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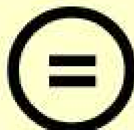

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博士學位論文

Signal transduction pathway involving caspase-2, -4 and -12

朝 鮮 大 學 校 大 學 院

生 物 新 素 材 學 科

Giri Raj Dahal

케스페이즈 -2, -4, -12의 세포사멸 신호전달 체계

Signal transduction pathway involving caspase-2, -4. -12

2007年 8月 24日

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TABLE OF CONTENTS

LIST OF TABLES..........IV

LIST OF FIGURES..........V

ABSTRACT..........VI

INTRODUCTION..........1

I-1. Caspases..........1

I-2. Classification of caspases..........2

I-3. Caspase-2..........3

I-4. Caspase-4..........4

I-5. Caspase-12..........5

I-6. Outline of thesis..........7

II. MATERIALS AND METHODS..........9

II-1. Materials..........9

II-2. Construction of recombinant caspases..........9

II-3. Purification of caspases..........10

II-4. Enzyme assay and determination of kinetic parameters....11

II-5. Cell culture and Cell death assay..........12

II-6. Caspase activity assay..........12

II-7. Nuclear DNA fragmentation assay..........13

II-8. Cell extract preparation and western blotting..........13

II-9. Analysis of cytochrome c release..................................14
II-10. In vitro DFF cleavage assay..14
II-11. DNA fragmentation assay...14
II-12. In vitro apoptosis induction..15
II-13. Caspase cleavage assay...15

III. RESULTS AND DISCUSSION..16
III-1. Caspase-2 mediates nuclear DNA fragmentation and cell death without caspase-3 activation..16
III-1-1. Construction of cell permeable Tat-reverse-caspase-2 and its biochemical characterization.......................................18
III-1-2. Tat-reverse-caspase-2 mediated cell death in cultured mammalian cells..21
III-1-3. VDVADase but not DEVDase and IETDase activity was detected in Tat-reverse-caspase-2-treated cells 21
III-1-4. Nuclear DNA fragmentation occurred..........................24
III-1-5. Release of cytochrome c without Bid cleavage...........27
III-1-6. Tat-reverse-caspase-2 cleaved DFF45/ICAD without caspase-3 activation and induced DNA fragmentation *in vitro* 27
III-2. Substrate specificity of caspase-4 in comparison with caspase-12, as effecter molecules in endoplasmic reticulum-dependent apoptosis..32
III-2-1. Kinetic Assay of Caspases-4 and -12......................32
III-2-2. Caspase-4 can induce DEVDase activity in vitro and

processes caspases-3 and- 7..35
III-2-3. Caspase-4 processes ICAD/CAD and releases CAD....35
III-2-4. Caspase-4 process Bid weakly................................39
III-3. Related studies and prospects of further research..........43
III-3-1. Tat-reverse-caspase-4 and -12 induced cell death...43
III-3-2. Checking the procaspase-9, -12 and Bid cleavage by caspases..44
III-3-3. Checking the cleavage of APE by caspases................44

IV. REFERENCES..49
V. ACKNOWLEDGEMENTS..57
VI. ABBREVIATIONS...58
VII. 적 요..59

LIST OF TABLES

Table 1 Kinetic parameters of caspase-2 and Tat-reverse-caspase-2 20
Table 2 Kinetic parameters of caspase-4 33

LIST OF FIGURES

Fig. 1. Purification of recombinant caspase-2's........................19

Fig. 2. Tat-reverse-caspase-2-induced cell death in HeLa and SHSY5Y cells..22

Fig. 3. VDVADase but not DEVDase activity was observed in Tat-reverse-caspase-2-mediated cell death......................23

Fig. 4. Nuclear DNA fragmentation assay..................................25

Fig. 5. Cytochrome c release and DFF40/CAD-DFF45/ICAD cleavage occurred without Bid cleavage and caspase-3 activation...26

Fig. 6. Tat-reverse-caspase-2 and caspase-2 cleaved DFF40/CAD-DFF45/ICAD in vitro and caused DNA fragmentation..29

Fig. 7. Caspase-12 but not caspase-4 can cleave caspase-12...34

Fig 8. Caspase-4 induce DEVDase activity in SK-N-BE(2) cell extact...36

Fig. 9. Caspase-4 processess caspase-3 and-7.......................37

Fig. 10. Caspase-4 processes CAD/ICAD and release CAD.........38

Fig. 11. Caspase-4 processess Bid weakly...............................40

Fig. 12. Three major apoptotic pathways in mammalian cells.......42

Fig. 13 Tat-reverse-caspase-4 induced cell death......................45

Fig. 14. Tat-reverse-caspase-12 induced cell death...................46

Fig. 15. In vitro cleavages of procaspase-9, procaspase-12 and Bid by different caspases..47

Figure 16. In vitro cleavages APE by caspases..........................48

ABSTRACT

Signal transduction pathway involving caspase-2, 4 and -12

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The signal transduction pathway of caspase-2 has not been known well. In this report, cell permeable Tat-reverse-caspase-2 was employed to induce caspase-2-specific cell death with high efficiency. Cell death occurred as early as 2 hr, and caspase-2-specific VDVADase activity was detected but not other caspase activities including DEVDase and IETDase. Interestingly, nuclear DNA fragmentation occurred without activation of caspase-3. Consistently, DFF45/ICAD was cleaved inside the cell and also *in vitro* by caspase-2, suggesting that the caspase could transduce the apoptotic signal to the downstream through nuclear DNA fragmentation without involvement of caspase-3.

Caspase-12 is endoplasmic reticulum (ER)-specific caspase in Rodent. In human, however, caspase-4 appeared to play the similar roles. This study was carried out to compare the enzymatic characteristics of caspase-4 and caspase-12. Ac-WEHD-AMC is found to be the best caspase-4 substrate in

terms of k_{cat}/K_M, followed by Ac-LEHD-AMC, Ac-LEVD-AMC, Ac-IETD-AMC, Ac-VEID-AMC and Ac-DEVD-AMC while caspase-12 showed no activity with any of these substrates. Caspase-4 could induce DEVDase activity in SK-N-BE (2) cell extract and could cleave downstream caspases such as caspases-3 and -7. Caspase-4 could also induce DNA fragmentation upon incubation with ICAD/CAD and cleave proapoptotic Bcl-2 family protein Bid, although they were weak. None of the above proteins, however, were cleaved by caspase-12. These results suggest that caspases-4 and -12 transduce the apoptotic signal to the downstream pathway through different pathway in spite of their similar physiological role in ER stress-mediated apoptosis.

I.Introduction

I-1. Caspases

Caspases are cysteinyl aspartate proteinases that cleave their substrates following an Asp residue [1]. There are 11 caspases described in human, 10 in mouse, 4 in chicken, 4 in zebra fish, 7 in *Drosophila melanogaster* and 4 in *Caenorhabditis elegans* [2]
Caspases reside in almost all healthy cells as inactive precursors called zymogens with little catalytic activity and kept in check by a variety of regulatory molecules [1]. They undergo proteolytic processing to generate two subunits that comprise active enzyme [3]. The cleavage of the zymogen is not always an obligatory requirement for caspase activation, but all activated caspases can be detected as cleaved fragments in apoptotic cells [3]. Mature caspases is a heterotetramer, composed of two heterodimers derived from two precursor molecules.

A molecular hallmark of apoptosis is the activation of caspases- specific proteases that execute cell death through cleavage of multiple protein substrates [4]. There are two well characterized apoptotic pathways in mammalian cells; extrinsic pathway and intrinsic pathway [4 . The extrinsic pathway exemplified by the elimination of unwanted cells during animal development is initiated by ligand induced activation of death receptors at the plasma membrane [5]. The intrinsic cell death pathway on the other hand is triggered by cellular stress signals such as DNA damage [5]. The onset of apoptosis requires a cascade of sequential activation of initiator and effector caspases. [6].

I-2. Classification of Caspases

Caspases are classified based on their prodomain structure or primary function. Caspases-1, -2, -4, -5, -9, -11 and -12 contain a long prodomain with a caspase activation and recruitment domain (CARD). Among these CARD-containing caspases, caspase-1, -4, -5 and -11 have functions in the inflammatory response, while caspases-2, -9 and -12 play roles in apoptosis. Caspases-8 and -10 possess two death effectors domains (DEDs) in their prodomains, and are also apoptotic family members. Caspases-3, -6, -7 and -14 has short prodomains and are effector caspases in the apoptotic pathway[7].

The first method, utilizing the structural characteristics of each caspase, places the mammalian family members into two main categories: long prodomain and short prodomain. Caspases-1, -2, -4, -5, -8, -9, -10, -11 and -12 belong to the former category. Each has a long prodomain that encompasses structural motifs in the death domain super family including the caspase activation and recruitment domain (CARD) or death effector domains (DEDs). These motifs enable caspases to associate with other proteins via homotypic interaction mechanisms. Caspases-3, -6, -7 and -14 fall into the latter category. These caspases bear short prodomains and are activated upon proteolytic cleavage by other caspases.

The second method of classification divides the caspases into two main streams on a functional basis, distinguishing between inflammatory and apoptotic caspases .Caspases-1, -4, -5, and -11 have been reported to play roles in cytokine maturation and inflammatory responses. The remaining family members are primarily involved in apoptotic signaling pathways. These apoptotic caspases can be further divided into 'initiators' (caspases-2, -8, -9, -10, -12) and 'effectors' (caspases-3, -6, -7, -14). Caspases in general can be categorized in two categories: initiator or apical caspase and

effector or executioner caspase [6]. Initiator caspases function upstream within apoptotic signaling pathways. They are capable of activating downstream caspases (effector caspases) either directly, through proteolysis, or indirectly via a secondary messenger mechanism. Upon activation by an initiator caspase, effector caspases are immediate ‘executioners’ of the apoptotic program, cleaving certain cellular substrates to cause demolition of the cell. Interestingly, these two methods of classification yield a close structure-function relationship among the caspases: all initiator apoptotic caspases contain a large prodomain whereas all effector caspases have a short prodomain.

Initiator caspases are activated through catalytic intrachain cleavages[3,6].Although the intrachain cleavage is essential for the activation of some initiator caspases, it has only modest effect on the catalytic activity of other initiator caspases and may not be required for their activation. The activation occurs on specific adaptor protein complex ([8-10]. The activation of caspase-2 in mammalian cell depends on PIDDosome [11], caspase-8 on DISC and caspases-9 on apoptosome [12].

