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ORIGINAL PAPER

Multiple alphaII-spectrin breakdown products distinguish calpain and caspase dominated necrotic and apoptotic cell death pathways

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Abstract Apoptosis and oncotic necrosis in neuronal and glial cells have been documented in many neurological diseases. Distinguishing between these two major types of cell death in different neurological diseases is needed in order to better reveal the injury mechanisms so as to open up opportunities for therapy development. Accumulating evidence suggests apoptosis and oncosis epitomize the extreme ends of a broad spectrum of morphological and biochemical events. Biochemical markers that can distinguish between the calpain and caspase dominated types of cell death would help in this process. In this study, three chemical agents, maitotoxin (MTX), staurosporine (STS) and thylenediaminetetraacetic acid (EDTA), were used to induce different types of cell death in PC12 neuronal-like cells. MTX-induced necrosis, as determined by the increased levels of calpain-specific cleaved fragments of spectrin by antibodies specific to the calpain-cleaved 150 kDa αII-spectrin breakdown product (SBDP150) and 145 kDa αII-spectrin breakdown product (SBDP145). In this paradigm, there were no detectable SBDP150i and SBDP120 fragments as determined by antibodies specific to the caspase-cleaved specific fragments similar to those seen in the EDTA-mediated apoptotic PC-12 cells. In contrast to the calpain specific MTX necrosis treatment and the caspase EDTA apoptotic treatment is the STS treatment which induced both proteases as shown by the increase in all the SBDP fragments. Furthermore, compared to SBDP150, SBDP145 appears to be a more specific and sensitive biomarker for calpain activation. Taken together, our results suggested calpains and caspases which dominate the two major types of cell death could be independently discriminated by specifically examining the multiple αII-spectrin cleavage breakdown products.

Keywords αII-spectrin · Breakdown product · Calpain · Caspase · Apoptosis · Oncosis · Necrosis · Proteolysis · Biomarker

Abbreviations

SBDP	Spectrin breakdown product
MTX	Maitotoxin
STS	Staurosporine
EDTA	Ethylenediaminetetraacetic acid
TBI	Traumatic brain injury
PCD	Programmed cell death

Introduction

Programmed cell death (PCD) in neuronal and glial cells has been documented in many neurological diseases, such as traumatic brain injury (TBI), stroke and Alzheimer's disease [1–4]. Generally oncotic necrosis of neurons has been identified in the acute post-traumatic period within contused or hemorrhaging regions while the apoptotic neurons were localized in regions further from the site of impact in the days and weeks after trauma [1, 5, 6]. The ability to distinguish the two major types of cell death

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(oncotic necrosis and apoptosis) in different neurological diseases should provide improved insight into the injury mechanisms of each specific pathoneurological condition. This can also help better diagnose patients and better pinpoint targets for therapy development [7–9].

Traditionally, morphological studies, including triphenyltetrazolium chloride (TTC), hematoxylin and eosin (H&E), and terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining have been used to address the role of apoptosis versus necrosis. Oncotic necrosis, from the Greek meaning "swelling and degenerating corpse," is characterized by cell and organelle swelling leading to nuclear degradation and, ultimately, to disruption of the cell membrane and cell lysis [10, 11]. It has been shown that when these rapid and active molecular and biochemical processes occur there is an increase in the activation of members of the proteolytic calpain family [7, 12].

Apoptosis, on the other hand, is characterized by a more well-defined series of morphological and biochemical traits. It typically includes the activation of a specific family of proteases known as caspases [13], although caspase-independent processes involving proteins such as AIF have been reported [14, 15]. Kerr et al. [16] first characterized this mode of cell death and labeled it "apoptosis", from the Greek meaning "falling leaves", to emphasize the cells' demise into small membrane bound particles. The features of apoptosis include chromatin condensation, internucleosomal DNA fragmentation, cell shrinkage and finally cell dismantlement into membrane-enclosed vesicles [17].

Accumulating evidence suggests apoptosis and oncosis epitomize the opposite ends of a broad spectrum of morphological and biochemical events. However under certain stress conditions neither oncosis nor apoptosis may predominate suggesting that a mixture of the two may occur either simultaneously or along a time scale. The cross-talk between the two cysteine protease systems involved in PCD, calpains and caspases, further blur the boundary between apoptosis and necrosis [18–22]. Furthermore, as more has become known about these two cell death processes morphological change can no longer be considered the most practical end-point measurement thus restricting its usefulness for clinical studies [23].

Finding biochemical markers which can distinguish between calpain and caspase dominated types of cell death is needed and has the potential of monitoring the endpoints of the therapeutic progress.

Recently, the degradation of αII-spectrin as a useful biomarker for the level and mechanism of injury has been documented in TBI, stroke and aneurysmal subarachnoid hemorrhage. [2, 24–27]. AlphaII-spectrin, a 280 kDa cytoskeletal protein found on the intracellular side of the plasma membrane, forms part of the membrane scaffolding that is cross linked with actin [28] thereby providing a cell with structure [29, 30]. In certain types of brain injury, such as diffuse axonal injury due to TBI, spectrin will be irreversibly cleaved by a member of one of two families of proteolytic enzymes, either calpains or caspases [31, 32].

