A novel apoptosis cascade mediated by lysosomal lactoferrin and its participation in hepatocyte apoptosis induced by D-galactosamine

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Abstract A new apoptosis cascade mediated by lysosomal lactoferrin was found in apoptotic liver induced by D-galactosamine. Caspase-3 and lactoferrin were increased in the apoptotic liver cytoplasm and serum transaminases were elevated. Recombinant lactoferrin stimulated procaspase-3 processing at 10⁻⁶–10⁻⁷ M to an extent similar to that by granzyme B in vitro. Lactoferrin changed procaspase-3 structure susceptible to the processing. Synthetic peptide Y⁶⁷⁹-K⁶⁹⁵ in lactoferrin molecule inhibited the processing of procaspase-3 by lactoferrin. Lactoferrin in lysosomes was decreased and lactoferrin released into cytoplasm was increased quantitatively in D-galactosamine induced apoptotic liver, and procaspase-3 in cytoplasm was processed to caspase-3.

Keywords: Apoptosis; Procaspase-3; Lactoferrin; Processing; Lysosome; D-Galactosamine

1. Introduction

Many apoptosis cascades have been reported, and mitochondrial factor mediated apoptosis cascades have been well established. Caspase-3 plays a central role in various apoptosis cascades as an executive enzyme [1–4]. In 1998, we found that an unknown protein extracted from lysosomes by digitonin enhanced procaspase-3 processing in liver cytoplasm [5,6]. After that, we determined that the activating factor was lactoferrin and suggested preliminary the existence of a new apoptosis cascade mediated by lysosomal lactoferrin [7]. This paper reports on the stimulation mechanisms of procaspase-3 processing by lactoferrin at the enzymological aspects in detail and the releasing mechanism of lysosomal lactoferrin into cytoplasm in D-galactosamine-induced apoptotic hepatocyte. The pathological aspects of severe liver injury induced by D-galactosamine have been well characterized by TUNEL staining and also DNA fragmentation [9–11]. We reported in a previous paper that caspase-3 in the cytoplasm of apoptosis liver induced by D-galactosamine was increased and the apoptosis was protected by epigallocatechin gallate in green tea which inhibited caspase-3 [11].

As the next step, we studied the molecular mechanism underlying increases in the activity of activated caspase-3 in the cytoplasm in vivo and found that a new procaspase-3 activating protein was released into the cytoplasm from lysosomes in D-galactosamine induced apoptotic hepatocytes. We determined that this activating protein was a lactoferrin originally located in lysosomes. Recombinant pure lactoferrin was found to strongly stimulate procaspase-3 processing to form active caspase-3 as same extent to that by granzyme B in vitro, and the activation mechanisms were studied at the molecular level. We reported that releasing mechanism of lysosomal lactoferrin into the cytoplasm in D-galactosamine induced apoptotic rat liver in vivo.

This paper reports on (1) stimulation mechanism of procaspase-3 processing by lactoferrin in vitro; (2) the mechanism by which lysosomal lactoferrin is released into the cytoplasm after D-galactosamine administration in vivo; (3) the existence of a novel apoptosis cascade mediated by lysosomal lactoferrin.

2. Materials and methods

2.1. Chemicals used

Caspase-3, procaspase-3, lipopolysaccharide (LPS), and lactoferrin were all recombinant pure proteins purchased from Sigma Co. (USA). The 17 residue peptide Y⁶⁷⁹-K⁶⁹⁵ (YEYKLGPQYVA-GITNLK) of the lactoferrin molecule was chemically synthesized by Asahi Techno-glass (Chiba, Japan) with 95% purity. Aspartylglutamylvalinylaspartyl-7-aminotrifluoromethylcoumarin (DEVD-AFC) was purchased from Peptide Institute Inc., Osaka, Japan.

2.2. Assay of procaspase-3 processing activity

Caspase-3 activity derived from procaspase-3 was determined from the fluorescence of AFC released from DEVD-AFC [5,11], and the enzyme activity was expressed in terms of AFC released in nmol/h/µg protein or nmol/h [5,6,11]. The DEVD-AFC cleavage reaction catalyzed by caspase-3 and procaspase-3 processing reaction had the same optimum pH. The rate of fluorescent AFC production from the substrate DEVD-AFC by active caspase-3 was much faster than the rate of processing reaction of procaspase-3 at the optimum pH of 7.5. AFC production could be expressed as the rate of procaspase-3 processing, because procaspase-3 processing is the rate-determining step. Negative staining of SDS-polyacrylamide gel electrophoresis (PAGE)
for the activator sample was performed basically according to the method of Fernandez et al. [12]. The processing activities were assayed in the 78-kDa band eluents after removing SDS with renaturing buffer.