The activation of effector caspases, such as caspase-3 and -7 is executed by an initiator caspases such as caspase-9, through proteolytic cleavage after a specific internal Asp residue to separate the large and small subunits of the mature. As a consequence of the intrachain cleavage, the catalytic activity of an effector caspase is enhanced by several orders of magnitude[13]. Once activated the effector caspases are responsible for the proteolytic degradation of a broad spectrum of cellular targets that ultimately lead to cell death. [4]

I-3. Caspase-2

Nedd2 was originally identified using subtraction cloning as developmentally

down-regulated gene in the mouse brain [14]. Using a murine Nedd2 cDNA a human foetal brain cDNA library was screened at low stringency and Ich-1, the human homologue of Nedd2 was identified[15].The mRNA that codes for caspase-2 is alternatively spliced into two forms one encoding a protein of 435 amino acids and other encoding a protein of 312 amino acids. Over expression of Ich-1_Lin some but not all cell types results in apoptosis, whereas over expression of Ich-1_S suppresses apoptosis induced by serum withdrawal suggesting that Ich-1 may play a role in both the positive and negative regulation of programmed cell death[16] .

Various studies have suggested the involvement of caspase-2 in tropic factor deprivation death[16], amyloid- induced neuronal cell death [17], TNFα [18] and Fas-induced, Ischemic neuronal death and DNA damage/Mitochondrial permeabilization [18-20] death pathways. Recently, a role of caspase-2 in metabolic regulation of oocyte cell death through the camKII-mediated phosphorylation of caspase-2[21.

Recent findings have demonstrated that the activation of procaspase-2 occurs in a large caspase-activating complex termed PIDDosome, which includes Rip-associated ICH-1/CED-3- homologous protein with a death domain RAIDD and P53 induced protein with a death domain PIDD [11]. The PIDDosome was described as analogous to apoptosome but its role is not clearly established as apoptosome.

I-4. Caspase-4

Three groups independently cloned ICreIII/TX/ICH-2(caspase-4[22, 23]. Caspase-4 belongs to caspase-1 subfamily. Caspase-4 along with caspase-1 and -5 in humans and caspase-1, 11 and -12 are categorized as inflammatory caspases (also known as group I caspases).Caspase-4 expression generally shows a similar tissue distribution being found in most

tissues examined with the exception of brain. Appreciable levels are found in both lung and liver and also in ovary and placenta where caspase-1 mRNA is barely detectable. Caspase-4 may be involved in the maturation of caspase-1[23]. The role of caspase-4 is not clear however caspase-5 together with caspase-1 was found to be components of the NALP1 inflammasome, a complex involved in the activation of caspase-1 [24] These findings reinforced the hypothesis that different inflammatory caspases may cooperate for full activity. Sequence comparison of the caspase domain and prodomains of the inflammatory caspases suggests that both caspase-4 and caspase-5 probably arose following the duplication of a caspase-11 ancestor gene [25].Little is known about the second possible caspase-11 orthologue caspase-4, although a few reports have suggested that caspase-4 may play a role in endoplasmic reticulum (ER) stress-induced apoptosis [26] a conclusion challenged by other studies [27].

I-4. Caspase-12

Caspase-12 was cloned from a murine L929r2 fibrosarcoma cDNA library in the year 1997.[28]. Caspase-12 gene is located in chromosome 9. The mRNA of caspase encodes a protein of 48 kDa. Based on primary sequence homology caspase-12 can be classified as an inflammatory caspase, since it shares the highest amino acid sequence identity with murine caspase-1 and -11 and human caspase-1, -4 and- 5.

In humans, caspase-12 has been dubbed ‘pseudo-caspase-12’as it contains coding sequence aberrations that prevent the translation of the putative full-length enzyme· This argues against the existence of a functional caspase-12 in humans and its hypothesized physiological roles in ER stress response. Interestingly, it has subsequently been reported that caspase-12 is naturally polymorphic and is expressed in its full-length form in 20% of

people of African descent. Individuals expressing full-length caspase-12 are more susceptible to sepsis as a result of attenuated inflammatory and innate immune response to endotoxins. These observations suggest that caspase-12 may have dual roles in apoptotic and inflammatory functions, which may also be species-dependent. Caspase-12 is specifically localized on the cytoplasmic side (outer membrane) of the ER and is thought to play a role in ER stress mediated cell death[29] .Caspae-12 is activated by ER stress stimuli such as tunicamycin, brefeldin A and thapsirgargin but not by member and or mitochondrial targeted apoptotic signals. ER stress mediated apoptosis is partly suppressed by caspase-12 deficiency suggesting involvement of caspase-12 in this apoptosis. Caspase-12 like most other members of the caspase family requires cleavage of the prodomain to activate its proapoptotic form. So far, several possible molecular mechanisms for the processing of caspase-12 have been postulated. A report suggested thatm-calpain another cysteine protease is responsible for cleaving procaspase-12 to generate active caspase-12 [29]. Caspase-12 is initially processed at the N-terminal region by calpain activated by ER stress. Caspase-12 is then activated and auto processed at D318. Thus calcium released from the ER may trigger a novel apoptotic pathway involving calcium mediated calpain activation and cross talk between the calpain and caspase families. Caspase-12 possesses a CARD domain [2] through which caspase-9 and Apaf-1 interacts and form apoptosis complexes. It may be possible that caspase-9 caspase-12 is activated by its association with an Apaf-1 like protein. GRP78 is involved in polypeptide translolcation across the ER membrane and also acts as an apoptotic regulator by protecting the host cell against ER stress induced cell death [30]. The other molecules which complexes with caspase-12 are TRAF-2. Caspase-12 is shown to be released from TRAF2 complexes by ER stress and is then auto processed via homodimerization [31].In response to ER stress TRAF2 plays crucial roles not

only in the signaling of the JNK pathway but also in the activation of caspase-12 to transduce signals form IRE1α. Thus TRAF-2 is a missing link in the ER stress induced apoptosis signaling pathway one which connects the stress sensor molecular IRE1α and the activation of caspase-12.

I-5. Outline of this thesis

The following chapters describe the studies on the characterization of caspase-2,-4 and -12. At first the study on caspase-2 signaling is presented. For this purpose, recombinant tat-caspase-2 was designedwhere protein transduction domain (PTD) of HIV-1 tat protein was fused with the caspase-2 protein. The protein was transduced to mammalian cells to check its signal transduction pathway. This study, for the first time revealed a novel pathway of caspase-2 signal transduction. Caspase-2 processes DFF40/45 complex releasing DFF40, which in turn causes the DNA degradation and ultimately cell death. DFF40/45 as a caspase-2 substrate was also supported by in vitro experiments.

Next, the study of substrate specificity of caspase-4 in comparison with caspase-12 as a molecule involved in Endoplasmic Reticulum-stress mediated apoptosis is presented. Enzyme assays using various peptide substrates showed that caspase-4 showed the activity towards 6 different substrates LEVD-AMC, DEVD-AMC, IETD-AMC, VEID-AMC, LEHD-AMC, whereas caspase-12 showed no activity with any of them. Caspase-4 induced DEVDase activity in cell extract prepared from BE (2) cells and processed different apoptotic proteins like caspase-3/-7, ICAD/CAD and Bid while none of these were processed by caspase-12. In conclusion, this study showed that though caspase-4 and -12 are implicated in ER stress-mediated apoptosis their physiological role is different.

Finally, some experiments apart from the mainstream research are

presented as they hold prospects for further research. The cell permeable tat-reverse-caspase-4 and tat-reverse-caspase-12 showed the cell death in SHSY5Y cells. A further study on all three caspases caspase-2, 4 and -12 to explore their signaling can be valuable. An invitro study showed the cleavage of Apurinic/apyrimidnic Endonuclease by caspase-3 and-9. Further study on the role of these caspases on APE activity can be of scientific interest. Additional studies that are not included here involve constructions of several plasmids, protein preparations and experiments on peptides.

II. MATERIALS AND METHODS

II-1. Materials

Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Life Technology Inc. (Grand Island, USA). DMEM/F-12 was from Welgene (Daegu, Korea). Caspase substrates were from A.G. Scientific Inc. (San Diego, USA). Ni-NTA and Hiram columns were from Amersham Biosciences (Piscataway, USA). Hepes, NaCl, $MgCl_2$, EDTA and EGTA were from USB (Cleveland, USA). DFF45 monoclonal antibody was purchased from Transduction Laboratories (Lexington, USA). Polyclonal antibodies to caspase-3 and cytochrome c were from Santa Cruz Biotechnology (Santa Cruz, USA). Polyclonal antibody against Bid was generated by injecting mice with purified recombinant Bid. dATP is from Promaga (Madison, USA). HEPES, NaCl, $MgCl_2$, EDTA, EGTA were from USB (Ohio, USA). All other chemicals were from sigma (St. Louis, USA).

II-2. Construction of recombinant caspases

The poor expression of Tat-reverse-caspase-2 in *E. coli* contributes to difficulties in its purification. To enhance soluble expression, trigger factor (TF, an *E.coli* chaperone) was utilized as a fusion partner. Ubiquitin was inserted between TF and Tat-reverse-caspase-2 to facilitate the efficient and specific cleavage by deubiquitylating enzyme, Usp2-cc. The prodomainless caspase-2 cloned in pET23b vector (Novagen bioscience Inc., Madison, USA) was used as a template to prepare reverse-caspase-2 and Tat-reverse-caspase-2. Briefly, caspase-2 small subunit amplified with PCR primers: 5'-GGATTCCATATGGCCGGTAAGAAAAGTTG-3' (sense), 5'-CGGATCCTGTGGGAGGGTGTCCTGG-3' (antisense) was cloned in

NdeI/BamHI site of pET21b TF-ubiquitin. The large subunit was amplified using single sense primer 5'-CGGATCCGGTCCTGTCTGCCTTCAAG-3'and three overlapping antisense primers (1) 5'-GCGTTT TTTGCGGCCATCTTGTTGGTCAACCCC-3', (2) 5'-ACGACGCTGGCGTTTTTGCGGCC-3' and (3) 5'-CCGCTCGAGACCACG ACGACGCTGGCGGCGTTT-3'. Thus amplified large subunit-Tat was then cloned in BamHI/XhoI site of pET21b TF-ubiquitin following caspase-2 small subunit that finally makes TF-ubiquitin-Tat-reverse-caspase-2.

To purify active caspase-4 we constructed full-length caspase-4 and caspase-4 (A94-N377) in which prodomain of caspase-4 is removed. pcDNA 3.1 caspase-4 (Kindly provided by Dr. Eguchi Osaka University, Japan) was used as template. For the construction of full-length caspase-4 and caspase-4 (A94-N377) Polymerase chain reaction (PCR) was performed caspase-4 CGGGATCCGGCAGAAGGCAACCACAG (sense) caspase-4 (A94-N377) CGGGATCCAGCTGGACCACCTGAGTCAG (sense) and CCGCTCGAGATTGCCAGGAAAGAGGTAG (antisense). The pcr products were cloned at Bamh1/Xho1 site of pET21b (-) vector

II-3. Purification of recombinant proteins

The recombinant caspases wereoverexpressed in *E. coli* BL21 (DE3) pLysS and protein induction was carried out with 0.4 mM isopropyl-β-D-thiogalactopyranoside at 30 ℃ for 4 hr. Proteins were initially purified in Ni-NTA column and after allowing the pooled fractions for cleavage with Usp2-cc at 37 ℃ for 2 hrs, the cleaved product was further purified by HiTrap Q and HiTrap CM column chromatography. Approximately 0.2 mg protein was obtained from one litre bacterial culture. Caspase-2 was purified using Ni-NTA and HiTrap Q columns.