A number of drawbacks have, until recently, limited the usage of total αII-spectrin degradation as a biomarker to distinguish calpain from caspase dominant necrotic and apoptotic neuronal cell death. For example, it is difficult to distinguish the calpain and caspase-generated SBDP150 and SBDP145 on the western blot membranes unless they are completely separated by SDS–PAGE gel electrophoresis [33–38]. Also, intact αII-spectrin lacks calpain and caspase dominant cell death specificity when used in ELISA endpoint measurements. Finally, calpain and caspase-3 each activation generate one αII-spectrin fragment at 150 kDa so that SBDP150 is often mistakenly considered to be a non-specific spectrin breakdown product (SBDP) [30].

In this study, we examined αII-spectrin and the cleavage products for both calpains (necrosis and apoptosis) and caspases (apoptosis) following treatment by various chemical agents. We demonstrated here that the two cell death pathways could be independently demonstrated by examining the αII-spectrin cleavage breakdown products. In the process of our study, a calpain-mediated SBDP150 and caspase-3 SBDP150 (SBDP150i) products could be distinguished using fragmental-specific antibodies. Lastly, our results suggest SBDP145 is a more specific and sensitive biomarker for calpain activation than SBDP150.

Materials and methods

BDP-specific antibodies

Antibody specific to calpain-generated αII-spectrin fragment SBDP150N (raised against new C-terminal QQQEV-COOH), SBDP150 (raised against new N-terminal NH_2-GMMPR), SBDP145 (raised against new N-terminal NH_2-SAHEV) and caspase-generated SBDP120 (raised against NH_2-SVEAL) as well as SBDP150i (NH_2-SKTAS) were synthesized. Briefly, custom-made peptides (California Peptide, Napa, CA) were coupled via C-terminal cysteine [C] to Keyhole Limpet Hemocyanin (KLH) protein using a sulfo-link crosslinking reagent (Pierce). The conjugated peptide (2 mg) was injected into two rabbits. After 3 months, serum samples from the rabbits were collected. The affinity-purified antibody was dialyzed against TBS (20 mM Tris–HCl, pH 7.4, 150 mM NaCl), concentrated and stored in 50% glycerol at −20°C.

Rat PC12 pheochromocytoma cell culture

Rat PC12 cells were grown on polystyrene tissue culture dishes (Falcon, Becton–Dickinson, Franklin Lakes, NJ, USA) in Dulbecco's modified Eagle's medium (Gibco, Invitrogen Corp., Grand Island, NY, USA) supplemented with 5% horse serum (Gibco), 1% Fungizone (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco) and kept at 37°C in a humidified 5% CO_2 incubator for 12–24 h before treatment.

Cell collection and preparation for immunoblots

The PC12 cells were treated for various time periods, washed twice with phosphate buffered saline (PBS), and solubilized with lysis buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor cocktail tablet (Roche, Indianapolis, IN). Samples (20 μg of protein) were resolved by Tris/glycine gel (Invitrogen Life Technologies, Carlsbad, CA, USA) sodium dodecyl sulfate–polyacrylamide electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) by the semidry method. Membranes were incubated with the primary antibody (mouse monoclonal or rabbit polyclonal) in 5% non-fat milk in TBST at 4°C overnight. Following a series of washes with TBST, membranes were incubated for one hour at room temperature with a biotinylated secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG—1:5,000 dilution). Following another series of washes, the membrane was incubated with avidin-conjugated alkaline phosphatase for 30 min. Proteins were visualized using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) phosphatase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA).

Primary antibodies included the general monoclonal anti-αII-spectrin (Affinity Research Products Ltd., Plymouth Meeting, PA; 1:5,000), activated caspase-3 (Cell Signaling, Danvers, MA; 1:1,000) and the cleavage specific antibodies produced by our laboratory including polyclonal anti-SBDP150N (1:1,000), polyclonal anti-SBDP150 (1:1,000) [39] and anti-SBDP145 (1:300) for calpains, polyclonal anti-SBDP150i (1:1,000) and anti-SBDP120 (1:1,000) for caspases and activated calpain-1 (1:300).

Pharmacologic intervention

In addition to untreated controls, the following conditions were used: maitotoxin (MTX) (1 nM; WAKO Chemical USA Inc., Richmond, VA) as a calpain-dominated challenge for 1 or 3 hours; the Ca^{2+} chelator ethylene diamine tetra-acetic acid (EDTA) (5 mM; Sigma–Aldrich, St. Louis, MO) for 24 h as a caspase-dominated challenge; and staurosporine (STS) (1 μM; Sigma–Aldrich) for 24 h as an excitotoxin challenge [40–42]. For pharmacologic intervention, cultures were pretreated 1 h before the MTX, EDTA or STS challenge with the calpain inhibitor SJA6017 (30 μM; Senju Pharmaceuticals, Kobe, Japan) [43, 44], or the pan-caspase inhibitor Z-VAD (OMe)-FMK (30 μM; R&D, Minneapolis, MN).

