A-processed pIL-1β was separated by SDS-PAGE, transferred to transblot membranes (Bio-Rad Laboratories, Zürich, Switzerland) and subjected to amino acid sequence determination using a pulsed liquid phase protein sequencer (477 A; Applied Biosystems, Foster City, CA) on-line phenylthiodyantoin-amino-acid analyzer (120 A; Applied Biosystems).

The peptide corresponding to the pIL-1β sequence Asn109 to Asp118 of human pIL-1β (Bachem, 10 μg) was incubated with purified granzyme A or B (1 μg each) at 37°C for 16 h, and the cleavage site was determined by laser desorption mass spectrometric analysis (20).

**Lysis by CTLs.** Bone marrow-derived macrophages from BALB/c mice (H2d) were stimulated with LPS (10 ng/ml) for 3 h, washed, and then added to the Plasmodium falciparum circumsporozoite protein peptide/H2d specific CTL clone PbCSF12 (21). The effector to target cell ratio was 4:1. After 4 h, cells were centrifuged and the cell-associated IL-1β was determined by Western blot analysis using a goat anti-mouse IL-1β polyclonal antiserum.

**Results**

As ICE and the lymphocyte granzymes are both crucial mediators of apoptosis, we investigated whether these proteases recognize and cleave common substrates. Potential cleavage of in vitro–translated human pIL-1β by granzyme A or B was investigated. As expected, the 31-kD precursor form of IL-1β was efficiently cleaved by ICE after a 10-h incubation into a fragment corresponding to mature IL-1β (Fig. 1 A). In spite of the shared substrate specificity of granzyme B and ICE, no cleavage of pIL-1β was observed with the former enzyme. However, incubation with purified granzyme A generated a fragment similar in size to the authentic 17-kD IL-1β. Cleavage of pIL-1β by granzyme A was rapid and already observed after 30 min (data not shown). The granzyme A-induced pIL-1β conversion was completely blocked by serine protease inhibitor DCI, whereas the ICE inhibitor YVA was inactive (Fig. 1 A). In turn, ICE activity was completely inhibited by YVA at identical concentrations (Fig. 1 A).

Efficient cleavage of pIL-1β by granzyme A was also observed when recombinant human pIL-1β produced in bacteria was used as a substrate (Fig. 1 B). Recombinant pIL-1β gave rise to two bands at ~31 kD by Western blot analysis, which were converted to two species of 16–17 kD upon addition of granzyme A. Both processed species showed an identical NH2 terminus, indicating that the recombinant protein was degraded at its COOH terminus. The conversion was again inhibited with DCI and IGA. Granzyme B had no effect on pIL-1β.

ICE cleaves between Asp116 and Ala117 of human pIL-1β to release the active cytokine. Sequence analysis of granzyme A-converted pIL-1β revealed Ser117 at its NH2 terminus, indicating that pIL-1β was cleaved after Arg120, in perfect agreement with its thrombinlike activity (Fig. 2 A). Three minor contaminants were found with NH2 termini corresponding to Leu144, Ser114, and Ala114. The Arg120–Ser117 cleavage site was also obtained when a synthetic peptide covering the ICE pIL-1β cleavage site (NEAYVHDAVRSLN) was offered to granzyme A (Fig. 2 B). In spite of comparable esterolytic activity on their respective substrates (BLT and BAT), in no case was cleavage detected after the authentic Asp116 by granzyme B.
We next asked whether this granzyme A–generated 17-kD fragment of IL-1β had any significant IL-1β biological activity. We have previously shown that, in an EL-4 T helper clone, EL-4–6.1, IL-2 receptor surface expression was strictly dependent on the presence of picogram quantities of IL-1β (19). Precursor 31 kD IL-1β had no demonstrable activity in this assay (Table 1), as demonstrated by others previously (22). Granzyme A alone was also inactive. FACS® analysis revealed induction of IL-2 receptor surface expression only when pIL-1β incubated with proteolytically active granzyme A was added. Compared to the recombinant human IL-1β, the activity was approximately three- to fourfold lower.

pIL-1β is synthesized as a cytosolic protein without signal peptide. Although ICE is a cytoplasmic protein, processed pIL-1β is not observed to be associated with intact cells but is only generated and released when cells are undergoing apoptosis (23). Only when rapid apoptosis of macrophages is induced by CTLs or ATP is intracellular conversion of pIL-1β observed (23). To evaluate whether this intracellular processing is due to granzyme A, LPS-activated macrophages were used as targets for the cytolytic T cells. The CTL clone used recognizes a peptide derived from the P. falciparum protein circumsporozoite in the context of the H2d class I antigen. When the conversion of pIL-1β was analyzed in macrophages during CTL attack, the anti-IL-1β antibodies detected a faint, cell-associated band of 17 kD in addition to the 31-kD precursor, thus confirming results by Hogquist et al. (23) showing that intracellular pIL-1β cleavage was occurring during the cytolytic attack. Processed intracellular pIL-1β was not present in cells not previously sensitized with the peptide (Fig. 3). When CTLs were pretreated for 30 min with IGA, intracellular processing of pIL-1β was abolished, suggesting that granzyme A may be involved in the rapid conversion of pIL-1β (Fig. 3).

Table 1. Activities of IL-1β

<table>
<thead>
<tr>
<th>Sample</th>
<th>Relative activity %</th>
</tr>
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<tbody>
<tr>
<td>Mature IL-1β (17 kD)</td>
<td>100</td>
</tr>
<tr>
<td>pIL-1β (31 kD)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ Granzyme A</td>
<td>30</td>
</tr>
<tr>
<td>pIL-1β (31 kD) + DCI</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ Granzyme A + DCI</td>
<td></td>
</tr>
<tr>
<td>pIL-1β (31 kD) + IGA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>+ Granzyme A + IGA</td>
<td></td>
</tr>
<tr>
<td>+ Granzyme A</td>
<td>&lt;1</td>
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</tbody>
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IL-1β activities were determined by the EL-4 assay described previously (19). The obtained activities were normalized to the activity of recombinant human IL-1β, which was arbitrarily set to 100%.