2.3. Detection of procaspase-3 activating protein using fluorescent reverse zymography

Our new double layer fluorescent reverse zymographic method was used for the detection of procaspase-3 activating proteins [21]. The activator sample was applied to a 15% polyacrylamide gel copolymerized with DEVD-AFC as the caspase-3 substrate, and the electrophoresis was performed at 13 mA for 120 min. After removing SDS from the gel with the renaturing buffer (2.5% Triton X-100), the gel was incubated with 100 μM procaspase-3 solution at 37°C for 30 min. The fluorescent AFC band formed by caspase-3 was detected using a UV-transilluminator [13].

2.4. Determination of amino acid sequence of the activating protein of 78-kDa

The intramolecular sequences of the activating protein isolated from the Zn-negative staining SDS-PAGE was determined using an HP G1005A protein sequencing system according to the Majima’s method [14]. The protein in the 78-kDa band was digested with lysyl-endopeptidase and the peptide fragments were separated with reverse-phase HPLC on a TSK gel ODS-120 T column (Tosoh) with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid. The amino acid sequences of the main separated peptides were also determined using Majima’s method [14].

2.5. Confocal immunohistochemical analysis of lactoferrin located in liver lysosomes

Confocal immunofluorescence analysis was performed on liver sections from C57BL/6 mice using FITC-labeled anti-lactoferrin pAb (Cappel, Turnhout, Belgium) and PE-labeled anti-Lamp-1 mAb (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

2.6. Method of administration of d-galactosamine with LPS in vivo

Rat liver apoptosis was induced by d-galactosamine treatment using Muntane’s method [8] by the intraperitoneal injection of d-galactosamine 0.5 g/kg or d-galactosamine 0.5 mg/kg plus LPS 50 μg/kg. Twelve hours after the injection, the rats were sacrificed to prepare the livers.

2.7. Preparation of lysosomes and cytoplasmic fraction of rat liver

The rat liver was gently homogenized with a Teflon pestle in 0.25 M sucrose-0.01 M Tris pH 8.0. The lysosomal and the cytoplasm were observed in DD-galactosamine dose-dependently. Data are shown as the means ± S.D.

2.8. Sample preparation for activity assay of the lysosomal procaspase-3 activating protein

The ML fractions of rat liver were extracted with 5 mL of digitonin solution. After concentration to 1 mL, 30 μL portion of the extract was applied to SDS gel to make SDS–PAGE. After removing the SDS, the 78-kDa fractions were eluted from the gel for determination of procaspase-3 activating activity or assay of the lactoferrin protein by the antibody. The activating activities were expressed as the AFC released nM/h/mg protein (cytoplasmic protein, lysosomal protein or SDS–PAGE band protein).

2.9. Statistical analysis

Values for expression are shown as means ± S.D. Quantitative differences between values were statistically analyzed by Dunnett’s multiple comparison t test P values <0.01 were considered to be significant.

3. Results and discussion

3.1. Detection of procaspase-3 activating protein in the cytoplasm of d-galactosamine treated apoptotic liver cells

When d-galactosamine was administered intraperitoneally to rats, caspase-3 activity in the liver cytoplasm was elevated in a dose-dependent manner 12 h after injection and cotreatment with LPS enhanced the apoptosis dramatically, as shown in Table 1. The liver injury markers, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were strongly elevated in the serum. Therefore, the increases of caspase-3 may participate in d-galactosamine induced liver injury [11].

In order to elucidate the mechanism of caspase-3 elevation during the apoptosis, the activation factor of procaspase-3 in the treated liver cytoplasm was determined by the following procedure. Same amounts of cytoplasmic fraction and lysosomal lactoferrin were observed in d-galactosamine dose-dependently. Data are shown as the means ± S.D.: n = 3–5, * P < 0.01.

