In vitro Investigation of Caspase-3 Activation and Its Proteolytic Targets in Adult Monkey Brain

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Abstract The major biochemical process of apoptosis involves the activation of a group of proteases (caspases) and the selective cleavage of a set of intracellular proteins that leads to the collapse of cell survival mechanism. Among the caspases identified, caspase-3 stands out because it is commonly activated by numerous death signals and cleaves a growing number of cellular components. In order to reveal potential targets of caspase-3 in primate neural tissue, an alternative cell free system based on adult monkey brain was established to reproduce the downstream part of apoptotic program, initiated by the addition of granzyme B. Through Western blot analysis, caspase-3 was found to become mature in a two-step manner and its activity was exhibited by the cleavage of the synthetic substrate, Ac-DEVD-pNA. Investigations on native proteins in the brain extract showed that poly (ADP-ribose) polymerase (PARP) was cleaved to an 85 ku fragment, suggestive of caspase-3 activity. And more intriguingly, a neuronal apoptosis inhibitory protein (NAIP)-immunoreactive fragment with molecular mass of approximately 40 ku was detected in granzyme B-treated brain extract and its production was not blocked by the caspase-3 inhibitor, Ac-DEVD-CHO. According to the substrate specificity of granzyme B and the size of cleavage product, putative cleavage site may be located immediately after the third DIR domain of NAIP. These data suggest that cleavage events involved in apoptosis can be reproduced in matured primate brain extract and NAIP is likely to be the target of granzyme B, but not of caspase-3, during apoptosis.

Key words apoptosis, granzyme B, caspase-3, poly(ADP-ribose) poly merase, apoptosis inhibitory protein, primate, brain

Apoptosis, the physiological process for killing cells, is critical for normal development and the maintenance of homeostasis in multicellular organisms. Abnormalities in cell death control can contribute to a variety of diseases such as cancer, autoimmune diseases and neurodegenerative disorders. Accumulated evidence demonstrated that apoptotic stimuli trigger apoptosis via several signaling pathways that converge on a common machinery of cell destruction——the activation of caspases[1]. Most of caspases are constitutively expressed and mainly located in the cytoplasm in the form of zymogen. During apoptosis, the caspase cascade is triggered and amplified through homo- and hetero-proteolysis among different family members. Activated downstream caspases (caspase-3, caspase-6 or caspase-7) then selectively cleave various intracellular proteins, including structural elements of the cytoplasm and nucleus, components of the DNA repair machinery and several types of protein kinase, therefore disrupting normal cellular homeostasis that culminates in cell death. Since caspase activation is a crucial step in the initiation and propagation of the apoptotic cascade, it is a very tightly controlled process. To date, it is known that the control is exercised via at least two mechanisms, Bcl-2 family and inhibitor-of-apoptosis proteins (IAPs). NAIP, mutated in several forms of spinal muscular atrophy, is the first IAP protein discovered in mammals[2]. Immunohistochemical staining demonstrated that NAIP-positive neurons were distributed broadly in mammalian central nervous system and was strongly expressed in anterior horn and motor cortex neurons of normal brains[3]. While NAIP was found to be capable of suppressing apoptosis in motor neurons, we have only limited insight into the molecular mechanism by which it may act.

Caspase-3 is a crucial executioner of apoptosis. Premature lethality accompanied by decreased neuronal apoptosis in caspase-3-deficient mice and its activation in acute brain injury or neurodegenerative diseases demonstrate the key role of caspase-3 in the apoptosis of both developing and mature brain[4]. It is responsible for the proteolysis of many intracellular proteins, such as PARP, the catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}), α-fodrin, during apoptosis[5]. Although at least 30 proteins have been identified as substrates of caspase-3, approach to search for other targets is still under way. Not only will it provide more information to understand the molecular mechanism of apoptosis, but also help revealing the function of target proteins in cells. In the present study, we attempted to establish a novel cell-free system based on the cerebral cortex of an adult rhesus monkey, to explore the activation of caspase-3 triggered by granzyme B, and to detect the proteolytic activities of caspases-3 on PARP and NAIP.