The Caspase-4 DNA cloned in pET21b (-) vector was over expressed in

E.Coli BL21 (DE3) pLysS. Protein expression was induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside at 30℃ for 3 hr. The proteins were purified using Ni-NTA column (amersham Pharmacia) and Hi-TrapQ column (Pharmacia). Approximately 0.5 mg per liter protein was obtained. Caspase-3, caspase-8, caspase-9, Caspase-12 rev△pro1(prodomain less caspase-12 where small subunit is followed by large subunit), Bid and ICAD/CAD were also purified accordingly.

Expression of caspase-12 alone in *E.coli* cell is low. To increase the expression trigger factor (TF, an *E.coli* chaperone) was fused with caspase-12 △pro1C298S (Prodomain less caspase-12 C298S) and cloned in pET21b(-) vector. This DNA was transferred to *E.coli* BL21 (DE3) pLysS and purified as described earlier. The protein was named as TF-caspase-12 C298S.

II-3. Enzyme assay and determination of kinetic parameters

The enzyme activity was performed in caspase assay buffer containing 20 mM Hepes-NaOH, pH 7.0, 20 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 10 mM dithiothreitol at 30 ℃ in a total reaction volume of 100 l. The release of fluorescence from AMCwas monitored at excitation and emission wavelengths of 360 nm and 480 nm respectively, using micro plate spectofluoremeter (Molecular Devices, CA, USA). The basic catalytic parameters $V_{\max}$, K_M and k_{cat}were determined by employing Michaelis-Menten equation:

$$\frac{1}{v} = \frac{K_M}{V_{\max}[S]} + \frac{1}{V_{\max}}$$

$$v = \frac{V_{\max}[S]}{K_M + [S]}$$

$$V_{\max} = k_{cat}.[E]_T$$

Where, $[S]$, substrate concentration; v, initial velocity; V_{max}, maximum velocity; K_M, Michaelis-Menten constant; k_{cat}, catalytic efficiency of an enzyme; E_T, total amount of enzyme used.

II-4. Cell culture and cell death assay

Human epithelial HeLa and neuroblastoma SHSY5Y and BE(2) cells were cultured in DMEM and DMEM with nutrient F-12 (1:1) respectively, in 5% CO_2 at 37 ℃. The media weresupplemented with 10% heat inactivated serum (FBS for HeLa and SHY5Y and CS for BE(2)) penicillin (100 units/mL) and streptomycin (100 μg/mL). The cells were seeded at 20,000/well in 96-well micro titer plates and incubated for 16 hr. Then the cells were replaced by fresh medium to be treated with indicated concentrations of proteins, protein purification buffer and STS. After incubation at 37 ℃ for indicated time, cell viability was assessed by colorimetric MTT assay at 570 nm on Spectra Max 190 (Molecular Devices, CA, USA).

II-5. Caspase activity assay

Cells were treated with the indicated chemical or protein as described above for 2 and 6 hr. After removing media, the cells were washed with ice-cold phosphate-buffer saline (PBS) twice. Then 40 μL of enzyme assay buffer (20 mM Hepes-NaOH, pH 7.0, 1 mM EDTA, 1 mM EGTA, 20 mM NaCl, 1 mM DTT, 1 mM phenylmethanesulphonylchloride, 10 μg/mL leupeptin, 5 μg/mL pepstatin A, 2μg/mL aprotinin, 25 μg/mL ALLN) was added into each well and incubated on ice for 20 min. During this period, 96-well plate was vortexed to detach the cells. Caspase activity assay was carried out as

described above, for 2 hr in 2 minute interval with three different substrates (30 μM VDVAD-AMC, 10 μM DEVD-AMC and 30 μM IETD-AMC final concentration). The results were expressed as a slope of total readings verses time.

II-6. Nuclear DNA fragmentation assay

HeLa cells (10^6) were treated with the indicated chemical or proteins for 6 hr. The PBS washed cells were incubated at 55 ℃ for 2 hr in lysis buffer (100 mM Tris-Cl, pH 8.0, 0.2 M NaCl, 5 mM EDTA and 1% SDS, 0.2 mg/mL Proteinase K). Then, after adding 1.5 M NaCl (final concentration), cell debris was spun down for 20 min at 14000 rpm. The supernatant was mixed with an equal volume of phenol-chloroform mixture and centrifuged for 3 min at 14000 rpm. Top layer was transferred to a new tube and precipitated with two volumes of absolute ethanol and incubated at -20 ℃ overnight. The precipitated DNA was washed with 70% ethanol, resuspended in 30 μL of buffer (10 mM Tris, pH 7.5, 1 mM EDTA and 1 μg DNAse free RNAse) and incubated at 37 ℃ for 2 hr. The DNA samples were run onto 1.5% agarose gel at 20 V for 6 hr and visualized with ethidium bromide staining under UV light.

II-7. Cell extract preparation and western blotting

Harvested cells were washed with ice-cold PBS and resuspended in enzyme assay buffer on ice for 20 min. The cell extract was obtained by centrifuging at 14,000 rpm for 20 min at 4 ℃. Equal amount of proteins, measured by Bradford assay, were separated in SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-rad laboratories, Hercules, USA). After blocking, blots were developed with indicated antibodies separately.

Finally, blots were hybridized with horseradish peroxides-conjugated secondary antibodies and visualized with ECL plus reagent kit (Amersham Biosciences, Piscataway, USA).

II-8. Analysis of cytochrome c release

Cells were washed with ice-cold PBS and resuspended in digitonin buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 190 μg/mL digitonin). After 5 min incubation on ice, cells were spun at 14,000 rpm at 4 C for 5 min. Supernatants were transferred to fresh tubes and pellets were resuspended in buffer containing 25 mM Tris-Cl, pH 8.0, 1% Triton X-100. Proteins from each sample were subjected to SDS-PAGE and proceeded for western blotting.

II-9. *In vitro* DFF cleavage assay

HeLa cell extract was treated with enzymes in caspase assay buffer in a total reaction volume of 20 μL. After incubation at 30 ℃ for 1 hr, the samples were resolved in 15% SDS- PAGE and western blotted with DFF45 specific antibody.

II-9. DNA fragmentation assay

Plasmid DNA was mixed with enzymes in DNA fragmentation buffer (20 mM Hepes, pH 7.0, 20 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol and 1 g bovine serum albumin). Total reaction volume was maintained to 20 μL and incubated at 30 ℃ for 1 hr. The reaction was stopped with 20 mM EDTA. The samples were loaded into 0.7% agarose gel and visualized with ethidium bromide staining under UV light.

II-11. *In vitro* apoptosis induction

Human neuroblastoma SK-N-BE (2) cells extract (150 μg) was mixed with either buffer only or with caspase-4; caspase-9, 10 μM cytochrome c and 1 mM dATP in buffer containing HEPES-NaOH, pH 7.0, 20 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 10 mM dithiothreitol. For control reaction caspase-9 and caspase-4 was incubated without cell extract in the same condition. Final reaction volume was maintained at 50 μL and incubated at 30℃ for 2 hr. After the reaction was over, 30 μL of the content was mixed with 10 μM Ac-DEVD-AMC to check the enzyme assay as described earlier.

II-12. Caspase cleavage assay

Cleavage reactions of TF- caspase-12 C298S, Procaspase-3 C163S, procaspase-7 C186S and Bid were performed in final reaction condition of 20 mM HEPES-NaOH, pH 7.0, 20 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 10 mM dithiothreitol. Substrate and enzyme were mixed in a final volume of 10 μL and incubated at 30 ℃ for 2 hr. The products were analyzed by 13.5% SDS-polyacrylamide gel electrophoresis (SDS- PAGE).

III. RESULTS AND DISCUSSION

III-1. Caspase-2 mediates nuclear DNA fragmentation and cell death without caspase-3 activation

Caspase-2 is unique among the caspase family members as it shares the properties of both initiator and executioner caspases. Like initiator caspases caspase-8 and -9, it possess long prodomain containing CARD protein-protein interaction motif [32] and shares sequence homology with fellow initiator caspases caspase-1, -4, -5 and -9 [33]. The similarity in activation mechanism with caspase-8, and -9, described as dimerization to be an initial activating event, has also assigned caspase-2 into the group of initiator caspases [34, 35]. But its cleavage specificity bears a resemblance with effector caspases caspase-3 and -7 [35, 36]. Further, unlike initiator caspases, caspase-2 neither processes nor activates executioner caspases directly [37] but it is cleaved by caspase-3although the physiological significance behind this cleavage is unknown [3, 33].

In terms of substrate specificity, caspase-2 has exceptional requirement of pentapeptide substrate for efficient cleavage dissimilar from other caspases, which have only tetrapeptides their optimal substrates. VDVAD and VDQQD, both providing hydrophobic residues at P5 position are favored amino acid sequences for its synthetic substrate. It showed a similar preference with caspase-3 and -7 in P4 position but in P3 Val was preferred over Gln like that of caspase-1 and -4, while in P2 position, a structurally wide range of amino acids were tolerated [35].

A wide range of studies has reported various proteins to serve as caspase-2 substrates. They are procaspase-2 itself, golgin-160 and αII-spectrin [33, 38, 39]. The cleavage of its own precursor, golgin-160 and PKCδ establishes caspase-2 as initiator caspase but the cleavage of

αII-spectrin takes it into executioner category [33]. There is still a controversy regarding Bid as caspase-2 substrate due to inconsistent reports. Recombinant purified Bid was cleaved by active protein on direct treatment but failed to reproduce such cleavage in liver cell mitochondria and jurkat cell extract [40].

Various hypotheses have been put forward to explain the caspase-2 signaling that can broadly be categorized into mitochondria-dependent pathway and DNA damage/p53-mediated pathway. Some studies have suggested that caspase-2 activation occurs upstream of cytochrome c release [18,19,41]. Active caspase-2 has been shown to induce Mitochondria Outer Membrane Potential (MOMP) in presence of Bid [37, 40, 42]. But, others have proposed MOMP to occur independently of Bid and cytosolic factors [43]. Interestingly, a report proposed the existence of nuclear/mitochondrial pathway showing the localization of caspase-2 in the nucleus and triggering mitochondrial dysfunction from the nucleus without relocalizing into the cytoplasm [18].