Immunocytochemistry

PC-12 cells were seeded onto LabTek II chamber slides (Nunc, Naperville, IL) followed by overnight incubation [45]. The next day, the medium was replaced and treated with MTX, STS or EDTA. Twenty-four hours following STS and EDTA treatment and 3 h for MTX, PC-12 cells were fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were incubated overnight in blocking solution at 4°C with anti-SBDP145 (1:300), anti-SBDP120 (1:1,000), anti-activated calpain-1 (1:300) or anti-activated caspase-3 (1:1,000) following a 1-h blocking step in 10% goat serum in TBST at room temperature. Alexa 488-conjugated goat-anti-rabbit or mouse secondary antibody (Molecular Probes, Eugene, OR) was added at a dilution of 1:1,000, followed by washing with PBS. The cells were mounted using medium with 4,6-diamidine-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Fluorescence images were viewed and digitally captured with either a 20× or 40× objective with an Axiovert 200 microscope (Zeiss, Thornwood, NY, USA) equipped with a Spot Real Time Slider high resolution color CCD digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Results

Design of αII-spectrin fragmental-specific antibodies

Analysis of immunoblots and immunocytochemistry for complete and fragmented peptides of non-erythroid αII-spectrin requires antibodies to specific cleavage sites of this protein which we have reported in previous studies and our new N-terminal microsequence [7, 39, 46, 47]. In Fig. 1 the cleavage sites, as highlighted on the partial sequence of αII-spectrin, indicate where the epitopes for the antibodies to these specific αII-SBDPs have been found. SBDP150 (Y*GMMPR) and SBDP145 (G*SAHEV) are calpain (Calp)-cleavage specific fragments while SBDP150i

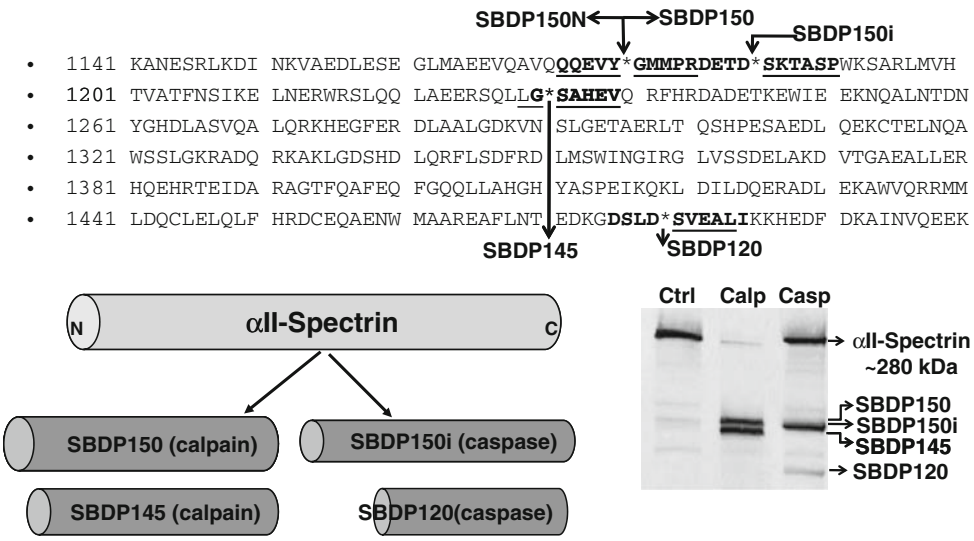

Fig. 1 Cleavage sites of αII-spectrin. The partial amino acid sequence of αII-spectrin pictured here highlights the cleavage sites of calpains and caspases and demonstrates the approximate sizes of the various SBDPs

(D*SKTAS) and SBDP120 (D*SVEAL) are caspase (Casp)-cleavage specific. Both proteases cleave αII-spectrin in repeat 11 near the calmodulin-binding domain (CaM) to produce 150 kDa SBDPs with a unique N-terminal region (SBDP150 N). Included in Fig. 1 is an example of an immunoblot with in vitro calpain-2 and caspase-3 digestion of rat brain lysates when probed with αII-spectrin (Biomol, Affinity, USA). As indicated, calpain-2 treatment generated αII-spectrin fragments 150 (SBDP150) and 145 kDa (SBDP145) and caspase-3 generates major fragments of 150 and 120 (SBDP 120). However, when compared to calpain-mediated SBDP150, caspase-3 generated SBDP150 (SBDP150i) appears to have a relatively lower molecular weight around 149 kDa.

Morphology following treatments

PC12 cells treated with either STS, MTX, or EDTA demonstrate the characteristic morphological changes of programmed cell death that accompanies the various treatments. While the control cells maintained the well-defined oval nuclear morphology with diffused DNA of a healthy cell, for the STS and EDTA treated cells the DNA condensation was accompanied by the collapse and shrinkage of the nucleus and eventual disintegration into membrane bound vesicles (Fig. 2). In the MTX-treated cells, a more nonspecific disintegration of the cells and nuclei with their DNA were observed (Fig. 2).