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1 Materials and Methods

1.1 Chemical reagents

Purified granzyme B and Ac-DEVE-pNA were purchased from Calbiochem (San Diago, CA). Recombinant human caspase-3 protein and Ac-DEVD-CHO, were products of Chemicon (Temecula, CA) and Clontech (Palo Alto, CA), respectively. Primary antibodies used in Western blotting included a rabbit polyclonal anti-caspase-3 antibody (H-277) from Santa Cruz Biotechnology (Santa Cruz, CA), a rabbit polyclonal anti-NAIP antibody (Ab-1) from Oncogene Research Products (San Diego, CA) and a mouse monoclonal anti-PARP antibody (clone C2~10) from PharMingen (San Diego, CA). Anti-rabbit and anti-mouse secondary antibodies conjugated with peroxidase were obtained from Santa Cruz Biotechnology and Transduction Laboratories (Lexington, KY), respectively. Ampholytes (pH 5~7 and pH 3~10), p*I* markers and prestained SDS-PAGE protein molecular mass standard were from Bio-Rad (Hercules, CA). An enhanced chemiluminescence detection system, ECL-plus kit, was purchased from Amersham-Pharmacia (Buckinghamshire, England). Other chemicals came in analytical grade purity from Sigma.

1.2 Brain tissue

Brain tissue was freshly collected from a 4-year-old female rhesus monkey (*Macaca mulatta*) in the Laboratory Animal Center of Chinese PLA General Hospital. All efforts were made to minimize the animal's suffering in accordance with the Helsinki Act. The animal was first anesthetized with ketamine hydrochloride (10 mg/kg, i.m.) and then sacrificed by exsanguination. Brain tissue blocks dissected from the motor cortex were immediately frozen by immersion in liquid nitrogen and then stored at -80℃ until use. As a positive control for the presence of caspase-3 in brain, rat brain tissue was taken from a two-week-old SD rat and was processed along with monkey brain samples.

1.3 Preparation of brain tissue extracts

Brain tissue block (wet weight 100 mg) was homogenized with a Dounce homogenizer in 0.5 ml of 50 mmol/L HEPES buffer (pH 7.4) containing 0.1 mol/L NaCl, 0.1% CHAPS, 10% sucrose, 1 mmol/L EDTA, 0.1 mmol/L phenylmethylsulfonyl fluoride, and 2 mg/L each of leupeptin, pepstatin A, and aprotinin. The homogenate was centrifuged for 30 min at 20 000 *g* and 4℃. The supernatant fraction obtained was either used immediately or stored at -80℃. Protein concentration was determined by Bicinchonicnic acid assay (BCA kit, Pierce).

1.4 Native isoelectric focusing (IEF) and 2-D electrophoresis combined with immunoblotting

In view of the facility in subsequent electrotransfer, freshly prepared polyacrylamide gels were used in native IEF. Briefly, 20 μg of total protein was loaded per lane onto a 5% polyacrylamide gel containing 2.4% ampholyte (0.4% for pH 3~10 and 2% for pH 5~7). Proteins were separated at 200 V for 3 h. A strip of gel loaded with p*I* marker proteins was stained in 0.1% Coomassie Brilliant Blue R250 and the other was immersed in 0.2% SDS for 30 s followed by a transfer (Semi-dry Transfer Apparatus, Bio-Rad) to PVDF (Millipore, Bedford, USA) at 10 V for 30 min in a discontinuous buffer system[6]. Blots were blocked and then probed with anti-caspase-3 antibody as described later in Western blot analysis. For 2-D electrophoresis, one strip of the IEF gel, 0.5 cm wide, was soaked in 10 ml of equilibration buffer (160 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 mmol/L EDTA, 1% dithiothreitol and 0.01% bromophenol blue) for 30 min at room temperature and was loaded onto a 12% SDS-polyacrylamide gel. Electrophoresis was performed for 1.5 h at 100 V followed by a semi-dry transfer and the immunodetection of caspases-3 as described below.