The activation of procaspase-2 is shown to occur in a large caspase-activating complex [44] termed PIDDosome, which includes Rip-associated-ICH-1/CED-3-homologous protein with a death domain (RAIDD) and p53-induced-protein with a death domain (PIDD) [11,45,46].The PIDDosome was described as analogous to apoptosome but its role is not clearly established as that of apoptosome.

In the present study, it is demonstrated that caspase-2 induces cell death without activating caspase-3 but by cleaving DNA fragmentation factor (DFF45)/Inhibitor of caspase- activated DNAse (ICAD). It is proposed that caspase-2 transduces apoptotic signals via nuclear DNA fragmentation.

III-1-1. Construction of cell permeable Tat-reverse-caspase-2 and its biochemical characterization

This study is an effort to elucidate the role of caspase-2 in apoptotic pathway. Although caspase-2 was the second mammalian caspase discovered [16, 47], it remains least understood in terms of its activation mechanism, target substrates and potential roles in apoptosis [40]. The previous experiments include gene knockout and siRNA and plasmid transfection to study physiological significance of caspase-2. Considering some demerits of these methods like inefficient, laborious, and time-consuming in many cases, cell permeable peptide (protein transduction domain, PTD) was preferred in this study. PTD-linked proteins are easy to use, transduce inside cells on simply adding it into the medium, and to be internalized in a rapid, concentration-dependent manner, achieving maximum intracellular concentration in less than 15 min. [48] Nearly uniform distribution of the fusion protein within a culture is easily achievable; reducing the background unaffected cells [49]. To circumvent the possible problems to arise due to cleavage during purification and transduction, a reverse form of caspase-2 (reverse-caspase-2) was constructed where small subunit was followed by large subunit to make constitutively active without processing. The reverse-caspase-2 was fused with Tat protein transduction domain (RKKRRQRRR) of human immunodeficiency virus type I [50,51]and glycine residue was inserted at the both ends of Tat sequence to enhance its flexibility. The previous study has already confirmed that Tat peptide itself does not have any toxic effects [51].

Following the purification of proteins (Fig. 1), enzyme activities of caspase-2 and Tat-reverse-caspase-2 were checked using three different substrates VDVAD-AMC (VDVAD-7-amino-4- methyl coumarin), DEVD-AMC and IETD-AMC to ensure that addition of Tat did not alter caspase-2 properties. The comparison of their catalytic parameters (k_{cat}/K_M) confirmed

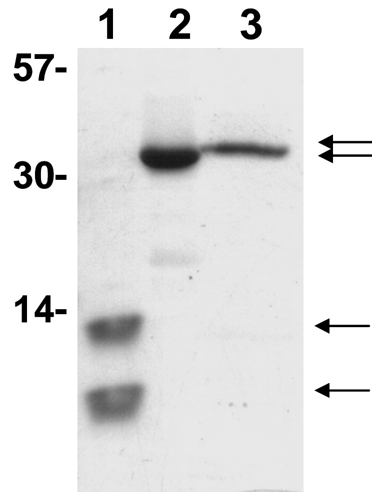

Fig. 1 Purification of recombinant caspase-2s. Three different caspase-2 constructs were designed and purified as described in materials and methods. The purified proteins were resolved in SDS-PAGE and the arrow on right indicates the protein bands. Lane 1; caspase-2, lane 2; reverse-caspase-2, lane 3; Tat-reverse-caspase-2.

Table 1 Kinetic parameters of caspase-2 and Tat –reverse-caspase-2

Substrates	Enzymes	$k_{cat}(S^{-1})$	$K_M(\mu M)$	$k_{cat}/K_M(M^{-1}S^{-1})$
VDVAD-AMC	Tat-reverse-caspase-2	1.25	14.5	86200
	Caspase-2	3.32	25.5	130000
DEVD-AMC	Tat-reverse-caspase-2	0.04	113	354
	Caspase-2	0.03	80.3	379
IETD-AMC	Tat-reverse-caspase-2	N.D.	N.D.	N.D.
	Caspase-2	N.D.	N.D.	N.D.

※ N.D. not detectable

Tat-reverse-caspase-2 possesses activity and specificity similar with native caspase-2 for all three tested substrates (Table 1). The VDVADase activity of Tat-reverse-caspase-2 and caspase-2 are comparable with previously reported ones but DEVDase activity is inconsistent with earlier report where no cleavage was detected up to 1 mM DEVD-pNA [35]. The reason for this discrepancy remains to be elucidated.

III-1-2.Tat-reverse-caspase-2 mediated cell death in cultured mammalian cells

The purified Tat-reverse-caspase-2 protein added directly into media resulted in cell death in both cell lines-SHSY5Y and HeLa. The cell death occurred in dose and time dependent manner (Fig. 2(a) and (b)). In HeLa cells, no cell death occurred up to 30 nM Tat-reverse-caspase-2 exposed for 6 hr and approximately 30% cell death occurred with 50 nM protein that increased to 50% with 100 nM. The 50 nM concentration of Tat-reverse-caspase-2 was then used to check time dependency of cell death. About 20% cell death was observed on 3 hr exposure that gradually increased to 30%, 50% and 70% in 6, 12 and 24 hr respectively. The similar pattern was found in SHSY5Y cells. For comparison, Tat-caspase-3 transduction produced a linear killing curve with 50% cell death at 4 hr exposure and reached nearly 100% in 16 hr [52]. On the basis of the above observations of time- and dose-dependency of cell death, short and medium time periods were selected for further experiments to ensure the cell death induced by caspase-2 itself but not by indirect activation of other caspases.

III-1-3.VDVADase but not DEVDase and IETDase activity was detected in Tat-reverse-caspase-2-treated cells

To verify the transduction of Tat-reverse-caspase-2 inside the cells and its

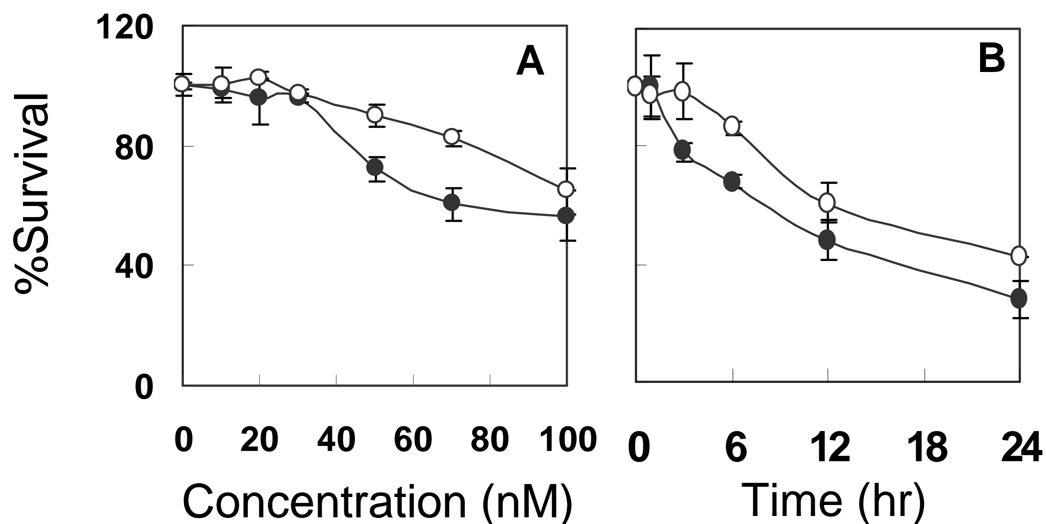

Fig. 2 Tat-reverse-caspase-2-induced cell death in HeLa and SHSY5Y cells. HeLa (●) and SHY5Y (○) cells were seeded for 16 hr at 37 ℃ prior to treatment. (A) The cells were exposed to 0, 10, 30, 50, 70 and 100 nM Tat-reverse-caspase-2 protein for 6 hr. (B) The cells were exposed to 50 nM Tat-reverse-caspase-2 and incubated for 0, 1, 3, 6, 12 and 24 hr. Cell survival rate at each time point was quantified by MTT assay. The result is an average of three independent experiments and the standard error bars are indicated.

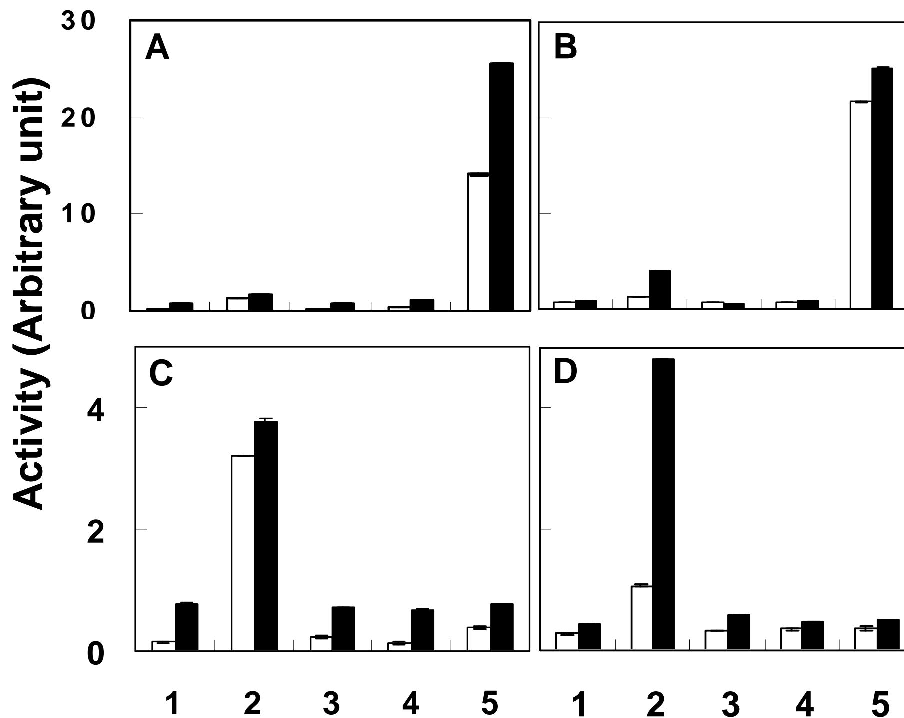

Fig. 3 VDVADase but not DEVDase activity was observed in Tat-reverse-caspase-2-mediated cell death.

