Caspase-1 activity is required for neuronal differentiation of PC12 cells: Cross-talk between the caspase and calpain systems ∗

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Abstract

Previously, we have found that caspase-1 activity is increased during myoblast differentiation to myotubes. Here we show that caspase-1 activity is required for PC12 differentiation to neuronal-like cells. Caspase-1 is shown to be activated (by immunoblotting and by assessing activity in cell extracts) in the PC12 cells following the initial stage of differentiation. The inhibition of caspase-1 arrests PC12 cells at an intermediate stage of differentiation and prevents neurite outgrowth in these cells; the inhibition is reversed upon the removal of the inhibitor. Calpastatin (calpain endogenous specific inhibitor, and a known caspase substrate) is diminished at the later stages of PC12 cell differentiation, and diminution is prevented by caspase-1 inhibition. The degradation of fodrin (a known caspase and calpain substrate) is found in the advanced stage of differentiation. Caspase-1 has been implicated in the activation of proinflammatory cytokines, and in cell apoptosis. The involvement of caspase-1 in two distinct differentiation processes (myoblast fusion and neuronal differentiation of PC12 cells) indicates a function for this caspase in differentiation processes, and suggests some common mechanisms underlying caspase roles in such processes.

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1. Introduction

Caspases have been mainly implicated in apoptosis [1,2], but may also play roles in processes not involving cell death [3–5]. The participation of caspases in nonapoptotic processes has been described in T lymphocyte activation [6–8], terminal differentiation and denucleation of erythrocyes, megakaryocytes and keratinocytes [3,9], differentiation of monocytes to macrophages [10], and skeletal muscle differentiation [11]. The caspases that appear to play a role in these processes belong to the subfamily involved in apoptosis, including initiators (caspase-2, -8, and-9) and effectors of cell death (caspase-3, -6, -7, and -14) [6–11]. A partial activation of the caspase apoptotic pathways has been assumed to be responsible for the participation of these caspases in nonapoptotic processes [3–5]. The other caspase subfamily, which includes caspases-1, -4, -5, -11, -12, and -13, has been implicated primarily in the activation of proinflammatory cytokines [3,12,13]. Little is known about roles of these caspases in noninflammatory, nonapoptotic events.

We have recently found that caspase-1 is required for myoblast fusion to multinucleated myotubes [14]. Myoblast differentiation and fusion to multinucleated fibers is involved in skeletal muscle formation and requires limited membrane protein degradation [15,16]. Calpain (Ca2+-dependent intracellular protease [17]) is involved in the

Abbreviations: Ac-DEVD-CMK, Ac-Asp-Glu-Val-Asp-CMK; BACMK, Boc-Asp(benzyl) chloromethylketone; DMSO, dimethylsulfoxide

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fusion-associated protein degradation [18–21]. In rat L8 myoblasts, we found that calpain levels did not change significantly during myoblast differentiation, whereas calpastatin diminished prior to myoblast fusion and reappeared after fusion [19,21–23]. The transient diminution in calpastatin in the differentiating myoblasts was found to be due to a transient activation of caspase-1 [14]. The inhibition of caspase-1 prevented calpastatin diminution and inhibited myoblast differentiation [14]. These results indicated an involvement of caspase-1 in myoblast differentiation, and raised the question of whether the activation of caspase-1 is limited to myoblast differentiation and fusion, or occurs in other types of cell differentiation.

PC12 cells, derived from rat pheochromocytoma, have been used extensively as a model for neural differentiation. The differentiation of PC12 cells (arrested proliferation and neurite outgrowth) can be achieved by treatment with the nerve growth factor (NGF) [24–26]. The involvement of the calpain–calpastatin system in PC12 cell differentiation has been described [27–29]. No information is available on the calpain–calpastatin system in PC12 cell differentiation has been described as a role for caspase-1 in neuronal cell differentiation. PC12 cells were grown in GM (0 h) or cultured in DM (NGF) (Alomone Labs, Jerusalem, Israel) (differentiation medium, DM). The time of cell replating in DM is defined as 0 h. The DM was replaced every 48 h.