1.5 Cleavage reaction and Western blot analysis

100 μg of monkey brain extract was incubated at 37℃ in the presence or absence of purified granzyme B (25 ng) and Ac-DEVD-CHO (0.1 μmol/L) in a final volume of 30 μl. At each time point, aliquots were collected and mixed with equal volume of 2× loading buffer (100 mmol/L Tris-HCl, pH 6.8, 4% SDS, 200 mmol/L dithiothreitol, 0.2% bromophenol blue and 20% glycerol) to terminate the reaction. All samples were boiled for 6 min prior to the submission to either 7.5% (PARP and NAIP) or 12% SDS-polyacrylamide gel (caspase-3). Electrophoresis was performed at 100 V for 90 min followed by a semi-dry transfer onto nitrocellulose membranes (Bio-Rad) at 10 V for 45 min. Blots were blocked for 1 h in PBST (100 mmol/L phosphate buffer, pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20) containing 5% non-fat dry milk (Bio-Rad) and then probed overnight at room temperature with the following primary antibodies diluted in blocking solution, anti-caspase-3 (1∶2 000), anti-PARP (1∶1 000) and anti-NAIP (1∶2 000). After a thorough wash for 30 min with several changes of PBST, blots were incubated for 1 h with peroxidase-conjugated secondary antibodies in blocking solution, followed by the detection with ECL-plus kit and the exposure to ECL Hyperfilm (Amersham).

1.6 Caspase-3 activity assay

Caspases-3-like activity was measured according to the method described by Chen[7] with modification. Brain extracts were incubated for 1 h at 37℃ in the presence or absence of granzyme B (1 mg/L) or Ac-DEVD-CHO (0.1 μmol/L). Then aliquots of 20 μg protein were added to the reaction buffer (25 mmol/L HEPES, 10% sucrose, 0.1% CHAPS, 5 mmol/L EDTA, 10 mmol/L

dithiothreitol and 200 μmol/L Ac-DEVD-*p*NA, pH 7.4) in a final volume of 100 μl and were further incubated for 15 min in a 96-well plate. Free *p* NA accumulation, which resulted from cleavage of the aspartate-*pNA* bond, was measured as the increase of absorbance at 405 nm (A_{405}) using a Vector-2 Multilabel Counter (EG&G, WALLAC).

2 Results

2.1 Procaspase-3 in adult monkey brain

Through Western blot analysis two bands with caspase-3 immunoreactivity were detected in monkey brain extract (Figure 1, lane *2*). Their molecular mass were approximately 64 ku and 32 ku, respectively. In contrast, there was only one band of 32 ku found in the brain of two-week-old SD rat (lane *1*). The 32 ku protein is in line with the reported caspase-3 zymogen in mammals. In order to determine the relationship between the two caspase-3-immunoreactive proteins in monkey brain, native IEF and 2-D gel electrophoresis were performed followed by immunodetection with anti-caspase-3 antibody. As shown in Figure 2a, the pattern of caspase-3 immunopositive bands after IEF appeared similar to that in Figure 1. Besides the bands with the same p*I* value for two species, another protein with a lower p*I* value was found in adult monkey brain (lane *2*). In

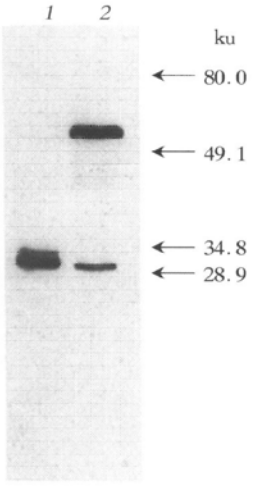

Fig. 1 Caspase-3 immunoreactive proteins in rat and monkey brains

Equal amount of total protein from either two-week-old SD rat (*1*) or adult monkey (*2*) brain tissue was separated in an SDS-polyacrylamide gel followed by the transfer and immunodetection using anti-caspase-3 antibody (H277) as described in **Materials and methods.**

combination with the result obtained from 2-D electrophoresis (Figure 2b), the band with a high p*I* value was corresponding to the small spot which showed its molecular mass at 32 ku, while that strongly stained band with a lower p*I* value in lane *2* of Figure 2a was to the large spot with molecular mass approximately at 64 ku. These results suggest that the 64 ku band is an unknown protein present in adult monkey brain and unlikely to be any form of caspase-3.