### Table 1

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Enzymes</th>
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<td></td>
<td>In liver cytoplasm</td>
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<tr>
<td></td>
<td>Caspase-3 (AFC nM/mg/hr)</td>
</tr>
<tr>
<td>Normal</td>
<td>76.50 ± 9.42</td>
</tr>
<tr>
<td>d-GalN(300 mg/kg)</td>
<td>1000.0 ± 360</td>
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<tr>
<td>d-GalN(500 mg/kg) + LPS(50 μg/kg)</td>
<td>5159.73 ± 1250.37</td>
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0.5 g/kg of the d-galactosamine plus 50 mg/kg of LPS was intraperitoneally injected to rats, and the 12 h after the injection, the rats were sacrificed. The procaspase-3 activating activities (lactoferrin) translocated from the lysosomes into the cytoplasm in the apoptotic hepatocytes were assayed using the method as follows. The quantitative amounts of the digitonin extracts of the lysosomes or the cytoplasm preparations were applied to make SDS–PAGE, and the procaspase-3 activating activities in the 78-kDa fractions and those in the 35-kDa fractions from the lysosomes, and also those from the cytoplasm were assayed at the same time. Furthermore, these activating protein amounts in the cytoplasm corresponded to the released lactoferrin protein amounts using anti-lactoferrin antibody [18]. The activating activities were expressed as the AFC released nM/mg protein in the extracts of cytoplasmic protein or lysosomal protein. The reciprocal movements of the lactoferrin (procaspase-3 processing activities) in the lysosomes and the cytoplasm were observed in d-galactosamine dose-dependently.
somal extract of liver were applied separately to SDS gels and electrophoresis was performed. After removing the SDS from the PAGE with renaturing buffer, the procaspase-3 processing activities in the eluents were detected in the 78-kDa fraction and the 35-kDa fraction using caspase-3 formation from recombinant procaspase-3. The procaspase-3 activating protein in the 78-kDa fraction was increased in the cytoplasm and the activity in the lysosomes was decreased in a reciprocal manner, as shown in Table 1. Sufficient amounts of procaspase-3 were present in the normal cytoplasm, but active caspase-3 was not detected in the normal cytoplasm. These data suggest that the procaspase-3 activating protein is originally located in the lysosomes under normal conditions and that only the 78-kDa activating protein was released into the cytoplasm by d-galactosamine administration.

We then purified the 78-kDa activating protein from the liver lysosomes to allow identification. The partially purified activating protein from the digitonin extracts of liver lysosomes was applied to SDS gels to separate the proteins. Two kinds of activating proteins with molecular weights of 78-kDa and 35-kDa were detected using fluorescent reverse zymography for processing protease detection [13,21], as shown in Fig. 1(a) in lane 3. The 78-kDa band corresponds to recombinant lactoferrin, and the 35-kDa band is an unknown activating protein. The 35-kDa factor was not detected in the cytoplasm of the apoptotic liver, therefore, the 35-kDa factor does not participate in the apoptosis.

3.2. Purification and identification of the 78-kDa lysosomal activating protein as lactoferrin

A procaspase-3 activating protein was extracted with a 40 μM digitonin isotonic sucrose buffer from the purified lysosomes of bovine liver. The specific activity of the procaspase-3 processing enzyme in the digitonin extracts was 2.5 nmol AFC/h/μg protein (formed from DEVD-AFC). The extracted protein was fractionated with 40–60% ammonium sulfate and then heat treated at 70°C for 1 min. The supernatant was further fractionated with 40–50% ethanol at −20°C. The fraction was subsequently subjected to column chromatography using Superdex G75, Mono Q and then Hydroxyapatite CHT5-I BIO-RAD. The specific activity of the final active fraction was about 100 nmol AFC/h/μg protein. The purified sample had about the same activating activity as that of the recombinant pure lactoferrin. The purified fraction showed almost a single protein on the SDS-PAGE.

The intramolecular amino acid sequence of the purified 78-kDa activating protein eluted from the negative zinc staining band in the SDS–PAGE was determined [12]. The sample was hydrolyzed with lysyl-endopeptidase and the peptides produced were separated using reversed-phase HPLC. The amino acid sequences of the two different parts of the separated peptides were determined. The amino acid sequences of these two peptides were completely identical with those of the corresponding parts of recombinant bovine lactoferrin. The amino acid sequence of the purified 78-kDa activating protein was identical with that of lactoferrin.
acid sequences of these two domains from the purified sample were H\textsubscript{435}-P\textsubscript{444} (n-H-S-S-L-D-C-V-L-R-P-c) and N\textsubscript{653}-E\textsubscript{661} (n-N-L-L-F-N-D-N-T-E-c). The molecular weight (78-kDa) and the isoelectric point (5.4–5.7) of the purified activating protein were the same as those of the recombinant bovine lactoferrin. The processing product of procaspase-3 by the 78-kDa protein was the same as that by recombinant lactoferrin as demonstrated in Fig. 1(b). The same processing products of procaspase-3 by granzyme B were demonstrated.