The cells were left untreated (1) or treated with 1 μM STS (2), protein purification buffer (3), 50 nM caspase-2 (4) and 50 nM Tat-reverse-caspase-2 (5) for 2 hr (open bar) and 6 hr (closed bar). Following the treatment, caspase activity assay was performed. (A), (B) VDVAD-AMC activity of SHSY5Y and HeLa cells (C) and (D) DEVD-AMC activity of SHSY5Y and HeLa cells respectively. The results were expressed as a slope of readings verses time. The result is an average of three independent experiments and the standard error bars are indicated (in some cases the deviations were too small to be seen).

ability to trigger apoptotic cascades, various caspases activities were assayed. The exposure time was chosen as 2 hr to represent early phase of

apoptosis and 6 hr to analyze other apoptotic events appeared along with increment in cell death. VDVADase activity was observed in both time periods (Fig. 3(a) and (c)) while neither DEVDase nor IETDase activity was detected in both cells (Fig. 3(b), (d) and data not shown). In staurosporine (STS)-treated cells, DEVDase activity increased rather than VDVADase. These results indicate that no other caspases are involved in Tat-reverse-caspase-2-induced apoptosis at the used time points. The similar effects of Tat-reverse-caspase-2 transduction on cell death and caspase activity were observed in both HeLa and SHSY5Y cells. For this reason, in the following experiments, HeLa cells were mainly investigated to study the mechanism underlying caspase-2 signaling.

III-1-4. Nuclear DNA fragmentation occurred

Nuclear DNA into nucleosomal fragments is the most recognizable biochemical feature of apoptosis [53]. Nuclear DNA fragmentation was observed in HeLa cells treated with Tat-reverse-caspase-2 in a similar pattern as in STS-treated cells (Fig. 4). DFF45/ICAD-DFF40/CAD protein complex is essential for nuclear DNA fragmentation [54]and caspase-3 has been known to be the main enzyme to cleave this complex releasing DFF40/CAD which in turn fragmentizes the genomic DNA into nucleosomal fractions [55]. However, in our case, even in the absence of caspase-3-like activity, nuclear DNA fragmentation occurred, suggesting a possibility that Tat-reverse-caspase-2 can directly mediate the DNA fragmentation to cause cell death.

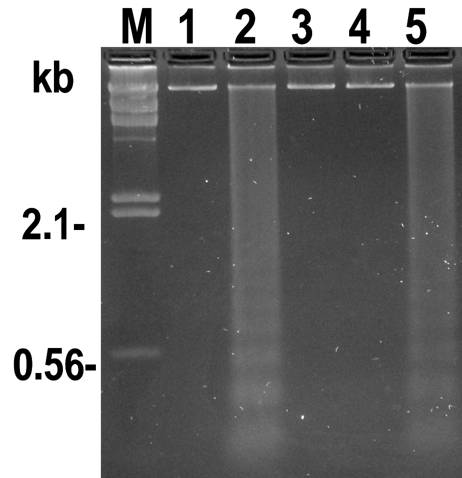

Fig. 4 Nuclear DNA fragmentation assay.

HeLa cells were left untreated (1) or treated with 1 μM STS (2), protein purification buffer (3), 50 nM caspase-2 (4) and 50 nM Tat-reverse-caspase-2 (5). Following treatment, cells were harvested and genomic DNAs were analyzed. The result is a representative of three independent experiments.

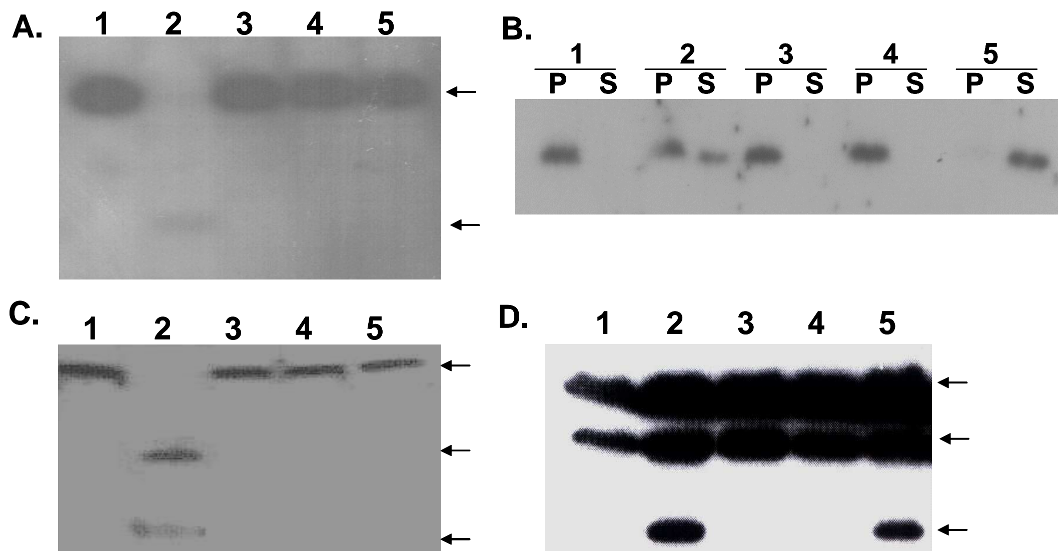

Fig. 5 Cytochrome c release and DFF45/ICAD-DFF40/CAD cleavage occurred without Bid cleavage and caspase-3 activation.

HeLa cells were left untreated (1) or treated with 1 μM STS (2), protein purification buffer (3), 50 nM caspase-2 (4) and 50 nM Tat-reverse-caspase-2 (5). Cell extracts were prepared from each group, equal amount of cell extracts were separated in SDS-PAGE and analyzed by western blotting with Bid (A), cytochrome c (B), caspase-3 (C), and DFF45 (D) antibody, respectively. In (B) the cells were separated into subcellular fractions and equivalent amountsof supernatant (S) and Pellet (P) fractions of each group were analyzed. Arrows indicate the uncleaved protein and corresponding cleavage products.

III-1-5.Release of cytochrome c without Bid cleavage

Bid is a proapoptotic Bcl-2 family protein that plays an important role in apoptotic cell death [56]. It is a substrate of caspase-8 that once processed, translocates to mitochondria and potently induces cytochrome c release [57, 58]. Bid has been also described as caspase-2 substrate by some reports [37, 57]. However, processing of the protein was not detected in Tat-reverse-caspase-2-exposed HeLa cells (Fig. 5(a)). Unexpectedly, cytochrome c was released without Bid processing (Fig. 5(b)).

The release of cytochrome c, a component of mitochondrial respiratory chain, is observed during the early stages of apoptosis [59, 60]. Once in the cytosol,cytochrome c interacts with adaptor molecule Apaf-1, resulting in the recruitment, processing and activation of procaspase-9 [12], which in turn activates procaspase-3 and -7 leading to apoptosis [41]. Considering these reports, it was also unexpected that caspase-3 was not activated (Fig. 2(c), 2(d) and 5(c)) in Tat-reverse-caspase-2-mediated cell death. At the moment, the mechanism for cytochrome c release without Bid cleavage and failure of caspase-3 activation is not known. We did not pursue these pathways further because from the above results it was speculated that this mightnot be the direct cause of cell death.

III-1-6.Tat-reverse-caspase-2 cleaved DFF45/ICAD without caspase-3 activation and induced DNA fragmentation *in vitro*

The nuclear DNA fragmentation occurred in Tat-reverse-caspase-2-treated HeLa cells (Fig. 4) and consistently, DFF45/ICAD was processed in the same pattern as STS- treated cells (Fig. 5(d)). Although caspase-3 was identified as an essential protease to release the active DNase from DFF45/ICAD that produces DNA fragmentation [61], no any caspase-3 (DEVDase) activity was detected during our study (Fig. 3). The result indicates that it occurred

through non-caspase-3-mediated pathway. One speculation for the DNA fragmentation might be that added Tat-reverse-caspase-2 can directly process the nuclear proteins. Actually, both caspase-2 and Tat-reverse-caspase-2 could process DFF45/ICAD resulting in similar products with that of caspase-3 although the former was much weaker (Fig. 6(a)).It was further confirmed by DNA fragmentation as Tat-reverse-caspase-2 indeed fragmentized the plasmid DNA, which is roughly 3 times weaker than that of caspase-3 (~50 ng of caspase-3 induced a complete DNA degradation, data not shown) but 2 times efficient than that of caspase-2 (Fig. 6(b)). These differing capabilities to cause DNA fragmentation suggest that in a certain optimal condition, caspase-2 can mediate the nuclear DNA fragmentation as efficiently as caspase-3.

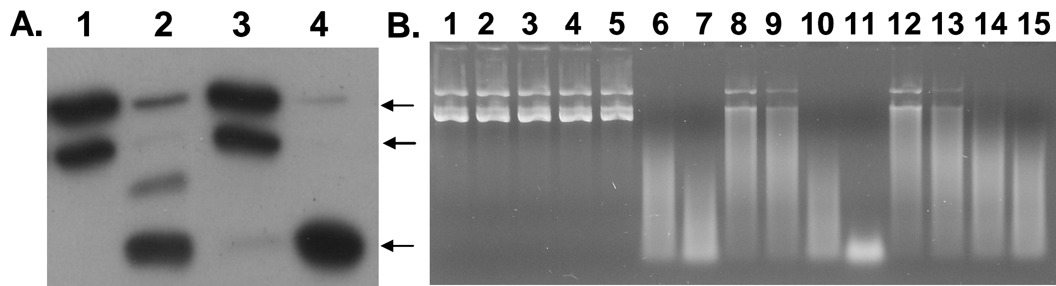

Fig. 6 Tat-reverse-caspase-2 and caspase-2 cleaved DFF45/ICAD-DFF40/CAD *in vitro* and caused DNA fragmentation

(A) HeLa cell extract was left untreated (1) or treated with 50 ng of caspase-3 (2), 100 ng of caspase-2 (3), 200 ng of Tat-reverse-caspase-2 (4) for 1 hr at 30 ℃. Each reaction mixture was separated in SDS-PAGE and western blotted with DFF45-specific antibody. (B) Plasmid DNA was incubated alone (1), mixed with 30 ng caspase-3 (2), 200 ng Tat-reverse-caspase-2 (3), 200 ng caspase-2 (4), 20 ng DFF45/ICAD-DFF40/CAD (5), 20 ng DFF45/ICAD-DFF40/CAD with 10, 30 ng caspase-3 (6, 7), 20 ng DFF45/ICAD-DFF40/CAD with 30, 50, 100, 200 ng Tat-reverse-caspase-2 (8, 9, 10 and 11), 20 ng DFF45/ICAD-DFF40/CAD with 30, 50, 100, 200 ng caspase-2 (12, 13, 14 and 15) at 30 ℃ for 1 hr.

