Promotion of cathepsin L activity in newt spermatogonial apoptosis induced by prolactin

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Abstract We previously showed that prolactin (PRL) induces apoptosis in newt secondary spermatogonia and indicated that caspase activity is involved in the apoptosis. Since it was recently reported that Z-VAD-fmk, a pan-caspase inhibitor, blocks activity of cysteine cathepsins as well, we examined whether cathepsin is involved in the newt spermatogonial apoptosis. We found cathepsin L activity in the testis that was elevated by PRL in organ culture of testis, while E-64d, a lysosomal cysteine protease inhibitor, and Z-VAD-fmk suppressed it and chromosomal condensation. These results suggest that cathepsin L activity play a pivotal role in PRL-induced spermatogonial apoptosis. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cathepsin; Prolactin; Apoptosis; Spermatogenesis; Newt

1. Introduction

Germ cell death is a common feature during spermatogenesis [1] and occurs preferentially during certain developmental stages [2,3]. In some seasonally breeding animals, germ cell death is often observed at transition stages from spermatogonia to spermatocytes [4]. In the Japanese red-bellied newt, spermatogonial cell death is caused by the elevation of plasma prolactin (PRL) concentrations following transfer of animals to low temperatures [5]. This finding is consistent with the observation in nature that spermatocytogenesis ceases in late autumn when the environmental temperature goes down [5]. We demonstrated in vitro that PRL induces spermatogonial apoptosis by direct action on newt testis [6].

There are several reports indicating that members of the caspase family, known as apoptotic executioners, play key roles in germ cell apoptosis during spermatogenesis [7–9]. In rat testis, caspase-3 expression is associated with spermatocyte apoptosis that results from a reduction of plasma testosterone concentrations [9]. Suppression of caspase activities by Z-VAD-fmk, a broad-spectrum inhibitor, resulted in inhibition of apoptosis as assessed by the appearance of apoptotic nuclear morphology and oligonucleosomal DNA fragmentation, the hallmarks of apoptosis [11]. In newt testis, PRL induces elevation of caspase activity upon induction of spermatogonial apoptosis [10]. Treatment of the testis with Z-VAD-fmk inhibited PRL-induced caspase activity and morphological apoptotic changes in the spermatogonia [10]. Recently, however, it was reported that Z-VAD-fmk also efficiently blocks cathepsin B activity in vitro [12,13] and in WEHI164 cells [13] at concentrations commonly used to demonstrate involvement of caspases. From these results, it is assumed that Z-VAD-fmk inhibits apoptosis by blocking a variety of caspase and cathepsin activities.

In the current study, we measured cysteine cathepsin activities in newt testis and examined the effect of cathepsin inhibitor on PRL-induced spermatogonial apoptosis. We found that cathepsin L activity was elevated by PRL in organ culture of the testis and that treatment of the testis fragments with cathepsin inhibitors blocked the apoptotic changes of the spermatogonial nuclei. These results indicate that cathepsin participates in PRL-induced spermatogonial apoptotic events such as chromatin condensation and DNA fragmentation.

2. Materials and methods

2.1. Animals

Adult newts, *Cynops pyrrhogaster*, were purchased from a dealer (Hamamatsu Seibutsu Kyozai, Hamamatsu, Japan), kept at 22°C and fed frozen *Tubifex*.

2.2. Reagents

Carbobenzoxy-Phe-Arg-4-methyl-coumaryl-7-amide (Z-FR-MCA), carbobenzoxy-Arg-Arg-4-methyl-coumaryl-7-amide (Z-RR-MCA), carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk) and E-64d (epoxy-succinyl-L-leucylamido-3-methyl-butane ethyl ester) were purchased from the Peptide Institute (Osaka, Japan). All other chemicals were obtained from Nakalai Tesque, Kyoto, Japan.

2.3. Organ culture of testicular fragments

All operations were carried out in sterile conditions. The immature part of each testis containing a boundary region between the spermatogonia–spermatocyte stages was cut into 2 mm diameter fragments so that each fragment contained the boundary region. Five fragments were placed on a Nuclepore filter (pore size 0.2 μm; diameter, 25 mm; Coaster, Cambridge, MA, USA) that was floated on culture medium in a 35 mm plastic dish (Falcon, Lincoln Park, NJ, USA; #1008). The fragments were cultured at 22°C in humidified air. The basal culture medium consisted of Leibovitz L-15 medium supplemented with 10 mM HEPES, adjusted to pH 7.4 with 1 N NaOH. To induce apoptosis, testicular fragments were cultured in the presence of ovine PRL (5 μg/ml; Sigma, St. Louis, MO, USA). E-64d was dissolved in DMSO (100 mM stock solution) and aliquots were added to the culture medium to obtain final concentration of 100 μM. Incubation with 100 μM E-64d was started 1 h before addition of PRL and continued throughout the culture period.