To study the effects of protease inhibition, the following cell permeable inhibitors were used: the selective caspase-1 inhibitor BACMK [30]; the selective caspase-3 inhibitor Ac-DEVD-CMK [31]; the selective calpain inhibitor calpeptin [29,32] (Calbiochem, La Jolla, CA, USA). The inhibitors were dissolved in DMSO (5.0 mM stock solution for BACMK, 25 mM for Ac-DEVD-CMK, and 25 mM for calpeptin); they were added to the DM at 0 h at a final concentration of 5 μM BACMK, or 25 μM Ac-DEVD-CMK, or 25 μM calpeptin and replenished every 24 h. Some samples were treated with DMSO (at a final concentration of 0.1%), to check for any effect of the solvent on PC12 differentiation. Cell morphology was evaluated by confocal or phase contrast microscopy. Four experiments were carried out for the evaluation of BACMK-treated cells, and three experiments of calpeptin-treated cells as compared to morphology of control cells.

2.2. Preparation of cell extracts for SDS-PAGE, and immunoblotting analyses

PC12 cell lysates were prepared using 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 2.5 mM EGTA, and protease inhibitors cocktail set III (Calbiochem) (buffer C). Lysates were kept on ice for 30 min, centrifuged and aliquots of supernatants were mixed with Laemmli sample buffer for SDS-PAGE. The Laemmlli sample buffer consisted of 10% SDS, 0.32 M Tris–HCl, pH 6.8, 25% glycerol, 0.025 M EDTA, pH 7.4, 0.05 M dithiothreitol (DTT) and bromophenol blue. One volume of Laemmlli sample buffer was mixed with four volumes of cell lysate.

SDS-PAGE was carried out according to standard procedures (using 12%, 10% and 6.5% acrylamide for caspase, calpain/calpastatin and fodrin, respectively). Samples containing 20–40 μg of PC12 cell proteins were electrophoresed for the immunoblotting of various proteins (amounts were dependent on the antibody affinity, with the same amount of proteins loaded into each well of any single gel). Following electrophoresis, the samples were transferred to nitrocellulose membranes (Schleicher and Schuell). Immunoblotting was carried out as previously described [22], using polyclonal anti-caspase-1 antibody p20 (M-19):Sc-1218 (Santa Cruz Biototechnology, Santa Cruz, CA, USA) (1:500); monoclonal anti-μ-calpain antibody (1:1000); and polyclonal anti-m-calpain antibody (1:500) [22]; monoclonal anti-calpastatin antibody (R19):Sc-7561 (Santa Cruz) (1:500); monoclonal anti-β tubulin antibody (Sigma, St Louis, MI, USA) (1:1000); monoclonal anti-α-fodrin antibody (Affiniti Research Products, Mamhead, UK) (1:1000). The appropriate peroxidase-conjugated secondary antibodies were used, and the detection of bands was carried out with ECL (Pierce), as previously described [22]. Membranes were stripped off and reprobed with anti-β tubulin antibody for the estimation of loading. Bands were quantified by densitometry. Three independent experiments were carried out for the analysis of each protein.

2.3. Measurement of caspase-1 activity in PC12 cell extracts

PC12 cells were grown in GM (0 h) or cultured in DM for 48–120 h. Cells, harvested at 0, 48, 72, 96 and 120 h, were washed and lysed in 10 mM Tris–HCl, pH 7.4, 130
mM NaCl, 10 mM Na Pyrophosphate, 1% Triton X-100, 10 mM NaH2PO4/Na2HPO4, pH 7.4 (buffer A). Lysates (prepared from cells of four independent experiments) were kept on ice for 30 min, then centrifuged and the protein concentration in the supernatants measured (protein assay reagent kit, Pierce Biotechnology, Rockford, IL, USA). Supernatants were kept at −20 °C prior to the estimation of caspase activity. Caspase activity in the supernatants was estimated according to published methods [33] in 20 mM Hepes, pH 7.4, 2 mM dithiothreitol (DTT), 10% glycerol (buffer B), using the caspase-1 fluorogenic substrate Ac-YVAD-AMC (Alexis Biochemicals, Lausen, Switzerland), in the absence and presence of the selective caspase-1 inhibitor YVAD-CHO. Amc release was monitored at 460 nm (excitation at 380 nm), using an FL 600 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA).