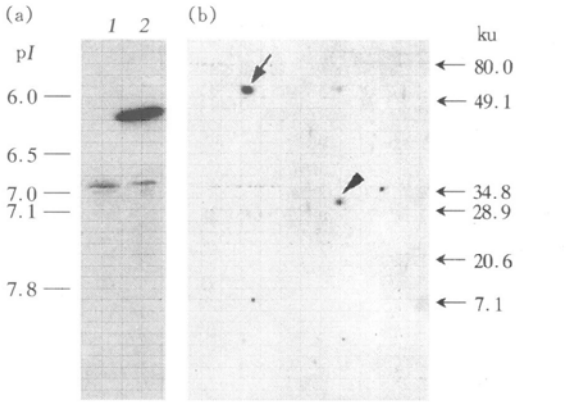

Fig. 2 Native IEF and 2-D electrophoresis combined with immunoblotting of caspase-3

(a) native IEF of brain extracts from two-week-old rat (*1*) and adult monkey (*2*). (b) 2-D electrophoresis of proteins in adult monkey brain extract. Two caspase-3 immuonreactive spots were detected, a large one (arrow) and a small one (arrowhead). The other two small spots with high density and sharp edge were false positive signals.

2.2 Processing and activation of procaspase-3 by exogenous granzyme B

As shown in Figure 3, procaspase-3 in untreated primate brain extract remained intact during the whole incubation period. Upon exposure to purified granzyme B, the 32 ku preform of caspase-3 was cleaved into three fragments, i.e. 20 ku, 19/17 ku and 12 ku (data not shown for the last one). If the caspase-3 selective inhibitor, Ac-DEVD-CHO, was present prior to the addition of granzyme B, the formation of 19/17 ku product was completely blocked while the 20 ku product accrued at the expense of the diminishing of 32 ku preform. These results indicated that procaspase-3 in monkey brain extract was efficiently processed by exogenous granzyme B in a two-step manner in which the full length procaspase-3 was firstly processed to the 20 ku intermediate and then cleaved into the 19/17 ku fragment. The second step was sensitive to caspase-3 inhibitor, so it was an autocatalytic process. In addition to the maturation of caspase-3 molecule, the matured caspase-3 was proven to be active through the cleavage of Ac-DEVD-*p*NA. As shown in Figure 4, the caspase-3-like activity increased significantly in brain extracts treated with granzyme B and Ac-DEVD-CHO was capable of blocking the activity ($P < 0.01$).

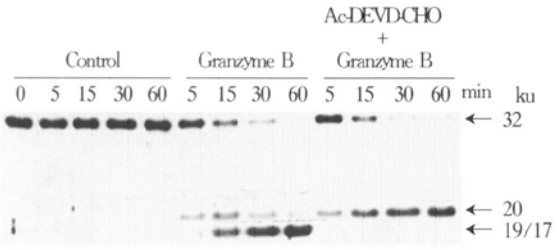

Fig. 3 Maturation of procaspase-3 in granzyme B-treated monkey brain extracts

Monkey brain extracts were incubated in the presence or absence of granzyme B or Ac-DEVD-CHO for indicated periods of time and then analyzed using Western blotting for caspase-3.

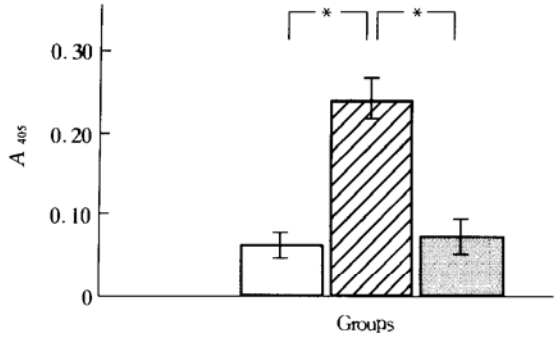

Fig. 4 Caspase-3 cleavage activities in monkey brain extracts

Brain extracts were incubated with Ac-DEVD-*p*NA for 15 min at 37℃ in the presence or absence of granzyme B (GrB) or Ac-DEVD-CHO (DEVD-CHO) as described in **Materials and methods**. The absorbance at 405 nm (A_{405}) was measured and results were expressed as $\bar{x} \pm s$. * $P < 0.01$. □: control; ▨: GrB; ▣: GrB + DEVD-CHO.