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2.4. Histology

Five fragments from each culture dish were fixed with the filter in Bouin's solution after culture. The samples were dehydrated in an ethanol series and embedded in a block of paraffin (paraplast plus tissue embedding medium, Oxford Labware, St. Louis, MO, USA). The blocks were sectioned serially (5 μm) and stained according to the Delafield hematoxylin-eosin method.

2.5. Measurement of cathepsin activity

The preparation of testis lysates was performed by the method of Yazawa et al. [10]. Enzymatic activity of the lysate was measured by using 20 μM Z-FR-MCA (for cathepsin L) and Z-RR-MCA (for cathepsin B) as substrates. Assays were performed at 25°C in 100 mM sodium acetate buffer (pH 4.5) containing 10 mM DTT and 2 mM EDTA. Substrate hydrolysis was continuously monitored at intervals of 3 min during a 30 min period in a fluorometer at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Fluorescence measurements were carried out on a Tecan Spectrafluor Plus (Wako, Osaka, Japan). For inhibition assays, the reaction mixture was preincubated with 10 μM E-64d or Z-VAD-fmk at 25°C for 15 min. One unit of the enzyme activity was defined as a cleavage of 1 pmol of each substrate per minute.

2.6. Determination of DNA cleavage in situ by TUNEL

Testes were fixed in Bouin's solution and routinely processed for paraffin embedding and sectioning. The sections were treated with in situ apoptosis detection kit (Takara, Osaka, Japan) according to the manufacturer's instructions and counterstained with hematoxylin.

2.7. Statistics

The result was analyzed by using the Student's *t*-test. A probability level of <0.05 indicated a statistically significant difference.

3. Results and discussion

3.1. Cathepsin activity in extracts of newt testis

To determine the molecular species of cathepsin which may function in newt testis, we first measured proteolytic activities of cathepsins in extracts derived from the testes (Fig. 1). The testis extracts displayed significant proteolytic activity against a synthetic peptide, Z-FR-MCA, which is an optimal substrate for cathepsin L, while there was no proteolytic activity against Z-RR-MCA, a substrate for cathepsin B. These results indicate that cathepsin L activity is present in newt testis.

3.2. Elevation of cathepsin L activity by PRL

To investigate whether cathepsin activity is involved in PRL-induced apoptosis, testis fragments containing germ cells

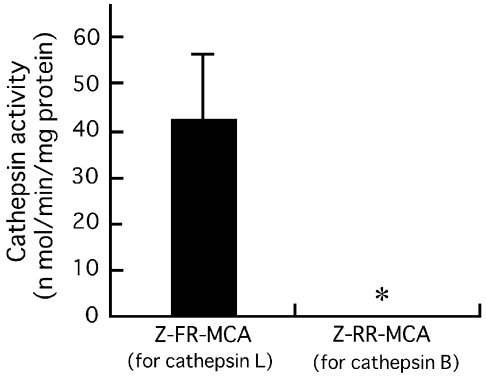

Fig. 1. Cathepsin activity in newt testis. Testis extracts were analyzed for proteolytic activity on Z-FR-MCA and Z-RR-MCA. Error bars represent mean ± S.E.M. from three independent experiments. An asterisk means that there was no detectable activity present.

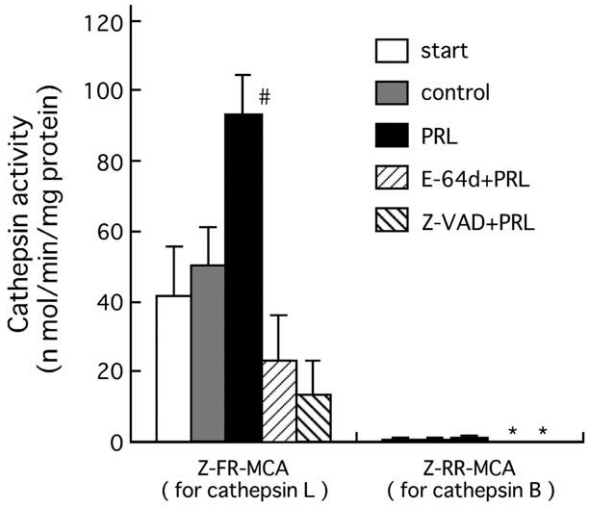

Fig. 2. Elevation of cathepsin activity by PRL and effect of peptide inhibitors on the activities. Testis fragments were cultured in the absence or presence of PRL for 48 h. Testis extracts were prepared and analyzed for proteolytic activity on Z-FR-MCA and Z-RR-MCA with and without E-64d or Z-VAD-fmk. Error bars represent mean ± S.E.M. from three independent experiments. An asterisk means that there was no detectable activity present. #Significantly different from the control values ($P<0.05$).