AlphaII-spectrin biomarker analysis of treatments

The breakdown products of the protein αII-spectrin (SBDPs) are known biomarkers and are able to distinguish, indirectly, between calpain and caspase activation [7, 47, 48]. The results show that treatment for 1 h with MTX, a

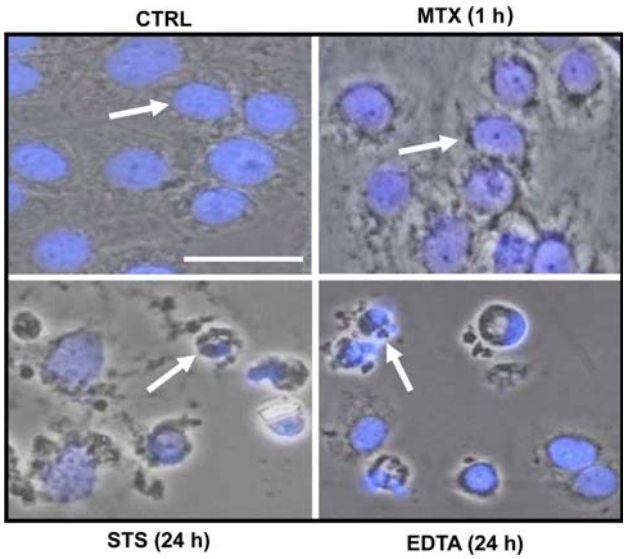

Fig. 2 PC12 cell morphology after chemical treatment. PC12 cells were treated with MTX (1 nm), STS (1 mM), and EDTA (5 mM). The cells were observed under phase contrast with DAPI stain, for detecting nuclear morphology, after the noted times. The *arrows* highlight the typical structure of cells, except control (CTRL) cells, undergoing cell death following treatment. Magnification = 200×, scale bar = 20 mm

Ca^{2+} influx stimulator, was able to induce the calpain-specific cleavage of αII-spectrin in the PC12 cells (Fig. 3). The antibodies specific to the SBDP150, SBDP150 N and SBDP145, all calpain-cleaved fragments, highlighted increased levels of the fragments over control strongly suggesting calpain activation. Contrast to this is the lack of detectable SBDP150i and SBDP120 fragments indicative of caspase activation such as is seen with the calcium chelator EDTA treated (24 h) PC12 cells (Fig. 3).

For STS treatment, the results after 24 h revealed that both calpain and caspases were activated. STS was

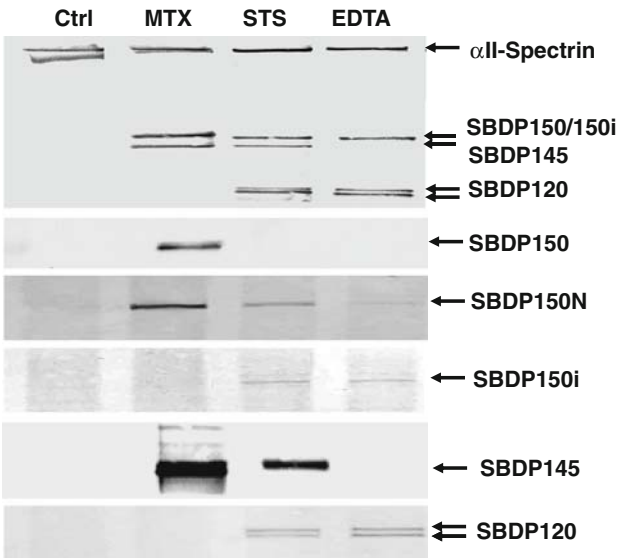

Fig. 3 αII-SBDPs after treatments. PC12 (rat) neuronal-like cells were treated with MTX (1 nm—1 h), STS (1 mM—24 h), and EDTA (5 mM—24 h). The cell lysates (20 mg) were separated by 6% SDS–PAGE gels. The breakdown products were probed with a general antibody to spectrin (top blot) and with specific antibodies to the breakdown products particular to each family of proteases, SBDP150, SBDP150N and SBDP145 for calpains and SBDP150i and SBDP120 for caspases. All the experiments are done five times independently

generally considered as a non-selective protein kinase inhibitor to function as an inducer of apoptosis. Here it was evidenced by the generation of the caspase-cleaved SBDP150i and SBDP120 fragment. Additionally, STS treatment also generates the calpain-cleaved fragments as the increased levels of SBDP145 demonstrate. Interestingly, no SBDP150 was detected except SBDP150N (Fig. 3).