2.3 Cleavage of PARP and NAIP in granzyme B-treated brain extracts

As granzyme B cleaved procaspase-3 into its active form, it is reasonable to assume that the activated caspase-3 in the extract could subsequently result in the proteolysis of either Ac-DEVD-*p*NA or intracellular components in primate brain. PARP, a known substrate of caspase-3, was found not only in the nucleus but also in the cytoplasm of neurons[8]. The intact form of PARP is a 116 ku protein. Once exposed to granzyme B, PARP in the brain extract was efficiently cleaved into an 85 ku fragment (Figure 5). The production of this fragment is indicative of caspase-3 cleavage activity. As for NAIP, its degradation also occurred in the presence of granzyme B (Figure 6). In contrast to the control subjects in which the intensity of 164 ku full length NAIP remained unchanged throughout the whole period of incubation, a decrease of the intensity could be observed during the incubation with granzyme B and became remarkable at 120 min. Concurrently, a small band with molecular mass at 40 ku or so appeared at the beginning of incubation and its intensity gradually increased in all granzyme B-treated groups. Another small band just beneath the 40 ku one was found in almost all lanes regardless of the presence of granzyme B. That may be a nonspecifically degraded product or other proteins cross-reactive to the anti-NAIP antibody. The 40 ku fragment represents the N-terminal part of NAIP molecule at which the recognition site of anti-NAIP antibody is located. In addition, the production of this fragment is independent of caspase-3 activity, as it was not blocked by Ac-DEVD-CHO.

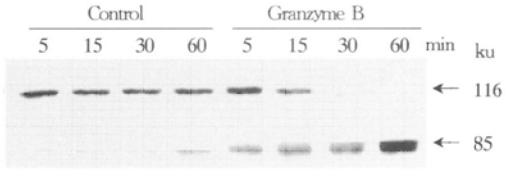

Fig. 5 Cleavage of PARP in granzyme B-treated monkey brain extracts

Brain extracts were incubated with or without granzyme B at 37℃ for indicated periods of time and then analyzed through Western blotting for PARP.

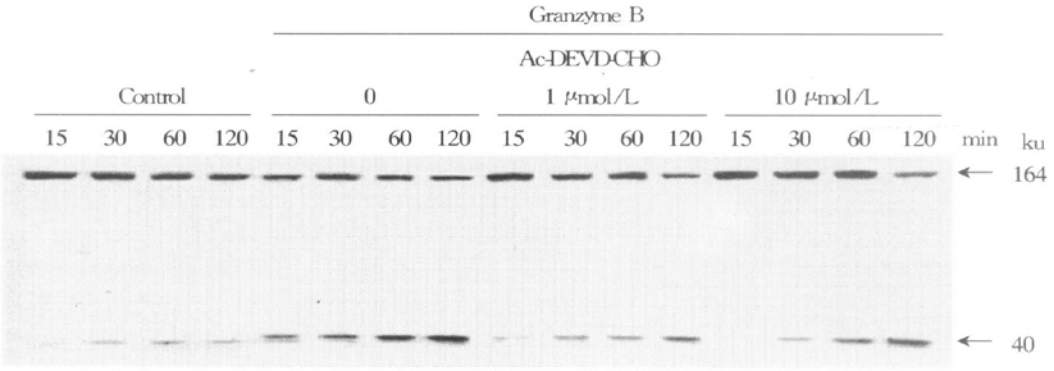

Fig. 6 Cleavage of NAIP in granzyme B-treated monkey brain extracts

Brain extracts were incubated at 37℃ for indicated periods of time in the presence or absence of granzyme B or different concentrations of Ac-DEVD-CHO. Proteins were analyzed through Western blotting for NAIP. The experiment was repeated in three occasions with the same result.