3.3. Subcellular localization of lactoferrin in liver lysosomes
A two-phase Ficoll partition centrifugation method in 1 mM CaCl\textsubscript{2} was used to determine the subcellular localization of the activating protein in rat liver [15]. The activating activity was detected only in the lysosomal fraction, coinciding with the location of cathepsin L, while the activity was not detected in the swollen mitochondrial fraction, coinciding with glutamic dehydrogenase (data not shown). The confocal immunohistochemical staining of mouse liver, using a monoclonal anti-lactoferrin antibody and a PF-labeled anti-Lamp-1 antibody as lysosomal markers, was used to determine the subcellular localization of the lactoferrin (see Section 2). The lactoferrin was detected only in the lysosomal particles located in the cell membrane area, as Fig. 2 shows.

3.4. Activation mechanism of procaspase-3 by lactoferrin
The activation mode of the procaspase-3 processing reaction mediated by pure lactoferrin was analyzed. Procaspase-3 was originally processed slowly via autocatalytic reaction in Tris–HCl buffer at pH 7.5 and 37 °C in vitro, and \(1 \times 10^{-7}\) M lactoferrin strongly accelerated the autocatalytic processing to several-fold, as shown in Fig. 3(a). The procaspase-3 processing rate mediated by \(1 \times 10^{-6}\) M lactoferrin was about 7000–8000 nM AFC formed per hour, while the autocatalytic rate was about 1000–1600 nM AFC formed per hour. Specific activity of procaspase-3 processing reaction catalyzed by lactoferrin was 432 nM AFC formed/h/mg protein, while that by granzyme B was 515 nM AFC formed/h/mg. Both catalytic activities showed about the same level. The lactoferrin mediated procaspase-3 activating reaction was not inhibited by various cysteine protease inhibitors, including E-64 or serine protease inhibitors, and anti-granzyme B antibody also did not inhibit the activation reaction (data abbreviated). Since both apo-lactoferrin and holo-lactoferrin had the same activation function and \(1 \times 10^{-7}\) M lactoferrin showed the enough activation (Fig. 3(b)), the iron atom itself and the domains of the iron atom binding did not participate in this activation reaction. The processing products of recombinant procaspase-3 mediated by lactoferrin were the same as those mediated by granzyme B by Western blotting using the anti-caspase-3 antibody as shown in Fig. 1(b).

With regard to the activating mechanism of procaspase-3 processing by the lactoferrin, a lactoferrin–procaspase-3 complex may be formed as an intermediate step. The lactoferrin may play a chaperone-like role to alter the tertiary structure of the procaspase-3 and render it more susceptible to being processed. The binding affinity for making the complex was shown in Fig. 3(c), and the \(K_m\) is about \(1 \times 10^{-11}\) M. The lactoferrin did not have any effect on the caspase-3 assay reaction. The domain which participates in the binding of procaspase-3 to the lactoferrin molecule was estimated to be Y\textsubscript{679}-K\textsubscript{695} (YE-KYLGPQYVAGITNLK) in lactoferrin. We reported previously that this domain was an inhibitory site of the

![Fig. 2. Subcellular localization of lactoferrin in rat liver using confocal immunohistochemical staining. (a) Hematoxylin-eosin staining of hepatocytes. (b) Lactoferrin localization in lysosomes using an anti-lactoferrin antibody in green. (c) Lysosome staining by PF-labeled anti-Lamp-1 antibody in red. (d) Merged profile of lactoferrin and lysosomal marker Lamp-1. The lactoferrin was stained in the lysosomes located in the cell membrane area.](image-url)
lactoferrin for cysteine proteases [16]. This domain is highly homologous with a common active site of the cystatin family. The synthetic peptide Y_679-K_695 strongly inhibited not only the activation reaction of the processing mediated by lactoferrin, but also the autocatalytic processing reaction, as shown in Fig. 4. This peptide may disturb the binding of procaspase-3.

Fig. 3. Activating mechanisms of procaspase-3 processing reaction mediated by lactoferrin. The procaspase-3 processing activity was assayed by the formed caspase-3 activity and the formed caspase-3 activities from procaspase-3 are expressed as AFC formed nM/h in the vertical axis. Panel (a): Reaction time course of procaspase-3 processing with lactoferrin. Panel (b): Dose-dependent activations of the procaspase-3 processing reaction mediated by holo-lactoferrin or apo-lactoferrin. Apo-lactoferrin was prepared from holo-lactoferrin by the treatments at pH 2.0. The iron atoms were released into the supernatant and no iron atoms were detected in the precipitated lactoferrin. Panel (c): Kinetical studies of the affinity of procaspase-3 for lactoferrin and the $K_m$ value.
to lactoferrin. Therefore, the domain Y_{679-K_{695}} of the lactoferrin may play an important role in the acceleration function by participating in the binding of procaspase-3 to lactoferrin. To explain the practical allosteric structural changes, the X-ray co-crystallographic analysis of these complexes is required. We previously reported a similar type of chaperone-like functioning protein, a chondroitin-sulfate proteoglycan, which is a potent enhancer of the autoprocessing of procathepsin L to form the active mature cathepsin L [17]. We propose that these kinds of specific accelerator proteins, or “enzymoids”, may participate in various post-translational processing reactions in general.