Discussion

Our results show that, although both granzyme B and ICE exhibit aspase activity and are crucial mediators of apoptotic cell death, they do not act on identical cellular target proteins. pIL-1β, the only known substrate for ICE, is resistant to the proteolytic action of granzyme B. Even when the ICE pIL-1β cleavage site is offered as a 14-mer peptide, no cleavage after the critical Asp residue is observed. These observations indicate that the presence of an Asp residue alone may not be solely sufficient for the efficient processing of pIL-1β at the Asp116–Val117, and other structural constraints may be required. Today no proteinaceous substrate for granzyme A has been reported.
zyme B has been found, in spite of its known high estero-
lytic activity on synthetic thioester substrates. The absence
of pIL-1β processing by granzyme B cannot exclude, how-
ever, the possibility that other proteins, such as those acti-
vated during apoptosis, are substrates shared by ICE and granzyme B.

Unlike granzyme B, the second lymphocyte protease
granzyme A expressed in vivo can generate an active IL-1β from
the inactive precursor form. Cleavage occurs between Arg120
and Ser121, yielding a cytokine four amino acids smaller in
length than the one generated by ICE. Activity of the IL-1β
diminishes rapidly as the cleavage site moves away from the
genuine Asp16 cleavage site. Mast cell chymase and chymo-
trypsin generate molecules that are two and three residues
longer with no change in activity (24). Trypsin and elastase,
in contrast, generate polypeptides that are 13 and 41 amino
residues longer than authentic IL-1β but result in almost complete
loss of activity (22).

The biological activity of the granzyme A–processed pIL-1β
was decreased relative to the recombinant one, in agreement
with the reduction of activity observed when Arg120 is re-
moved or replaced by Asp (25), although structure–activity
studies with truncated proteins expressed in COS cells have
shown that IL-1β retains full functional activity even when
shortened to Met136 (26). By contrast, using in vitro–trans-
lated IL-1β, removal of Arg120 resulted in decreased receptor
binding (27).

Precursor IL-1β is predominantly found in intact cells. In
cells undergoing apoptosis, processing by ICE results in the
release of the active cytokine, in contrast to necrosis, in which
IL-1β is released almost exclusively in the precursor form (23).
Extracellular proteases such as mast cell proteases may then
be expected to cleave the 31-kD precursor IL-1β in areas of
necrotic damage and thereby to release substantial amounts
of IL-1β. Intracellular processing of pIL-1β, however, has
been observed in macrophages that are attacked by CTLs (23).
CTLs cause rapid apoptosis induced by the action of gran-
zyme B, granzyme A, and Fas ligand, a membrane protein
with structural homology to TNF (15, 28, 29). Granzymes
are believed to enter the target cell and act on intracellular
substrates whose cleavage leads to apoptosis (30). This may
account for the rapid intracellular processing of pIL-1β. In-
deed, treatment of CTLs with a granzyme A inhibitor abol-
ished intracellular conversion of pIL-1β in the target cell. Al-
though the granzyme A inhibitor displays high specificity
for granzyme A, it also blocks other proteases with similar
specificity, such as thrombin (our own unpublished data).
Whether the absence of intracellular conversion is due to the
inactivation of granzyme A or to other factors remains there-
fore to be shown.

This lymphocyte protease–generated IL-1β is interesting
from the perspective that some cells known to synthesize
pIL-1β lack the corresponding converting enzyme (31). For
example, fibroblasts and keratinocytes have been shown to
produce pIL-1β mRNA but lack IL-1β cytokine activity (31,
32). Other secreted proteases may also play a role in processing
precursor IL-1β. Inflammatory responses of diverse origins
may, in turn, be a consequence of this process. Mast cell
chymase generates an active cytokine and is proposed to play
a critical role in the initiation of the inflammatory response
in the skin (31). Cathepsin G, which also displays pIL-1β-
converting activity, is present in synovial fluids and lung la-
vage from patients with inflammatory polyarthritis (32). A
streptococcus-derived cysteine protease produces biologically
active IL-1β, indicating that bacteria are able to modulate the
inflammatory response during pathogenesis (33). The gran-
zyme A–generated IL-1β may directly contribute to the inflam-
atory response in areas of lymphocyte tissue infiltration.
IL-1β may recruit T cells to the site of antigenic challenge
caused by viruses and parasites and, in turn, activate Th lym-
phocytes and CTLs whose efficient activation is dependent
on the IL-1β costimulatory signal. This would lead to mac-
rophage and neutrophil sequestration to remove cellular mem-
brane debris caused by CTL action. In an effort to support
this model, others have attempted to identify the NH2 ter-
minal of IL-1β at inflammatory sites to determine the pro-
cessing site, but, because of the low concentration of IL-1β
present at inflammatory sites or as a result of proteolytic degra-
dation, they have been unsuccessful to date (32). The obser-
vation that precursor IL-1β may be processed in vivo by pro-
teases other than ICE may have important implications in the
future development of therapeutic approaches. In partic-
ular, drugs currently developed to inhibit the pIL-1β pro-
cessing in monocytes by interfering with ICE activity may
be unable to inhibit IL-1β production in vivo.

The authors gratefully acknowledge A. Allegrini, S. Aslan, and A. L. Quipnerez for technical assistance,
and E. Magnenat for protein sequencing. We thank Dr. C. Kamel for helpful comments.
References