2.4. Measurement of calpain activity in PC12 cell extracts

Calpain activity in the PC12 cells was assessed by determining the Ca2+-induced degradation of endogenous fodrin. PC12 cells, grown in GM (0 h), were homogenized in 50 mM Tris–HCl, pH 7.4, containing 1.0 mM EDTA and 0.1 mM PMSF. Aliquots (of two independent experiments) were incubated at 37 °C for 60 min with and without 1.0 mM Ca2+, and in the presence or absence of 2.0 mM EDTA; other aliquots were preincubated for 15 min with 10 μM of calpeptin prior to the addition of Ca2+. Samples were then mixed with Laemmli sample buffer, sonicated for 10 s, electrophoresed and processed for identification of fodrin by immunoblotting, as described above in Section 2.2.

3. Results

3.1. PC12 differentiation and effects of protease inhibitors

PC12 cells were cultured in DM, in the absence and presence of the protease inhibitors, as described in Material and methods. Under control conditions, PC12 cells exhibited the beginning of neurite growth at about 48 h. Neurite outgrowth and elongation in the differentiating cells continued to about 96–120 h of culture (Fig. 1A), with the cells maintaining this morphology for at least additional 72 h (not shown). Neurite growth was observed in about 60–80% of the cultured cells. DMSO (0.1%), used to solubilize the protease inhibitors used, did not affect the morphology of the cultured cells (not shown). To find out about the effects of caspase inhibition on PC12 cell differentiation, we used two cell penetrating caspase inhibitors, the selective caspase-1 inhibitor BACMK and the selective caspase-3 inhibitor Ac-DEVD-CMK. BACMK inhibited PC12 neurite outgrowth at a concentration of 5 μM; the cells appeared to be arrested at a stage corresponding to that shown by the control cells at about 48 h. Upon the removal of BACMK from the cultured cells, the inhibition was reversed and the cells proceeded to differentiate, with neurites observed after an additional 48–72 h (Fig. 1B), indicating that under the conditions used the inhibitor was not toxic to the cells. The caspase-3 inhibitor Ac-DEVD-CMK did not inhibit PC12 cell differentiation at a concentration of 25 μM (results not shown). The cells treated with the calpain inhibitor calpeptin at a concentration of 25 μM exhibited enhanced neurite growth in the initial and intermediate stages of differentiation, as compared to the control cells (Fig. 1C); the calpain inhibitor-treated cells appeared similar to the control cells at later stages of culture in DM (not shown).

3.2. Caspase-1 in differentiating and in inhibited PC12 cells

The activation of caspase-1 in the PC12 cells was assessed by immunoblotting and by measuring the activity of cell extracts on caspase substrates. Little, if any, caspase-1 activity was observed in dividing PC12 cells. In differentiating PC12 cells, the activation of caspase-1 was indicated by the presence of a band of 20–22 kDa [34], especially noted starting at 72 h of culture (Fig. 2A); little activation was observed in cells inhibited by BACMK, with activation shown following the removal of the inhibitor (Fig. 2B). The activity of caspase-1 in cell extracts increased during differentiation; the addition of the caspase-1 inhibitor Ac-YVAD-CHO to cell extracts resulted in the inhibition of the activity (Fig. 2C). These results indicate that caspase-1 is activated in the differentiating PC12 cells.

3.3. Calpain and calpastatin in PC12 cells during differentiation, and effects of inhibitors

Calpastatin protein levels increased during the initial stages of PC12 cell differentiation (up to about 48 h), then declined, as shown by immunoblotting (Fig. 3A). Means±S.E. for 48 h and 120 h were 149%±12.3 and 73%±6.2, respectively, with significant difference between each of them and the initial calpastatin levels at 0 h (P<0.015). Calpastatin diminution in differentiating cells was prevented in BACMK-treated cells (Fig. 3B) (means±S.E. were 67±4.7 for the control cells, and 102±5.2 for the BACMK-treated cells). Calpeptin did not prevent calpastatin diminution (not shown). The results indicate that following some increase in calpastatin levels in the initial phases of PC12 cell differentiation, the diminution of calpastatin at a later stage of differentiating PC12 cells is due to caspase activity, specifically caspase-1. No change was found in μ-calpain protein levels during the differentiation of PC12 cells (Fig. 4). Similarly, m-calpain level did not change during PC12 cell differentiation (not shown). These results are consistent with those of previous studies that showed no change in calpain protein levels [27] but diminution in calpain activity [29].
3.4. Protein degradation in differentiating and in protease-inhibited PC12 cells