This study has revealed that caspase-2 can directly process DFF45/ICAD and it may be one of the critical events to mediate caspase-2-induced cell death. DFF45/ICAD has not been described as a substrate for caspase-2 before. Interestingly, the cleavage sites of DFF45/ICAD by caspase-3, ^{219}VDAVD^{223} and ^{113}VDETD^{117} are similar with caspase-2 recognizable sequence VDVAD or VDQQD, respectively. The somewhat similarity in these sequences might explain the DFF45/ICAD cleavage by caspase-2. As cell permeable Tat-reverse-caspase-2 was utilized here, it is crucial to maintain the substrate specificity and catalytic efficiency of this enzyme with that of native caspase-2. The unaltered biochemical characteristics between caspase-2 and Tat-reverse-caspase-2 were confirmed by determining the catalytic parameters for caspase-2, -3 and -8 specific substrates (Table 1) Furthermore, both Tat-reverse-caspase-2 and caspase-2 could process DFF45/ICAD *in vitro* (Fig. 6(a)) although the latter was weak in terms of its cleavage efficiency. Consistently, *in vitro* DNA fragmentation assay with plasmid also illustrated that processing efficiency of Tat-reverse-caspase-2 and caspase-2 was comparable, with approximately two times better for the former (Fig. 6(b)).All data suggest that the signal transduction pathway exhibited by Tat-reverse-caspase-2 will in fact reflect that of native caspase-2.

The reason behind the higher activity for Tat-reverse-caspase-2 over normal caspase-2 for DFF45/ICAD is not known but some explanations are possible. It was reported that caspase-2 formed complex called PIDDosome to get activated [11,45,46]. This implies that caspase-2 activity in the cell may be different from what has been observed with the purified recombinant enzyme. This case is well exemplified in caspase-9 where the enzymatic activity increased by ~1000 times in the Apaf-1 apoptosome protein complex [62].It is possible that the addition of Tat peptide to caspase-2 might help to mimic its original activity as in the complex. In this context, the difficulty

for assigning roles of caspase-2 in the previous reports may be due to the ‘altered activity’ of the recombinant enzyme. With this assumption, searching for new caspase-2 substrates is underway using Tat-reverse-caspase-2. Especially, our focus is on some nuclear proteins to reveal the possible substrates of caspase-2 as the enzyme is known to be predominantly localized in the nucleus (~35%) [38].

Another interesting observation of the present study was the release of cytochrome c without processing of Bid (Fig. 5(b)). This result is inconsistent with the reports that caspase-2 processes Bid resulting in cytochrome c release [37, 57]. Some studies have indicated that although Bid was not a good substrate of caspase-2, small amount of cleaved Bid could induce the process of cytochrome c release [63]. Other lines of reports, however, have shown that caspase-2 directly interacted with the outer mitochondrial membrane to trigger the release of cytochrome c without the involvement of Bid [43]. Moreover, considering the report that DNA damage resulted in cytochrome c release from the mitochondria, it is also possible that nuclear DNA fragmentation as shown in Fig. 3 may be responsible for the release. Due to the huge diversity in the pathways of cytochrome c release, it is not clear which mechanism(s) are applicable for the results in this study. At this point the effect of cytochrome c release is not clear either, because caspase-3 was not activated. As the relevance of cytochrome c release and cell death remains to be established, no further research was presented here. The speculations and other possibilities are under investigation. In conclusion, on investigating the signal transduction pathway of caspase-2 with the aid of its cell permeable version, DFF45/ICAD was found to be its substrate. The occurrence of cell death accompanied by DFF45/ICAD cleavage consistent with nuclear DNA fragmentation in the absence of caspase-3 activation demonstrated a novel physiological role of caspase-2 in apoptosis.

III-2. Substrate specificity of caspase-4 in comparison with caspase-12, as effecter molecules in endoplasmic reticulum-dependent apoptosis

Caspase, a family of cysteine proteases, are critical mediators of programmed cell death [64] that are activated in a sequential cascade of cleavage by other caspase family members [65]. Caspase-8 mediates signal transduction downstream of death receptors, while caspase-9 mediate apoptotic signal after mitochondrial damage [12, 66]. Caspase-12 has been found to mediate endoplasmic reticulum (ER)-specific apoptosis pathway and contribute to amyloid -induced neurotoxicity in rodents [29]. Human caspase-12, however, doesn't seem to function but caspase-4 appeared to be primarily activated in ER stress-induced apoptosis that may be involved in pathogenesis of Alzheimer's disease [26] Caspase-4 has 48% homology with caspase-12 that belongs to the group I family of caspases with caspases-1, -5, -11, and -13 [67]. Considering these reports, we compared the enzymatic properties of caspase-4 with that of caspase-12 to understand the signal transduction pathways of two molecules involved in ER-dependent apoptosis.

III-2-1.Kinetic Assay of Caspases-4 and -12

To examine the substrate specificity of caspases-4 and -12 with different peptide substrates, a fluorometric assay was carried out using 7 different substrates. Ac-WEHD-AMC was the best substrate for caspase-4in terms of k_{cat}/K_M, followed by Ac-LEHD-AMC, Ac-LEVD-AMC, Ac-IETD-AMC, Ac-VEID-AMC and Ac- DEVD-AMC (Table 2). No activity was detected with Ac-VDVAD-AMC, the specific substrate for caspase-2. Caspase-12 showed no activity with any of these substrates but could cleave only caspase-12 itself (Fig. 7). On the other hand Caspase-4 did not cleave caspase-12.

Table 1 Kinetic parameters of caspase-4

Substrate	k_{cat} (S^{-1})	K_M (M)	k_{cat}/K_M ($\times 10^3 M^{-1} S^{-1}$)
WEHD-AMC	3.67	577	6.37
LEHD-AMC	4.89	1440	3.30
LEVD-AMC	0.37	165	2.25
IETD-AMC	0.39	540	0.80
VEID-AMC	0.81	1120	0.73
DEVD-AMC	0.19	301	0.64
VDVAD-AMC	N.D.	N.D.	N.D.

N.D. not detected

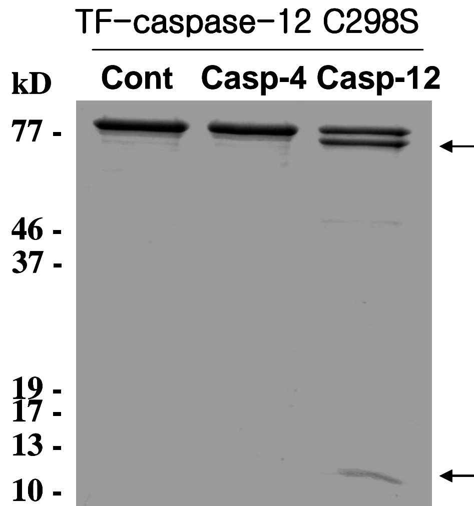

Fig. 7 Caspase-12 but not Caspase-4 can cleave Caspase-12.

TF-caspase-12 C298S (4 μg)was mixed with buffer, caspase-4 (200 ng) and caspase-12 (200 ng) and incubated at 30 ℃ for 2 hr and analyzed by 13.5% SDS-PAGE. Cont indicates the group without any enzymes. Relative molecular size (kD) and cleaved protein bands were indicated on left and right, respectively.

III-2-2. Caspase-4 can induce DEVDase activity in vitro and processes caspases-3 and- 7

Caspase-4 induced DEVDase activity in cell extract prepared from the human neuroblastoma cell line SK-N-BE (2). The activity was weaker, compared with groups of cytochrome c with dATP or caspase-9 (Fig. 8). The activity should be due to the processing of caspase-3 and -7 because the amount of added caspase-4 was not enough to result in the level of activity (Fig. 2). To confirm the argument, it was examined whether caspase-4 can directly cleave caspase-3 and caspase-7. Recombinant procaspases-3 and -7 bearing active site cysteine mutated to serine were used as substrates. Caspase-4 cleaved procaspase-3 into ~19 kD and ~14 kD and procaspase-7 into ~23 kD and ~14 kD, consistent with the expected sizes after their cleavage into larger and smaller subunits (Fig. 9). Unlike caspase-4, caspase-12 couldn't cleave either procaspase-3 or -7 (data not shown).

III-2-3. Caspase-4 processes ICAD/CAD and releases CAD

CAD/DFF40, the enzyme that causes DNA fragmentation during apoptosis, is complexed with its inhibitor (ICAD/DFF45) as latent form in non-apoptotic cells. Upon apoptotic stimuli activated caspase-3 cleaves ICAD/DFF45, which causes the release of CAD/DFF40 that degrades chromosomal DNA in nuclei [68-70]. Since caspase-4 itself has weak DEVDase activity (Table 2), it was examined whether caspase-4 could induce DNA fragmentation. Purified ICAD/CAD was treated with increasing concentrations of caspase-4 and the activity was assessed by the degradation of plasmid DNA. Caspase-4 could cause DNA fragmentation although it was weaker than caspase-3 (Fig.10),

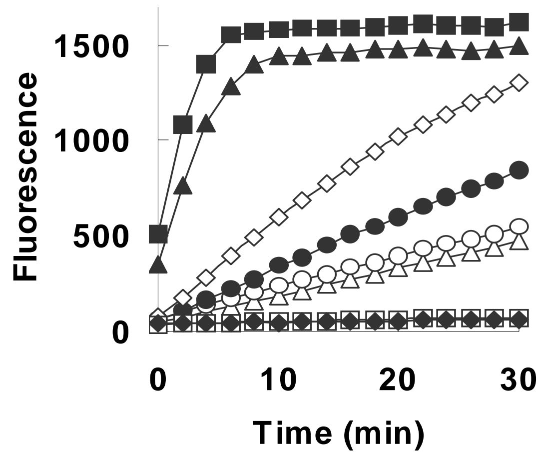

Fig. 8 Caspase-4 induce DEVDase activiy in SK-N-BE (2) Cell Extract.

SK-N- BE (2) cell extract (150 μg) was incubated with the buffer (△), 1 mM dATP and 10 M cytochrome c (▲), 0.5 μg caspase-9 (■), 0.5 (○), 1.0(●), 1.5 μg caspase-4(◇) at 30 ℃ for 2 hr. For controls, 0.5 μg caspase-9 (□) and 1.5 μg caspase-4 (◆) without cell extract were also incubated in the same. Afterwards 10 μM Ac-DEVD-AMC was added and fluorescence reading was taken.

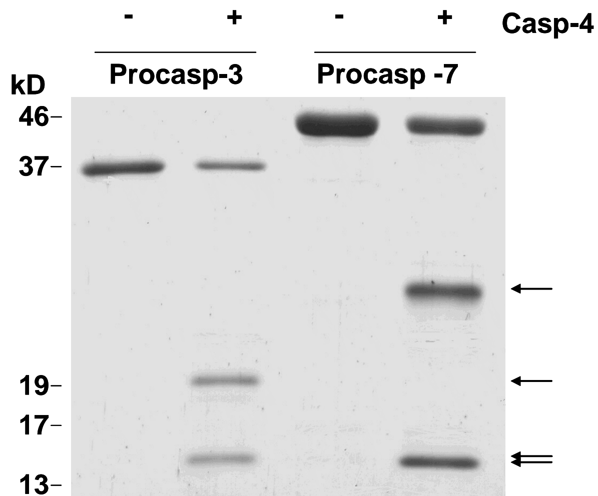

Fig. 9 Caspase-4 processess caspase-3 and -7. Procaspase-3 C163S (0.7 μg) and procaspase-7 C186S (3 μg) were incubated with buffer or caspase-4 (200 ng) at 30 ℃ for 2 hr and analyzed by 13.5% SDS-PAGE. Relative molecular weights (kD) and cleaved protein bands were indicated on left and on right by arrows.