Correlate SBDPs to the activation of calpain-1 and caspase-3

To more directly assess the activation of calpains and caspases, PC12 cells were treated with MTX, STS and EDTA, with or without 1 h of pretreatment with either the pan-calpain inhibitor SJA6017 (SJA) or the pan-caspase inhibitor Z-VAD-FMK (VAD). In agreement with the αII-spectrin biomarker analysis, the immunoblot results illustrated that calpain-1 was activated following MTX and STS but not by EDTA, while caspase-3, the main caspase executioner, was activated by STS and EDTA treatment but not MTX (Fig. 4). The cells pretreated with the calpain inhibitor SJA showed a visible decrease in the levels of calpain-1 for the MTX-treated cells, an apparent decrease in the STS-treated cells, and it had no effect on the activated caspase-3. On the other hand, the caspase inhibitor VAD was effective in reducing (STS) or nearly eliminating (EDTA) the activation of the caspase-3 by reducing the levels of the 17 kDa, or large subunit, of the active caspase-3 molecule required for an active caspase-3. We further probed the multiple SBDPs and correlated them to the activity of calpain and caspase with either the specific calpain inhibitor SJA6017 or the pan-caspase inhibitor Z-VAD prior to chemical treatments. In Fig. 5, we found the production of SBDP145 and SBDP120 strikingly paralleled the activity of calpain and caspase-3. Calpain or caspase inhibitor successfully blocked SBDP150, SBDP145 and SBDP120 formation. However, it did not block SBDP150N. Even though calpain activation was detected in STS treatment, no SBDP150 could be found in the blots.

Our results also showed that the antibodies specific to the SBDP150i and SBDP120, both caspase-cleaved fragments in STS and EDTA treatment groups, exhibited increased levels over control and MTX treatment (Fig. 5). However, compared to the STS treatment, EDTA treatment generated less SBDP150i, and caspase-3 inhibitor efficiently prevent SBDP120 formation, but not SBDP150i. More interesting, we also noticed that one new band appeared below SBDP120 in the STS and EDTA treatment panels. This band can be recognized by both total αII-spectrin and the breakdown product specific antibody SBDP120. And, it has the same behavior as SBDP120 which responds to caspase-3 activation.

Immunocytochemical confirmation of increases in active proteosome and SBDPs following treatment

To confirm that activated calpain-1 and its αII-spectrin target fragment, SBDP145, was upregulated following MTX and STS treatments and that increased levels were analogous to the results noted previously using immunoblots (Figs. 4 and 5, respectively) we examined PC12 cells following treatment using immunocytochemistry (Fig. 6). There is a clear upregulation when compared to controls of SBDP145 and calpain-1 in MTX and STS-treated cells but not in the EDTA treated cells which showed similar levels of upregulation to that of the controls.

Likewise, to confirm that activated caspase-3 and its αII-spectrin target fragment, SBDP120, were upregulated following STS and EDTA treatments and that they, also, were analogous to the results noted previously using immunoblots (Figs. 3 and 4, respectively) we examined PC12 cells following treatment using immunocytochemistry (Fig. 7). There is a clear up-regulation of SBDP120 and caspase-3 in STS and EDTA treated cells, but only a barely detectable up-regulation of caspase-3 in the MTX treated cells and no increased levels in the controls.

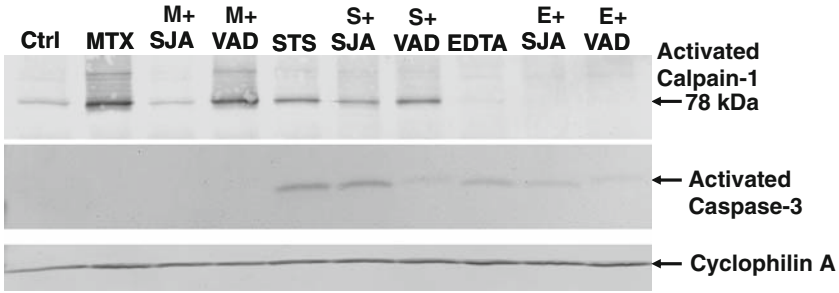

Fig. 4 Activation of calpain-1 and caspase-3 after treatments. PC12 neuronal-like cells were treated with MTX (M; 1 nm—1 h), STS (S; 1 mM—24 h), and EDTA (E; 5 mM—24 h) in the absence and presence of 1 h pretreatment with either the calpain inhibitor SJA6017 (SJA; 30 mM) or the pan caspase inhibitor Z-VAD-FMK (VAD; 30 mM).The cell lysates (20 mg) were separated by 4–20% gradient SDS–PAGE gels. All the experiments are done three times independently