3 Discussion

Cell free system, based on the cytosolic fraction of living cells in culture, has been proven to be a valuable tool in studying many key problems in apoptosis, such as the ordering of apoptotic signal transduction and determination of the mechanisms by which apoptosis regulating gene products or inhibitors/stimulants of the process act[9,10]. Recently, a couple of studies adopted developing brain extracts from rodents to investigate cytochrome c-triggered caspase activation pathway[11,12]. Our group found that unlike rodents, rhesus monkeys constitutively expressed caspase-3 zymogen in their brains throughout lifetime (manuscript in preparation). In order to ascertain if adult monkey brain extract can be used as an alternative cell-free system in studying caspase-mediated proteolysis, we applied the criterion of which the cleavage pattern in conventional cell-free system should be reproduced in the brain extract, i.e., upon exposure to a proper stimulus, the constitutively expressed procaspase-3 in the brain extract is cleaved and becomes active in processing its substrates. *In vitro* studies have shown that activating executioner caspases by initiators is efficient and fast, and granzyme B cleaves caspase-3 zymogen more efficiently than most, if not all, initiator caspases[13]. With IEPD (P_4-P_1) as its optimal tetrapeptide recognition motif, granzyme B preferentially cleaved the single chain caspase-3 zymogen at the site of IETD (D_{175}) between the large and small subunits. That resulted in the creation of a 12 ku small subunit and a 20 ku intermediate that is composed of large subunit and the prodomain. This step is prerequisite for the activation of caspase-3 because the separation of C-terminus of large subunit from N-terminus of small one makes it possible for the two subunits to interdigitate and to fold properly into the active conformation. Next, the 20 ku intermediate undergoes further cleavage at D_9 or D_{28} to generate a 19 ku or a 17 ku fragment, respectively. Studies using site-directed mutagenesis[14] or site selective inhibitors[15] confirmed that cleavage occurred through autocatalysis at both sites. However, factors that determine the cleavage at either D_9 or D_{28} is yet unknown. Recently, Kamradt *et al.*[16] reported that a small heat shock protein, αB-crystallin, can interrupt both the mitochondrial and death receptor pathways by binding and inhibiting the intermediate of caspases-3. This indicates that the autocatalytic process in the maturation of caspase-3 is also subject to regulation.

Up to now, more than 40 intracellular proteins undergo specific cleavage in apoptotic cells or under cell-free conditions by caspases and non-caspase proteases and the number is still growing. In this study, we observed the cleavage of PARP to an 85 ku fragment. However, the 64 ku product, indicative of the cleavage by granzyme B, was not seen (data not shown). Froelich *et al.*[17] found that the 64 ku fragment, though much less abundant than the 85 ku fragment, transiently appeared 30 min after the addition of granzyme B and perforin into the medium of Jurkat cells. The transient appearance of 64 ku fragment could be explained by the access of granzyme B to nuclei that are abundant in PARP and the subsequent rapid cleavage of the fragment by caspase-3. In our cytosol-like extract, where the level of PARP is much lower, the 64 ku fragment is probably too little to be detectable. So caspase-3 exhibits a much higher activity than granzyme B in processing PARP.

In addition to PARP, we focused on the fate of NAIP, an intracellular anti-apoptotic component, in the brain extract treated with granzyme B and found that the full-length NAIP was cleaved to produce a small fragment of approximately 40 ku. The cleavage was not blocked by Ac-DEVE-CHO and so unrelated to caspase-3 activity. This result is, to some extent, consistent with the finding of Roy that recombinant caspase-3 and NAIP did not bind tightly *in vitro*[18]. Despite the lack of any other reports on the relationship between granzyme B and NAIP, the cleavage site in NAIP could be deduced on the basis of proteolytic features of granzyme B and the size of cleavage product found in our present study. As shown in Figure 7, there are two sites in NAIP molecule containing Asp residue (D_{353} and D_{376}), at each site the cleavage may release a fragment of approximately 40 ku. One of the cleavage sites is located within BIR3 domain and the other immediately downstream to it. The latter one has the sequence VTPD that is very similar to VDPD at the granzyme B cleavage site in PARP[19]. So it is more likely that granzyme B cleaves NAIP after D_{376}.