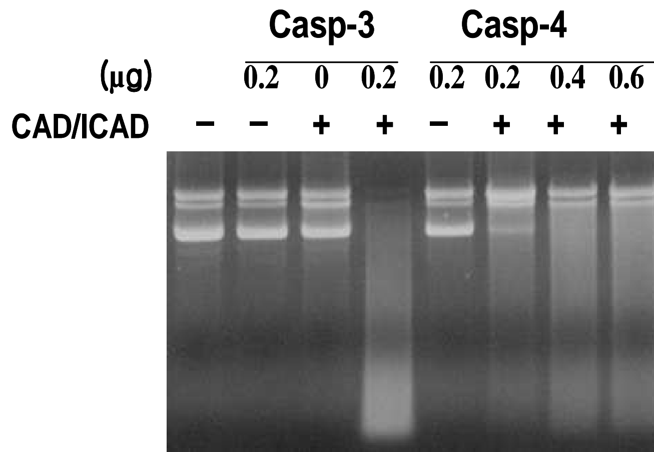

Fig. 10 Caspase-4 processes ICAD/CAD and releases CAD.

Plasmid DNA (0.4μg) was incubated with no protein, ICAD/CAD (20 ng), and ICAD/CAD plus caspase-3(0, 0.2 μg), caspase-4 (0, 0.2, 0.4, 0.6 μg) at 30 ℃ for 2 hr and analyzed by 0.7% Agarose gel.

indicating the possibility that caspase-4 may induce DNA fragmentation directly. Unlike caspase-4, caspase-12 could not induce the DNA fragmentation with ICAD/CAD (data is not shown).

III-2-4 Caspase-4 process Bid weakly

The Bcl-2 family proteins are the central regulators of apoptosis because they integrate diverse survival and death signals [71]. Bid is a proapoptotic member of Bcl-2 family proteins. The cleavage of Bid by caspase-8 induces mitochondrial damage during death receptor-mediated apoptosis[57, 58, 72]. Caspase-4 could process the recombinant Bid protein giving the same size products as caspase-8, albeit weakly compared with the latter enzyme (Fig.11). On the other hand, caspase-12 did not process Bid at all (data not shown).

Recent studies have suggested that caspase-4 might be the homologue of caspase-12, which is inactive in humans [26]. The both caspases primarily reside in ER and mediate in ER-specific apoptosis and A-induced cell [26, 29]. The downstream pathways of the two caspases, however, have not been clearly defined. In an attempt to address the questions, we compared the both enzymes in terms of substrate specificity. Caspase-4 can cleave synthetic peptide substrates such as Ac-WEHD-AMC while no such substrate is available for caspase-12. Caspase-4 is able to induce DEVDase activity *in vitro*in cell free system, which should be caused by cleavage of caspase-3/-7 and can also process ICAD/DFF45 as a result. These results

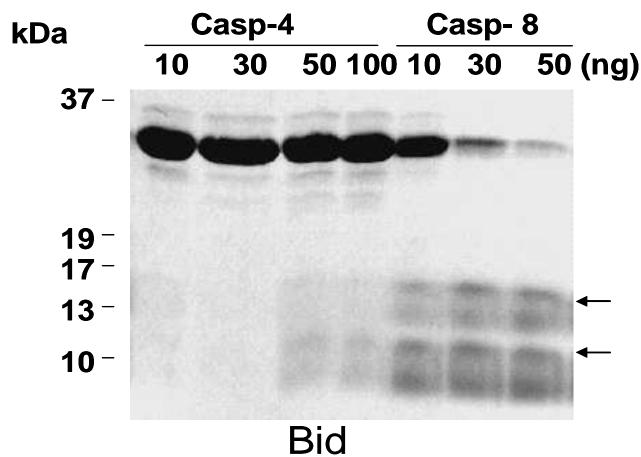

Figure 11. Caspase-4 processes Bid weakly.

Recombinant protein Bid (1.5 μg) was incubated caspase-4 (10, 30, 50, 100 ng) and caspase-8 (10, 30, 50 ng) at 30℃ for 2 hr and the cleavage were analyzed by 13.5% SDS-PAGE. Relative molecular weight marker (kD) is indicated on left and cleaved proteins bands by arrows on right.

imply that caspase-4 may act as an upstream caspase; upon ER stress transduces the signal to downstream caspases and other proapoptotic proteins leading to cell death. ICAD/DFF45 and Bid were also cleaved by caspase-4 but they were quite weak, compared with those of caspases-3 and -8. The implication of the observation remains to be elucidated. Caspase-12, on the other hand, could not cleave any of these substrates, indicating that it is unable to directly pass the signal through these substrates. In conclusion, although caspase-4 shares its physiological role with caspase-12 in ER-dependent apoptosis, it's biochemistry in signal transduction pathway should be different.

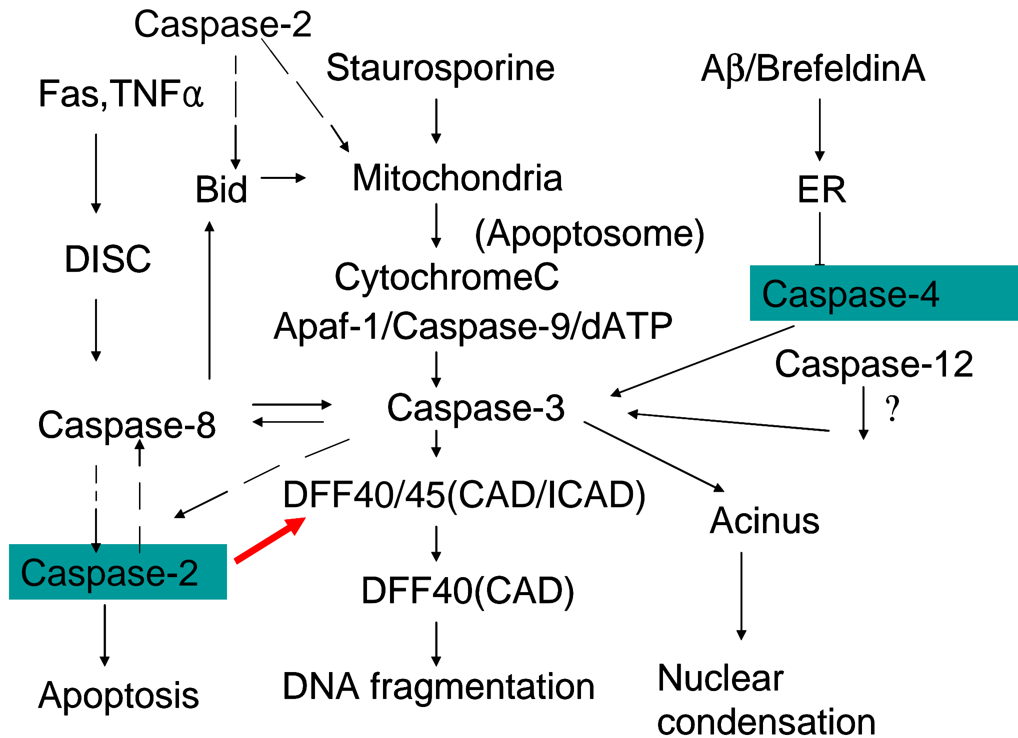

Fig. 12 Three major apoptotic pathways in mammalian cells.

III-3. Related studies and prospects of further research

III-3-1. Tat-reverse-caspase-4 and -12 induced cell death

Tat-caspases are difficult to purify due to low expression and solubility. Hence, a novel vector system was designed using trigger factor (TF) which is a molecular chaperone. The vector containe Trigger factor followed by ubiquitin and tat-caspases. A deubiquilating enzyme Usp-2cc can cleave the ubiquitin to get tat-caspase proteins. Three proteins caspase-2,-4 and -12 was purified in this way.

Concentration dependent cell death was checked for the Tat-reverse-caspase-4-treated SHSY5Y cells. The result showed that approximately 40% cell death from the 200nM Tat-reverse-caspase-4 over 6 hr periods (Fig. 13). Next, the cell death induced by Tat-reverse-caspase-12 were tested in human neuroblostoma cell line SHSY5Y cells. The result showed that tat-reverse-caspase-12 causes cell death in dose and time dependent fashion (Fig. 14). The minimum dose to cause cell death for Tat-reverse-caspase-12 was found to be 50 nM which is similar with the Tat-reverse-caspase-2-induced cell death. About 50% cells were dead after 6hr exposure of 100 nm Tat-reverse-caspase-12. This concentration was used to check the time dependent cell death. The result found that cell death started after 3 hr exposure and a increased sharply over the time causing 50% in 6 hr, 70% in 12 hr and 24 hr. It showed that after 12 hr it reaches a saturation curve after that cell death doesn't increase sharply.

The potency of cell death of Tat-reverse-caspase-4 is low compared to Tat-reverse-caspase-2 and Tat-reverse-caspase-12. The reason might be because the protein was not pure as the other two and also the LEVD activity of Tat-reverse-caspase-4 was lower than the native caspase-4. The cause behind this discrepancy is unknown.

III-3-2. Checking the procaspase-9,-12 and Bid cleavage by caspases

Caspases are generally defined as initiator and effector category. Initiator caspases are the first to get activated by the death stimuli which in turn cleaves the effector caspases to flow the signal downstream. The imitator caspases are at the apex and effector remains below the initiators in caspase cascade. But there are many exception effector caspase like caspase-3 can activate the initiators like caspase-8 to amplify the signal. Here two caspases caspase-9 and -12 are used as substrate their cleavage by other caspases including themselves. For this experiments substrate gene containing plasmid were invitro translated using invitro translation kit and enzymes were added into it in buffer condition optimum for the reactions. The results showed that caspase-3,-7, 8 and -9 can cleave po caspase-9.(Fig 15A) while caspase-3,-6,-7-8,-9 and -12 were able to cleave procaspase-12 (Fig 15B). Similarly caspase-2,-3,-8, and -9 wre able to cleave Bid (Fig15C)

III-3-3. Checking the cleavage of APE by caspases

Invitro experiments were carried out to check whether caspases can process APE, the results found that caspase-3,-6 and -9 can cleave it (Fig. 16 A).Among them cleavage of caspase-3 and -9 were significant. Hence, further experiments were done for using different concentration of caspase-3 and-9 and found that 500ng of both able for complete cleavage (Fig.16 B,C)

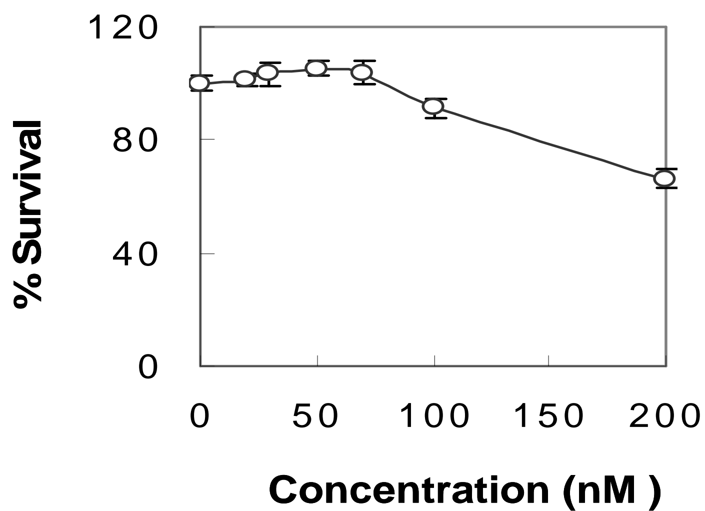

Fig. 13 Tat-reverse-caspase-4 induced cell death

SHSY5Y cells were exposed with Tat-reverse-caspase-4 (0, 20, 30, 50, 70, 100 and 200 nM) at indicated concentration for 6 hr and cell deaths were assayed by MTT assay. The % survival was calculated against untreated cells.