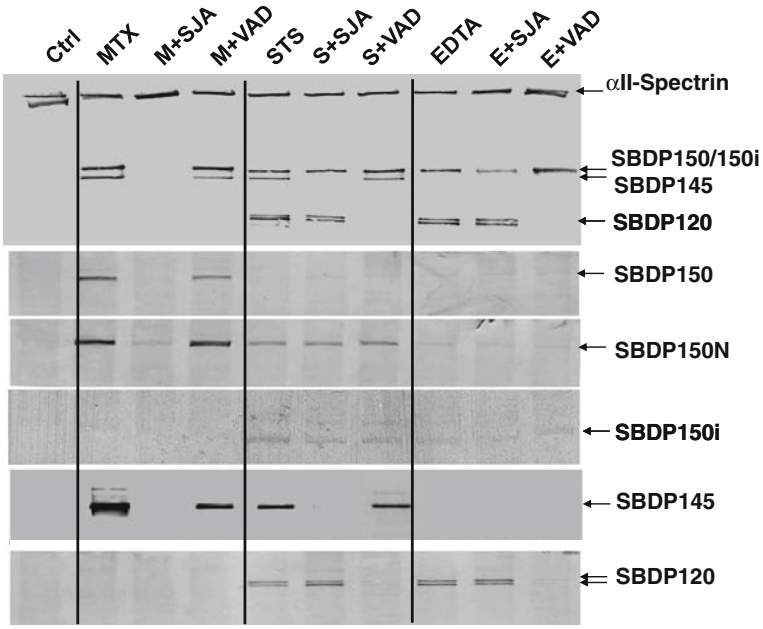

Fig. 5 Effects of calpain and caspase inhibitors on specific SBDP generation pattern. The treated PC12 cell lysates were probed with αII-spectrin (top blot) and specific SBDP-specific antibodies. Paralleled αII-spectrin (top) proteolysis and multiple SBDP were monitored as the marker of calpain or caspase activity. Calpain inhibition preserved the SBDP150 and SBDP145, while caspase inhibition preserved SBDP120. All the gels used in this figure are 6% SDS–PAGE gels except SBDP150i (4–20%). All the experiments are done three times independently

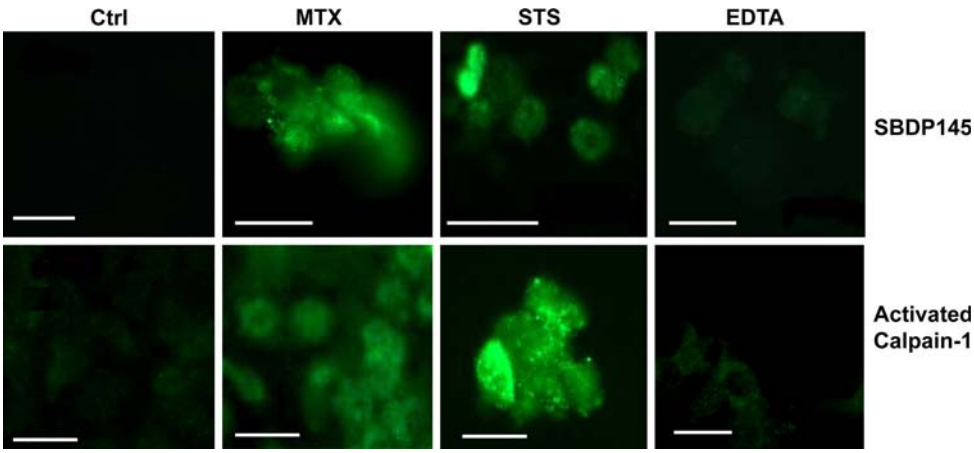

Fig. 6 Activated calpain-1 and its target SBDP145 in PC12 cells challenged with MTX, STS, or EDTA. The PC12 cells were treated with MTX (1 nm—3 h), STS (1 mM—24 h), and EDTA (5 mM—24 h) and then probed with the indicated antibody. Calpain-1 is activated under MTX and STS treatments, but not by EDTA treatment. This is supported by the increased presence of SBDP145. Magnification = 400×, scale bar = 20 mm

Discussion

This is the first systematic study to characterize αII-SBDPs in different cell death models with fragment-specific SBDP antibodies. In this study, three distinct chemical challenged cell death models were used to induce calpain-dominant necrosis, caspase-dominant apoptosis and an active mixture of the two (Fig. 8).

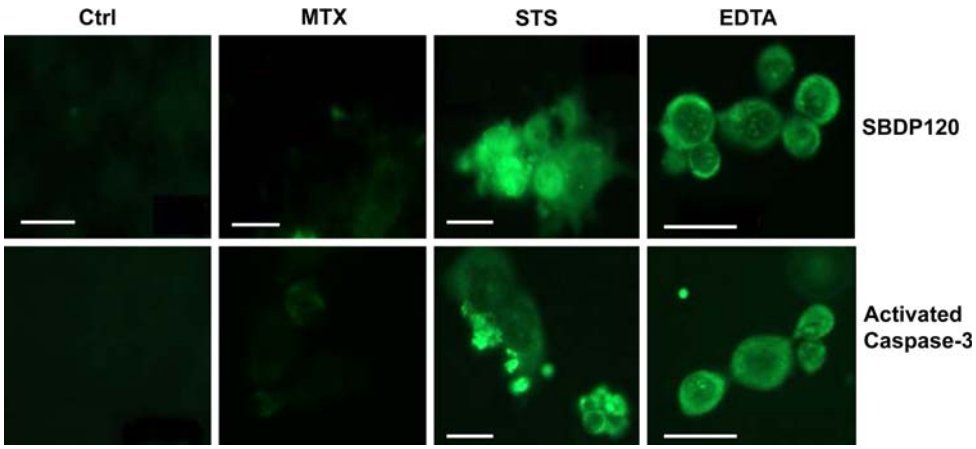

Fig. 7 Activated caspase-3 and its target SBDP120 in PC12 cells challenged with MTX, STS, or EDTA. The PC12 cells were treated with MTX (1 nm—3 h), STS (1 mM—24 h), and EDTA (5 mM—24 h) and then probed with the indicated antibody. Caspase-3 is activated under STS and EDTA treatments and this is supported by the increased presence of SBDP120, but little active caspase-3 or its target SBDP120 is seen in the MTX-treated cells. Magnification = 400×, scale bar = 20 mm