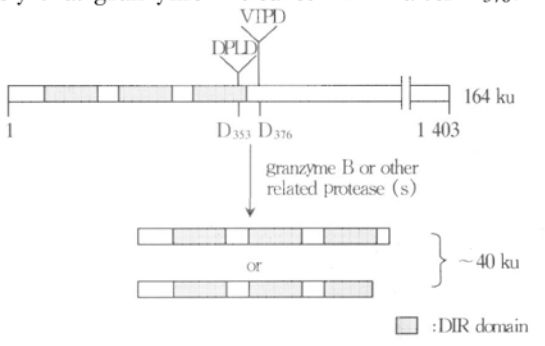

Fig. 7 Putative cleavage sites in NAIP protein

Human NAIP is a 164 ku single chain protein containing three DIR domains near its N-terminus. The recognition site of the polyclonal anti-NAIP antibody used in this study is located at the N-terminal end of NAIP. According to the size of the cleavage product, proteolysis is likely to occur after either D_{353} or D_{376} in NAIP molecule.

The possible link between granzyme B and NAIP suggests that a totally different mechanism might exist in granzyme B-triggered apoptotic signaling pathway in addition to activating executioner caspases and cleaving some of caspase substrates directly. The cleavage of NAIP by granzyme B is reminiscent of those findings that Bcl-2, Bcl-X_L, and c-IAP1 could be cleaved by caspases-3-like caspases and the cleavage resulted in the conversion of these proteins from anti-apoptotic to pro-apoptotic molecules[20,21]. However, NAIP appears unique in both its structure and function compared with other IAP family members. Since NAIP lacks the C-terminal ring domain in its primary structure and this domain is believed to contribute to the pro-apoptotic feature of cleaved IAP1, NAIP cleavage by granzyme B may lead to an alternative outcome. Further efforts should be taken to investigate whether NAIP is cleaved *in vivo* and what effects of the cleavage will have on the function of NAIP and on the process of cell death.

In conclusion, caspase-3 exists in normal adult monkey brain in its dormant preform. Granzyme B can trigger caspase-3 activation in a simple cytosolic fraction from adult monkey brain tissue as in living cells or commonly used cell-free systems. This extract has been proven useful in pursuing downstream events associated with active caspase-3, or granzyme B. Activated caspase-3 in granzyme B-treated brain extract can process PARP but not NAIP, whereas granzyme B may cleave NAIP directly or through a hitherto unknown intermediate protease. These results suggest that the substrate spectrum of granzyme B is different from that of caspase-3 in neural cells and granzyme B may utilize a means of destroying endogenous inhibitors of apoptosis to ensure a rapid destruction of its target cells.

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猕猴脑胱天蛋白酶-3活化及其靶蛋白的体外研究

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摘要 凋亡的主要生化过程包括胱天蛋白酶的活化及其对细胞内蛋白质的选择性切割. 在已知的胱天蛋白酶中，可被多种凋亡刺激信号激活的胱天蛋白酶-3备受注目. 为进一步揭示灵长类动物神经组织中未知的胱天蛋白酶-3靶蛋白，采用成年猕猴脑组织粗提物作为无细胞体系，通过加入granzyme B引发凋亡途径的部分反应，如胱天蛋白酶-3的活化及随后发生的蛋白质水解. 经蛋白质印迹分析发现，与granzyme B共孵育后，猕猴脑胱天蛋白酶-3以两步方式从酶原转化为活性酶. 对猕猴脑组织自身蛋白质的进一步分析显示，多聚ADP-核糖聚合酶 (PARP) 被水解为长85 ku的片段，此片段提示胱天蛋白酶-3的特异切割活性. 此外，神经元凋亡抑制蛋白 (NAIP) 也被切割，产生长约40 ku的小片段，但是它的出现不被胱天蛋白酶-3特异性抑制剂Ac-DEVD-CHO阻断，因此可能是granzyme B直接作用于NAIP所致. 以上结果提示，凋亡相关酶切反应可在成年猕猴脑组织提取物中得到重现；NAIP可能是granzyme B而非胱天蛋白酶-3的作用靶点.

关键词 凋亡，granzyme B，胱天蛋白酶-3，多聚ADP-核糖聚合酶，神经元凋亡抑制蛋白，灵长类，脑

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