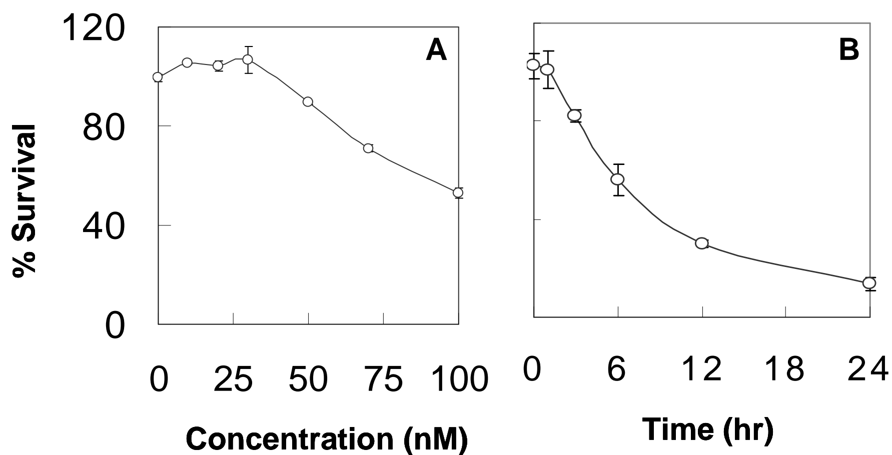

Figure 14. Tat-reverse-caspase-12 induced cell death. SHSY5Y cells were exposed with Tat-reverse-caspase-2 at indicated concentration for indicated time and cell survival rate were assayed by MTT assay.

A. Tat-reverse-caspase-12 (0,10, 30, 50,70,100 nM) at different was treated for 6hr.

B. 100 nM Tat-reverse-caspase-12 was treated for 0,1,3,6,12 and 24 hr.

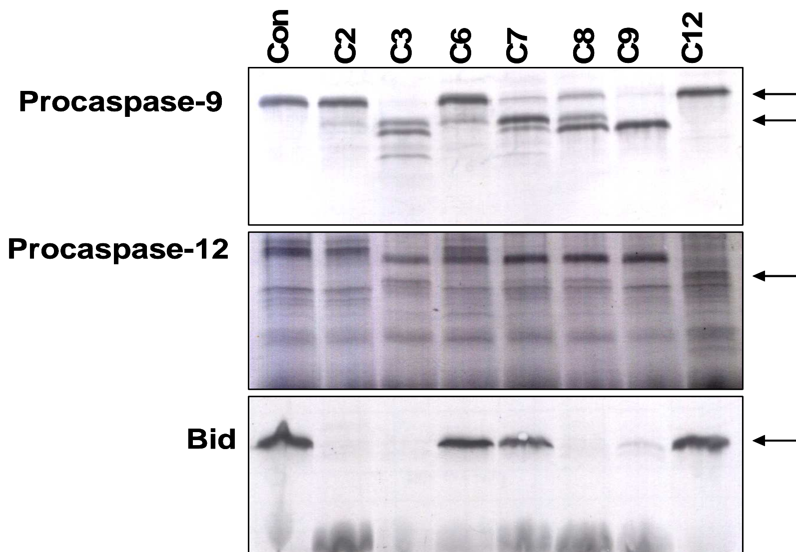

Fig. 15 In vitro cleavages of procaspase-9, procaspase-12 and Bid by different caspases. Plasmids containing caspase-9 (upper panel), caspase-12(middle panel) and Bid (lower panel) were invitro translated into protein containing S^{35} Methionine using invitro translation kit. Each each protein was treated with the different recombinant caspases as indicated for 2hr at 30 ℃. Following the reaction the proteins were run in SDS-PAGE. The gels were dried in vacuum drier and exposed to x-ray film for 3 days. The film was developed and visualized. Cleaved bands were shown by arrow.

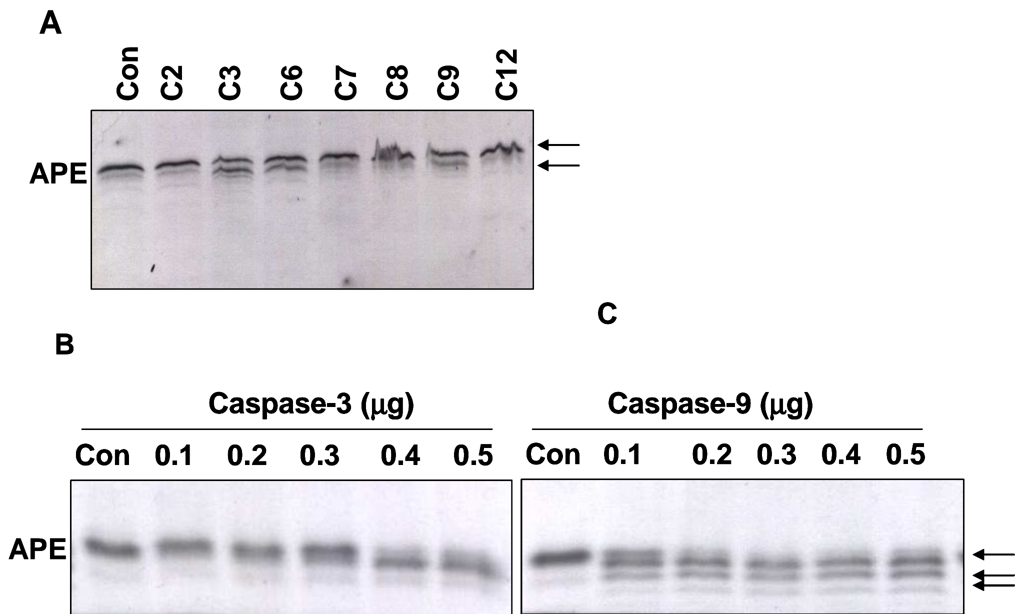

Figure 16. In vitro cleavages APE by caspases.

A. APE plasmids were invitro translated and reacted with caspases as indicated for 2hr at 30 ℃ and were analyzed as described above.

B. APE plasmids were invitro translated and reacted with increasing concentration of caspase-3 as indicated for 2hr at 30 ℃ and were analyzed as described above.

C. APE plasmids were invitro translated and reacted with increasing concentration of caspase-9 as indicated for 2hr at 30 ℃ and were analyzed as described above. Cleaved bands were shown by arrow.

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VI. ABBREVIATIONS

ER, Endoplasmic reticulum

DEVD-AMC,Asp-Glu-Val-Asp-7-amino-4-methylCoumarone

WEHD-AMC,Ac-Trp-Glu-His-Asp-AMC

LEHD-AMC, Ac-Leu-Glu-His-Asp-AMC

LEVD-AMC, Ac-Leu-Glu-Val-Asp-AMC

IETD-AMC, Ac-Ile-Glu-Thr-Asp-AMC

VEID-AMC, Ac-Val-Glu-Ile-Asp-AMC

VDVAD-AMC, Ac-Val-Asp-Val-Ala-Asp-AMC;

CAD, Caspase activated DNase,

DFF; DNA fragmentation factor,

ICAD Inhibitor of CAD

STS, staurosporine

HEPES, N (-2 hydroxyl ethyl) pauperizing-N'- (2-ethane sulfuric acid),

VII. 적 요

Caspase-2,-4,-12의 세포내 신호전달 체계

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Caspase 2의 신호전달 체계는 잘 알려져 있지 않다. 따라서 본 연구에서는 Caspase-2에 의한 특이적 세포 사멸의 효율을 높이기 위해 세포 침투성이 있는 Tat-reverse-caspase-2를 이용하였다. 세포 사멸은 2시간 이내에 빠르게 일어났고, caspase-2 특이적인 VDVADase의 활성은 관찰되었으나. 다른 caspase의 활성은(DEVDase, IETDase) 나타나지 않았다. 흥미롭게도 caspase 3의 활성화가 없이 nuclear DNA fragmentation이 일어난다. 또한 caspase 2에 의한 DFF45/ICAD의 절단이 in vivo 뿐만 아니라 in vitro에서도 일어나며, 이러한 현상은 caspase 2가 caspase 3의 활성화와 여부와 관계없이 세포사멸 신호를 세포내부로 전달함을 의미한다.

caspase-12는 설치류의 ER 특이적인 caspase로 human의 caspase-4와 그 역할이 유사한다. 따라서 본 연구에서는 caspase-4와-12의 효소학적 특성을 비교·분석하였다. Ac-WEHD-AMC는 caspase 4의 가장 좋은 기질인 반면 caspase-12는 Ac-LEHD-AMC, Ac-LEVD-AMC, Ac-IETD-AMC, Ac-VEID-AMC, Ac-DEVD-AMC 중 어느 기질과도 활성이 나타나지 않았다. SK-N-BE 세포 추출물에서 caspase 4는 DEVDase 활성화하고, caspase-3와 7을 절단함으로서 활성화 시킨다. 또한 caspase-4 는 ICAD/CAD와 반응시켰을 때 DNA fragmentation을 일으키고, 비록 약하긴 하지만 proapoptotic protein

(Bcl 2 family)인 Bid를 절단하지만 caspase-12에 의해서는 어떠한 단백질도 절단되는 것을 관찰하지 못했다. 이러한 결과는 caspase-4와 -12는 ER 스트레스에 의해 유도되는 세포사멸에서 생리학적으로는 유사한 역할을 보임에도 불구하고 세포 내부의 하부 신호전달 체계는 다르다는 것을 제시한다.

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	영문 Signal transduction pathway involving caspase-2, -4. -12				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의( ) 반대(0)

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