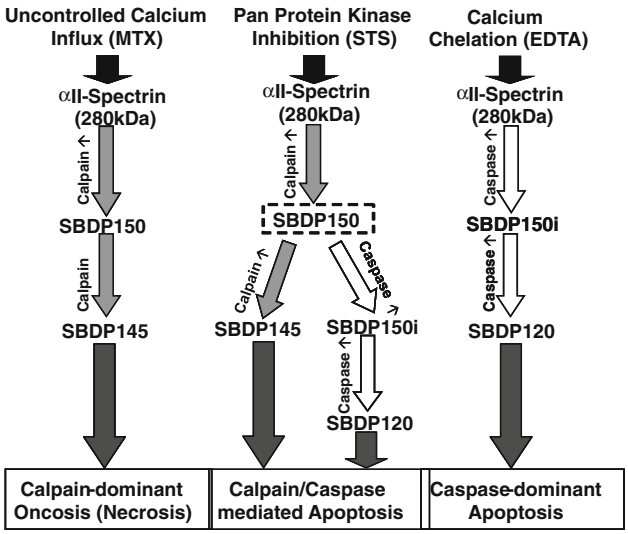

Fig. 8 Schematic of pathways leading to cell death when cells are challenged with MTX, STS, or EDTA. The schematic represents how the different treatments lead to different forms of cell death, oncosis (necrosis) or apoptosis. The processing of the biomarker αII-spectrin under the various treatments indicates which protease, calpain or caspase, is activated under the different paradigms

MTX, a highly potent marine toxin isolated from the dinoflagellate gambierdiscus toxicus, is an important molecular tool for the study of oncotic (necrotic) cell death [49, 50]. MTX, known for its ability to stimulate calcium influx into cells through a series of biochemical processes that include activation of both voltage-sensitive and receptor-operated calcium channels [51, 52], initiates calpain activation which eventually leads to cell death via cell lysis [40]. In our study, we confirmed that total αII-spectrin was degraded into SBDP150 and SBDP145 by calpain-dominant MTX-induced necrosis.

Staurosporine is an indolo[2,3-a]carbazole discovered in the course of screening extracts of the bacterium Streptomyces staurospores for constituent alkaloids with PKC-inhibitory activity. STS was shown to mobilize and increase [Ca^{2+}] from the thapsigargin-insensitive secretory granule Ca^{2+} stores by inhibiting protein kinase C [53]. Now identified as a potent, relatively non-selective, protein kinase inhibitor, recognized to block, by varying degrees, a number of different kinases, it is commonly employed as an intracellular stress inducer of apoptosis [54, 55]. However, it is controversial in that it not only induces caspase-dominant activation, but also calpain activation [56–58]. The precise mechanism underlying its activity is not known yet. In this study, our fragment-specific antibodies recognized SBDP150N, SBDP150i, SBDP145 and SBDP120 in STS-treated PC12 cells. Most interestingly SBDP150 could not be detected in STS challenged PC12 cells. In addition, although the calpain inhibitor did not prevent SBDP150N formation, it was able to prevent SBDP145. These results suggested that STS induces both calpain and caspase activation. STS appears to alter Ca^{2+} homeostasis of differentiated PC12 cells. Seo and Seo [59] found that the anti-apoptotic Bcl-2 family member Bcl-X_L prevented STS-induced neuronal cell death by interfering with Ca^{2+}-mediated apoptotic signals by inhibiting Ca^{2+} release from ER. This is consistent with the results obtained by Wang and colleagues [60] in rat hepatocytes who found that Bcl-X_L prevented cytochrome c release and caspase-3 activation in response to STS. It was also reported that less Ca^{2+} was released from the ER in Bcl-X_L expressing cells in response to apoptotic stimuli due to down-regulation of IP_3 receptors [61]. Moreover, a proteomic study by Short et al. [55] indicated that STS-induced apoptosis induces an unfolded protein response involving molecular chaperones,

co-chaperones and structural proteins indicative of ER stress. On the other hand Kim et al. [53] report that they did not find the ER to be the source of increased cytosolic Ca^{2+} concentration but instead they suggested secretory granules in the rat submandibular acinar cells to be the responsive elements to STS treatment [53]. Whether STS increases the cytosolic Ca^{2+} concentration from the ER Ca^{2+} stores, secretory granules or from external sources, Ca^{2+} appeared to play an important role in activating calpain and generating the calpain-cleaved fragments as the increased levels of SBDP145 demonstrated. This is not to overlook or minimize the crosstalk that may occur such as calpain contributing to caspase activation [58]. It has been reported that calpain-2 also cleaves caspase-3 and renders it more susceptible to full activation after neonatal hypoxia–ischemia [7, 62].