Fodrin is a known substrate for both caspase and calpain, with the fodrin fragment of 150 kDa indicative of caspase and calpain activities, 145 kDa considered to be due to calpain activity and 120 kDa considered to be due to caspase activity [35]. Little fodrin degradation was observed in cells grown in GM. Fodrin fragments, including mainly 150/145 kDa and some 120 kDa, were observed at the later stages of differentiation (Fig. 5A). Significant diminution in the fodrin fragments of 150/145 kDa and 120 kDa was observed in the BACMK-inhibited cells (Fig. 5A). Following the removal of BACMK, fodrin degradation was similar to that observed in the control cells (Fig. 5B). Little fodrin degradation was observed in either control or calpeptin-treated cells grown in DM for 48 h (Fig. 6A). Diminished fodrin degradation to fragments of 150/145 kDa was observed at the later stages of differentiation in cells treated with calpeptin, with diminution noted also of a fragment of

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Fig. 1. PC12 cell differentiation. Cells were cultured in DM in the absence and presence of protease inhibitors, as described in Materials and methods. (A) Differentiation of control PC12 cells. Confocal microscopy (Nomarski). (B) Effect of the caspase-1 inhibition on PC12 cell differentiation. Cells were cultured in the absence and presence of 5 μM BACMK. Some BACMK-treated cultures were freed of BACMK by washing the dishes at 96 h of culture and culture continued for additional 72 h. Photographs are from one experiment, representative of four experiments. Cells photographed as in Panel A. (C) Effect of calpain inhibition on PC12 cell differentiation. Cells were cultured in the absence and presence of 25 μM of calpeptin. Photographs are from one experiment, representative of three experiments. Phase-contrast microscopy.
about 180 kDa, and of the 120 kDa band (Fig. 6A). In order to verify that fodrin degradation in PC12 cells can be induced by calpain, lysate was prepared from non-differentiated cells, in which only minimal fodrin degradation is observed (as shown in Fig. 5A). The addition of Ca²⁺ to this cell lysate induced fodrin degradation, and the degradation was inhibited by calpeptin (Fig. 6B). These results indicate that Ca²⁺-promoted calpain activity causes fodrin degradation in PC12 cells, and that such a degradation is inhibited by calpeptin. The overall results thus show the degradation of fodrin at later stages of PC12 cell differentiation, and
indicate that the degradation is due to caspase-1 and calpain activities.

4. Discussion

The results presented here point to caspase, specifically caspase-1, as playing a role in the differentiation of PC12 cells. This conclusion is based on the effects of caspase inhibitors, on immunoblotting analysis and on estimation of caspase-1 activity in cell extracts. The activation of caspase-1 appears to allow the neurite outgrowth in the differentiating cells. Caspase-1 is present in some nerve cell bodies and in nerve fibers throughout the brain [36], and thus could play a role in the membrane and cytoskeleton reorganization required for the morphological alterations during nerve cell differentiation, neurite growth and dendritic dynamic modulation.

A variety of proteins are known to serve as substrates for caspases, including calpastatin [35,37]. The degradation of calpastatin at the later stage of PC12 cell differentiation, the prevention of degradation by caspase-1 inhibition, and the lack of calpeptin effect is consistent with its being degraded in these cells by caspase-1. The results also show that fodrin, known to be a substrate of caspase and of calpain [35], is degraded in the PC12 cells at the later stage of differentiation, and appears to be degraded by both caspase and calpain. Additional targets for caspase action in neuronal differentiation may be involved. The interleukins provide one such target. Caspase-1 is known to activate IL-1β and IL-18 and is involved in the expression of IL-1α [13]. In PC12 cells, IL-1α expression is enhanced by NGF [38], indicating a role for IL-1 in PC12 differentiation. Other interleukins may be indirectly involved (e.g., the synergistic induction of neurite outgrowth in PC12 cells by IL-6 and NGF [39]). The possibility that caspase-1 involvement in PC12 cell differentiation is via interleukin(s) requires further study.