in spermatogonia and primary spermatocyte stages were cultured in the presence of PRL (5 μg/ml) for 48 h and cathepsin activity in the extracts was measured. Proteolytic activity against the synthetic peptide, Z-FR-MCA, increased about twofold compared to their initial one (Fig. 2). This activity was inhibited by E-64d, a lysosomal cysteine protease inhibitor. On the other hand, cathepsin B-like activity was scarcely induced (Fig. 2). These results indicate that PRL activates cathepsin L protease in newt testis.

Consistent with recent studies that Z-VAD-fmk potently blocks cathepsin B activity in vitro and in cells [12,13], Z-VAD-fmk inhibited the cathepsin L activity as E-64d did (Fig. 2). Previously, we showed that nuclear apoptosis of newt spermatogonia is completely inhibited by Z-VAD-fmk but not by Ac-DEVD-CHO (inhibitor for caspase-3/-7) nor Ac-YVAD-CHO (inhibitor for caspase-1/-4) [10]. Therefore, the inhibitory effect of Z-VAD-fmk on the spermatogonial apoptosis may be partly due to its cathepsin-inhibiting property.

3.3. Suppression of PRL-induced apoptosis by cathepsin inhibitor

In newt testis, PRL induced spermatogonial apoptosis specifically in the penultimate stage of the spermatogonia before entrance into meiosis (Fig. 3B) [6]. To examine the role of cathepsins in PRL-induced spermatogonial apoptosis, testis fragments were treated with PRL+E-64d for 48 h, and then the nuclear morphological changes were observed; chromatin condensation of the spermatogonia was defective, though the nuclei lost spots of heterochromatin that are observed in normal spermatogonia, and looked homogeneous (Fig. 3C).

TUNEL labeling detects free 3′-OH ends and is widely used for detecting apoptotic cells. To examine whether cathepsin is involved in DNA fragmentation, testis fragments were treated with PRL+E-64d for 48 h, and TUNEL staining was performed. In the treated testis fragments, the nuclei of these cells were TUNEL-negative (Fig. 4B), whereas those in

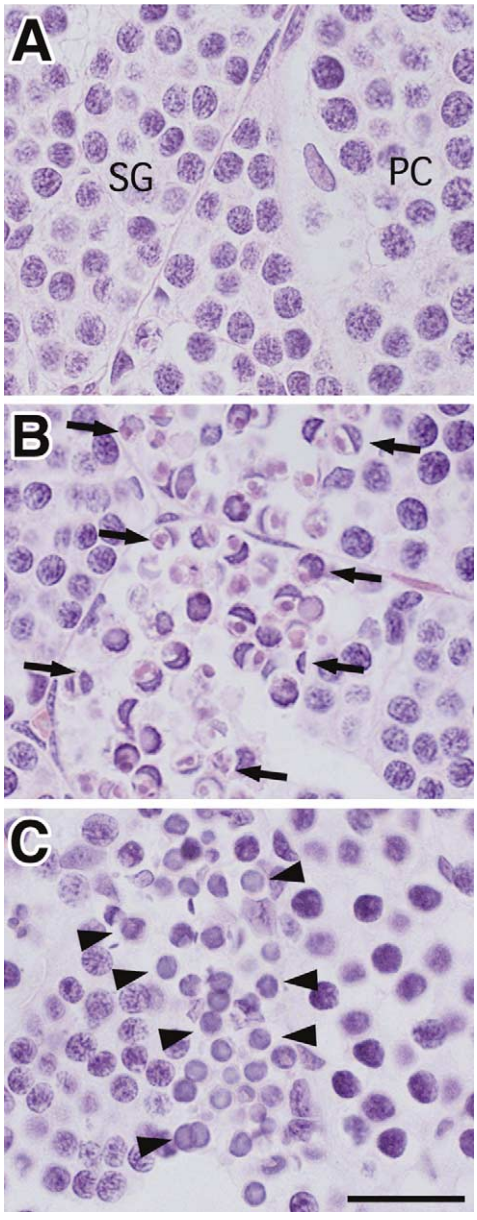

Fig. 3. Photomicrographs of hematoxylin-eosin stained sections of testis cultured in basal medium (A) and medium containing PRL (B) or PRL+E-64d (C) for 48 h. E-64d was added 1 h prior to the addition of PRL. PRL induced spermatogonial chromatin condensation (arrows). E-64d suppressed the PRL-induced chromatin condensation, but looked different from normal spermatogonia (arrowheads). SG = spermatogonia; PC = primary spermatocytes. Bar indicates 50 μm.