Based on our results, we propose that αII-spectrin first generates SBDP150N, SBDP150 and then SBDP145 under calpain-dominant STS challenge. Caspase-3 activation on the other hand cleaves SBDP150 to generate SBDP150i and then SBDP120. These results suggest SBDP145 is more sensitive and specific for calpain activation when compared to SBDP150 and SBDP150N. Conceptually we hypothesized that the SBDP150N antibody would behave similar to the SBDP150 antibody when we designed the peptides for it. Although our results suggest that SBDP150N generates the same patterns in calpain-dominant treatment (MTX) with or without calpain inhibition as SBDP150 and SBDP145; it, however, behaves differently in mixed calpain and caspase activated conditions (STS). It appears that calpain inhibition (SJA) is not able to prevent SBDP150N formation. The exact reaction underlying this phenomenon is unknown yet. We think that STS treatment generated a caspase dominant SBDP150i when the calpain inhibitor was added. Since there is only nine amino acids span difference between SBDP150N and SBDP150i, and based upon the native quaternary structure, it is possible that SBDP150N antibody picked up not only SBDP150N, but also some SBDP150i fragments under the calpain inhibition condition.

EDTA, a chemical compound also known as diaminoethanetetraacetic acid disodium salt, edetate, or versene, is a chelating agent. It forms coordination compounds with most monovalent, divalent, trivalent and tetravalent metal ions, such as silver (Ag^{+}), calcium (Ca^{2+}), Zinc (Zn^{2+}), copper (Cu^{2+}), iron (Fe^{3+}) or zirconium (Zr^{4+}). Ethylene glycol tetra-acetic acid (EGTA), a chelating agent related to EDTA, also has a high affinity for calcium and was previously found to trigger apoptosis in SH-SY5Y [41] and L929 mouse skin fibroblast cells [63]. Since EDTA is a calcium chelator and as such removes most if not all of the calcium in cells it triggers a stress response by the endoplasmic reticulum (ER) known as the unfolded protein response (UPR). The response is due to the disruption of cellular Ca^{2+} homeostasis disrupting the critical role Ca^{2+} plays as a co-factor in the synthesis and the proper folding of proteins, within the ER lumen [41, 64]. It has been also reported that EDTA induces apoptosis by Zn^{2+} chelation in Neuro-2A cell lines [65] or disrupting calcium-dependent cell adhesion molecules on intestinal epithelial cells [66]. However, those exact mechanisms are still under investigation.

Our results showed EDTA triggering a typical apoptosis like response in neuronal-like PC12 cells with caspase-3 activation. Typical of this response αII-spectrin was degraded to SBDP150i and SBDP120 after caspase-3 was activated. Consistent with our previous N-terminal microsequence study, this confirms our finding that calpain-mediated SBDP150 is different from caspase-3 mediated SBDP150i due to a nine residue span by fragment-specific antibodies.

Our results showed that SBDP150i and SBDP120 exhibited increased levels upon STS or EDTA treatment. The level of SBDP150i is less in EDTA treatment compared to STS groups. This might be due to more of it being converted from SBDP150i to SBDP120. However, compared to SBDP120, caspase inhibitor z-VAD does not efficiently block SBDP150i formation. This might due to several reasons: first, since SBDP150i is the first caspase cleavage site, it will be more susceptible and harder to block compared to SBDP120. Second, it has been reported that caspase-3 is not the only caspase involved. Other caspases including caspase-1, 2 4, 6 and 7 may also be involved in SBDP150i formation [47, 67]. However, SBDP120 is generated only by caspase-3. Caspase inhibitor Z-VAD at 30 μM appears most effective against caspase-3 and not as much as against the other caspases.

Another interesting phenomenon we observed is that an additional low molecular weight SBDP120 band appears upon ex vivo STS and EDTA treatment and it responds to the caspase inhibitor Z-VAD. The induction of the doublet of SBDP120 has also been occasionally noticed in the STS and EDTA treated human SY5Y and rat primary cortical neuronal cells (data not shown). However, we could not detect the doublet SBDP120 in any in vitro caspase-3 digested samples. Moreover, not all STS and EDTA treated cell lines can generate this doublet SBDP120. Based on the fact that our caspase-3 cleavage specific (D*SVEAL) antibody SBDP120 can detected both SBDP120 bands, we therefore assume that the generation of the lower molecular weight SBDP120 is a dynamic event. After active caspase-3 cleaves αII-spectrin to SBDP120, then caspase-3 or another apoptotic related proteases may further cleave spectrin at the C-terminal at some critical time point creating the slightly lower molecular weight SBDP120 (lower

band of SBDP120). The exact mechanism underlying the SBDP120 generation needs to be further clarified.

In addition to PC12 cells, we found that EDTA was also a better than average caspase-3 activator in a number of other cell types, including rat cerebrocortical and cerebellar granule neurons (data not shown). This suggests EDTA challenged neurons should provide a good model for studying caspase-3 related neuronal diseases.

Taken together, our studies have demonstrated that the multiple αII-SBDPs can be biomarkers capable of distinguishing calpain and caspase dominant necrotic and apoptotic cell death and is able to monitor the dynamic biochemical changes associated with calpain and caspase activation. Furthermore, the antibodies developed here, such as calpain specific SBDP145 and caspase-3 specific SBDP120 could be invaluable tools to monitor disease progression and the consequences of inhibitory potency of drugs that target calpain and caspase activation in different neuronal disease models such as ischemic brain injury, traumatic brain injury and Alzheimer's disease.

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