3.5. A new apoptosis cascade mediated by lysosomal lactoferrin

To confirm the translocation of the lactoferrin, the lactoferrin released in the cytoplasm was assayed quantitatively with antibodies to lactoferrin using Sanchez’s method [18]. The lactoferrin released in the cytoplasm increased to 7 μg/mL of cytoplasm upon treatment with 700 mg/kg D-galactosamine, while no lactoferrin was detected in the normal liver cytoplasm. It is possible to consider that the lactoferrin located in the lysosomes was released into the cytoplasm by D-galactosamine administration dose-dependently in vivo as shown in Table 1, although the releasing mechanisms are not known at the present. The released procaspase-3 activating activity in

Fig. 4. Inhibition profiles of procaspase-3 processing reaction mediated by lactoferrin by synthetic peptide, Y_{679-K_{695}}, in the domain of lactoferrin molecule. Lactoferrin concentration of 1 \times 10^{-6} M as an activator and synthetic peptide concentration of 1 \times 10^{-4} M as the inhibitor were used. The procaspase-3 processing reaction mediated by 1 \times 10^{-6} M lactoferrin (○-○) and inhibited by 1 \times 10^{-4} M of the peptide (●-●) are shown in this panel. Autocatalytic processing (□-□) and inhibition with 1 \times 10^{-4} M peptide (■-■) are also shown.

Fig. 5. Schematic illustration of a new apoptosis cascade mediated by lysosomal lactoferrin. Solid red line; the death signal transduction induced by D-galactosamine. Dotted blue line; a new apoptosis cascade mediated by lysosomal lactoferrin. LRF; unknown lactoferrin releasing factor.
the cytoplasm by d-galactosamine plus LPS treatment was 5000–6000 AFC nM/h, this activity is corresponding to the activity by $10^6$–$10^7$ M of lactoferrin (calculated by Fig. 3(b)). This amount (concentration) is enough to activate procaspase-3 in vitro. Sufficient amounts of procaspase-3 are located in the normal liver cytoplasm, but caspase-3 was not present in the normal cytoplasm. As a model experiment, the caspase-3 activity (DEVD-AFC cleaving activity) in a normal liver cytoplasm preparation was found to be strongly enhanced by the addition of recombinant lactoferrin in vitro. We reported in our previous papers that the caspase-3 activity in the normal liver cytoplasm was enhanced by the addition of digitonin extracts of lysosomes in vitro [5,6]. The releasing mechanism of lysosomal lactoferrin by amine treatment (data not shown). When a suitable death signal was added, the corresponding activating protein was translocated from the different organelles into the cytoplasm, resulting in increases in caspase-3 mediated apoptosis, in general [1]. The releasing mechanism of lysosomal lactoferrin by d-galactosamine treatment is not known. Quintero et al. reported that prostaglandin E1 protection against apoptosis induced by d-galactosamine is not related to the modulation of intracellular free radical production in primary culture of rat hepatocyte [19]. However, we have not any direct evidences on the releasing mechanisms of lysosomal lactoferrin into cytoplasm by d-galactosamine induced hepatocyte apoptosis. Fujita et al. reported that lactoferrin stimulated the apoptosis of azoxymethane-induced tumors and the elevation of active forms of caspases-3 and 8. But little is known about the mechanisms at the molecular level. The problem of this report is that the lactoferrin was administrated perorally with diet, it is difficult to consider that the 72-kDa lactoferrin is effectively absorbed from intestine [20]. The caspase-3 activity was dramatically elevated in the cytoplasm of d-galactosamine induced apoptotic hepatocytes and administration of epigallocatechin gallate which was strong inhibitor for caspase-3 activity suppressed the elevation of caspase-3 activity in the cytoplasm and protected the hepatocyte apoptosis [11]. Our proposed new apoptotic cascade mediated by lysosomal lactoferrin is illustrated schematically in Fig. 5.

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