Original Articles

PROCASPASES AND APOPTOTIC PROTEASE-ACTIVATING FACTOR-1 (APAF-1) IN BOVINE THYMUS, BRAIN, AND LIVER: PARTIAL PURIFICATION AND TISSUE DISTRIBUTION

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Abstract : We fractionated and highly purified a terminal pro-DEVDase, procaspase 9, and Apaf-1 from bovine thymus extracts by three steps of column chromatography. Using the purified factors, the essential factors of the mitochondria-mediated (type II) apoptotic pathway, we constructed a supplementation assay for each factor to examine the distribution in bovine thymus, brain, and liver. All the tissues expressed a class of procaspase 9 with a molecular size of 50 kDa. The distribution of proDEVDase, however, varied in the tissues. The chromatography of proDEVDase revealed the presence of four peaks in thymus, three in liver, and one in brain extracts. Among them, two major thymus proDEVDase (BT- proDEVDase-1 and -2) and a brain proDEVDase (BB- proDEVDase) were further purified. Although they were all procaspase 3-like judging from the substrate specificity and reactivity with anti-human caspase 3 antibody, they were significantly different from each other in molecular size and in reactivity with anti-human caspase 7, suggesting that they are different molecular species. All of these results revealed a diversity of tissue-specific expression of proDEVDase. Among the three factors examined, tissue distribution of Apaf-1 showed the most distinctive feature. The brain extracts showed essentially no caspase activation when incubated with cytochrome c and dATP but restored the activity when purified thymus Apaf-1 was supplemented. Essentially no Apaf-1 activity was detected in the extracts of bovine brain, suggesting that mature bovine brain lacks type II apoptotic pathway in spite of the essential role in the programmed cell death during the normal development of the brain tissue. Biological significance of these findings will be discussed.

Key words : apoptosis, Apaf-1, procaspase, bovine, thymus, brain, liver

INTRODUCTION

Apoptosis is an evolutionarily conserved process of cell death that plays a major role during normal development, homeostasis, and certain pathological conditions such as cancer, neurodegenerative disorders, and AIDS[1-6]. Recent progress in apoptosis research has elicited the central role for caspases in this process[7, 8]. Caspases belong to the family of cystein proteases that are expressed as inactive proenzymes in normal cells and activated during apoptosis[7-10]. Among 14 identified members of the family, caspases 3 and 7 are the key

executioners of apoptosis induced by various stimuli[7, 11]. Being activated, they cleave specific nuclear and cytoplasmic proteins essential for characteristic downstream events including chromatin condensation, internucleosomal DNA fragmentation, and the formation of apoptotic bodies[1, 8, 12].

Recent studies demonstrated that cleavage and activation of executioner caspases are mediated by either receptor-mediated (type I) or mitochondria-mediated (type II) apoptotic pathways[13-16]. The mitochondria-mediated pathway involves cytochrome c, dATP, Apaf-1, and procaspase 9 as the essential components[17-19]. In this pathway, the alteration in Apaf-1 conformation by binding of cytochrome c released from mitochondria is assumed to be the critical initiating event. Formation of the apoptosome composed of Apaf-1, cytochrome c, and procaspase 9 in the presence of dATP (or ATP) results in auto-processing and activation of the initiator caspase 9[20-23].

Recent studies on Apaf-1- and caspase 9-knockout mice have established an important role for the type II apoptotic pathway during brain development[24-27]. However, the role of this pathway in apoptosis in adult mammalian tissues including brain has not been convincingly identified. In this study, we partially purified Apaf-1, procaspase 9, and proDEVDase from bovine thymus. Using these purified factors, we constructed a supplementation assay for the activity of the factors involved in cytochrome c/dATP-dependent activation of terminal proDEVDases and examined the tissue distribution. The results demonstrate differential expression of the apoptosome components and, thus, a different efficiency of type II apoptotic pathway in adult tissues.

MATERIAL AND METHODS

Tissues Fresh thymus, liver, and brain of approximately 2-year-old oxen were kindly donated by Dr. Kori, Osaka Prefecture Meat Center, Japan. The tissues were divided into pieces of 100 g weight and stored at −85℃ until use.