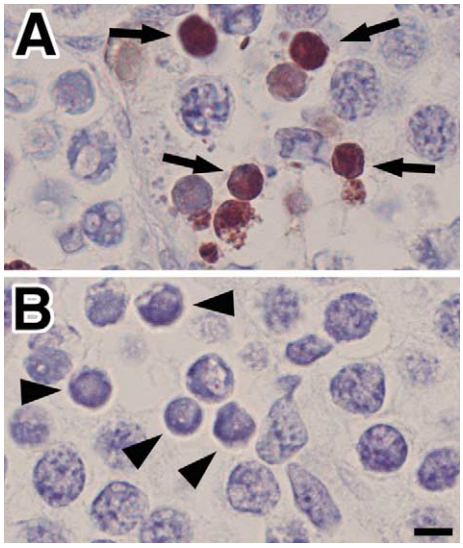

Fig. 4. Photomicrographs showing apoptosis in secondary spermatogonia detected by TUNEL methods. Testis fragments were cultured with PRL (A) or PRL+E-64d (B) for 48 h. E-64d was added 1 h prior to the addition of PRL. PRL induced apoptotic body formation (arrows) detected by TUNEL methods. Spermatogonia treated with E-64d were TUNEL-negative (arrowheads). Bar indicates 10 μm.

PRL-treated testis were positive (Fig. 4A). These results indicate that E-64d inhibits the PRL-induced chromatin condensation and DNA fragmentation in the spermatogonial apoptosis, suggesting that cathepsin is involved in chromatin condensation and DNA fragmentation during spermatogonial apoptosis.

Recent in vivo and in vitro studies suggest that cathepsins may function as mediators of apoptosis. Purified cathepsin B from rat liver lysosome activates caspase-11, and cathepsin B induces apoptotic chromatin condensation and DNA fragmentation in digitonin-permeabilized L929 cells [14], while cytosolic caspase-3 from rat liver is activated by purified cathepsin L [15]. Moreover, dihydrophenylalanine-induced apoptosis in HL-60 cells was dependent on the activation of lysosomal protease cathepsins B and L [16]. There are at least two steps in apoptotic chromatin condensation [17,18]: the first is a peripheral partial condensation that does not seem to require caspase activation. The second is a tight compaction of chromatin, which involves caspase activation. Several chromatin condensation factors have been identified. Among them, Acinus [19] and CAD [20] are known to be activated by caspases, and are likely to be involved in the second condensation step. However, it is not clear what factors are involved in the first step of chromatin condensation. In the current study, we showed that inhibition of cathepsin activities leads to blockade of the peripheral chromatin condensation. Therefore, this result may indicate that cysteine cathepsin is one of the factors required for the first step of chromatin condensation. However, further analysis is required to demonstrate whether caspase is involved in the second step of the chromatin condensation or whether cathepsin directly activates caspases in newt testis. On the other hand, as E-64d did not completely inhibit the morphological change of the spermatogonial nuclei, it is postulated that another factor exists for the first step of chromatin condensation upstream of cathepsin. Together with our previous result that treatment with Z-VAD-fmk can protect spermatogonia from PRL-induced apoptosis but not with other caspase inhibitors (for caspase-1, -2, -3, -7, -6, -9) nor mixtures of these [10], it is possible that this upstream factor may be a novel type of caspase or other protease(s) that are sensitive to Z-VAD-fmk.

In summary, we showed that cathepsin L-like activity was elevated during PRL-induced newt spermatogonial apoptosis, and that cathepsin inhibitor blocked the chromatin condensation and DNA fragmentation of apoptotic spermatogonia. Together these results suggest that cathepsin L activity plays

a pivotal role in PRL-induced spermatogonial apoptosis in newt testis. This is, as far as we know, the first report to demonstrate the involvement of cathepsin activity in the apoptosis of spermatogenic cells.

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