The results presented here point to roles for both caspase and calpain in PC12 cell differentiation. Several previous studies have shown that the calpain–calpastatin system is involved in PC12 differentiation [27–29]. It was shown that A- and m-calpain protein levels did not change during NGF-

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**Fig. 5.** Fodrin in differentiating PC12 cells. Cells were cultured in DM, in the absence and presence of caspase-1 inhibitor. Immunoblotting was carried out using antibody to α-fodrin, as described in Materials and methods. (A) Fodrin in control and in cells treated with 5 μM of the caspase-1 inhibitor BACMK. (B) Fodrin in BACMK-treated cells following the removal of BACMK. Some BACMK-treated culture dishes were freed of BACMK by washing the dishes at 96 h of culture and culture continued for additional 48 h. Representative of three independent experiments.

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**Fig. 6.** Effect of calpain inhibition on fodrin degradation in PC12 cells. (A) Cells were cultured in DM for 48 and 96 h, in the absence and presence of 25 μM calpeptin. Immunoblotting was carried out using antibody to α-fodrin, as described in Materials and methods. Representative of three independent experiments. (B) PC12 cells, cultured in GM, were homogenized, and aliquots incubated at 37 °C for 60 min, in the presence and absence of 1.0 mM Ca²⁺, 2.0 mM EDTA, and 10 μM calpeptin. Immunoblotting carried out using antibody to α-fodrin, as described in Materials and methods. Representative of two independent experiments.
induced PC12 differentiation, but that calpain activity was diminished; calpastatin-containing fractions from extracts of differentiating PC12 cells inhibited more calpain activity than did similar fractions from dividing cells [27]. It was suggested that the diminished calpain activity was due to an increase in calpastatin activity in the differentiating PC12 cells [27]. Initial neurite outgrowth was found to be promoted by cysteine protease inhibitors [28]. In another study it was shown that calpain activity was transiently diminished during the early stage of PC12 differentiation, with calpain activity increasing again at a later stage [29]. In that study, calpeptin promoted neurite growth, with the effect being transient, observed at the beginning of differentiation; at later stages, no difference in the neurites was noted between the control and calpeptin-treated cells [29]. Thus, calpain inhibition does not affect the overall neurite growth, but appears to play a role during the early stage of differentiation by enhancing neurite outgrowth ([28,29] and results of the present study).

Our results are consistent with and supplement previous results. The overall results indicate that at an early stage, when calpastatin is increased, calpain activity is diminished, possibly allowing the beginning of neurite growth. The increase in calpastatin at the early stage of differentiation may be due to signaling pathways involving NGF (via increased cAMP, known to promote calpastatin gene expression [40]), as suggested in the case of neural protection by NGF following cerebral ischemia [41]. At an intermediate stage of differentiation, caspase-1 is activated, and it degrades calpastatin and other proteins. The caspase-induced diminution in calpastatin would allow an increase in calpain activity in the differentiated cells. The activities of caspase and calpain may then be required for the modulation of the morphological changes and for protein turnover in these cells. The proposed steps are presented in the above scheme (Fig. 7). Previously, we found that caspase–calpain cross-talk occurs during myoblast differentiation via caspase-induced calpastatin degra-
dation [14]. Such cross-talk between the two protease systems has also been shown in events leading to cell death [42,43].

Overall, the participation of caspases in non-apoptotic processes may be ascribed to the partial activation of caspase cascades, so that some initial steps occur, but the reactions do not proceed to apoptosis [3–5]. Most studies have concentrated on caspase-3 as the downstream effector caspase in non-apoptotic events [6–11]. It would be of interest to carry out studies on the involvement of caspase-1 in such processes in various cell types. The fact that caspase-1 is involved in such two distinct differentiation processes (i.e., fusion in L8 myoblasts and neurite outgrowth in PC12 neuronal cells) suggests that this caspase plays a general role in non-apoptotic, differentiation processes. Caspase-1 activating proteins have been described in connection to cytokine processing [13]. The triggers for caspase activation in differentiation processes and pathways and factors involved remain to be studied.

References