Caspase substrates Acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (Ac-DEVD-MCA), Ac-Tyr-Val-Ala-Asp-MCA (Ac-YVAD-MCA), Ac-Val-Glu-Ile-Asp-MCA (Ac-VEID-MCA), Ac-Ile-Glu-Thr-Asp-MCA (Ac-IETD-MCA), Ac-Trp-Glu-His-Asp-MCA (Ac-WEHD-MCA), Ac-Leu-Glu-His-Asp-MCA (Ac-LEHD-MCA), Ac-Asp-Gln-Thr-Asp-MCA (Ac-DQTD-MCA), and Ac-Val-Asp-Val-Ala-Asp-MCA (Ac-VDVAD-MCA) were purchased from Peptide Institute, Inc., Osaka, Japan.

Protease-inhibitors Benzyloxy-carbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk) and benzyloxy-carbonyl-Phe-Ala-fmk (Z-FA-fmk) were purchased from Enzyme System Products (CA, U. S. A.); Ac-YVAD-aldehyde (Ac-YVAD-CHO) and Ac-VEID-CHO were purchased from Peptide Institute Inc. (Osaka, Japan); *p*-toluenesulfonyl-L-phenylalanine chloromethylketone (TPCK) and *p*-toluenesulfonyl-L-lysine chloromethylketone (TLCK) were obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Antibodies Goat polyclonal anti-human Apaf-1 (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA), rabbit polyclonal anti-human Apaf-1 (Chemicon International, Inc., Temecula, CA, USA), mouse monoclonal anti-human caspase 2 (Pharmingen, San Diego, CA, USA), rabbit polyclonal anti-human caspase 3 (Pharmingen), rabbit polyclonal anti-human caspase 6 (StressGen Biotech. Corp., Victoria, BC, Canada), mouse monoclonal anti-human caspase 7

(Transduction Laboratories, Lexington, KY, USA), rabbit polyclonal anti-human caspase 8, rabbit polyclonal anti-human caspase 9 (StressGen), and mouse monoclonal anti-human caspase 9 (MBL Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) antibodies were obtained from commercial sources as indicated in parenthesis.

Secondary goat anti-rabbit IgG, rabbit anti-mouse IgG, and rabbit anti-goat IgG conjugated with horse-radish peroxidase were obtained from DAKO Japan Co., Ltd.

Mediums Extraction medium contained 50 mM Tris-HCl buffer, pH 7.4, 2 mM EGTA (ethylene glycol bis(β-aminoethylether)-N, N, N', N'-tetraacetic acid), 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 0.15 M NaCl. Medium A (Med-A) contained 25 mM Tris-HCl buffer, pH 7.4, 1 mM DTT, 1 mM EDTA, 0.1 mM PMSF, 5 mM $NaHSO_3$, and 0.5 mM benzamidine-HCl and was used for the purification of caspases and caspase-activating factor throughout this study unless otherwise indicated. 1/2 Med-A was prepared by 2-fold dilution of Med-A with distilled water and used to dissolve caspases and for the assay of caspase activation.

Assay for caspase activity The activity of a DEVDase was assayed using a synthetic tetrapeptide substrate conjugated with 7-amino-4-methyl-coumarin (Ac-DEVD-MCA) and referred as DEVDase activity. The reaction mixture contained 20 μM Ac-DEVD-MCA, 25 mM Tris-HCl buffer, pH7.4, and 10 μl of enzyme sample in a total volume of 125 μl. Reaction mixtures were incubated at 30℃ for 30 min and terminated by the addition of 375 μl of 0.2% (w/v) sodium dodecylsulfate (SDS).

Cleavage of a substrate was monitored by measuring fluorescence using a Shimadzu RF5000 spectrofluorometer at excitation 380 nm and emission 460 nm. One unit of DEVDase was defined as the amount of the enzyme required for the hydrolysis of 14 pmol of the substrate. Activity assay for other caspases was also performed as described for DEVDase except that Ac-DEVD-MCA was replaced by other synthetic peptide specific to the respective caspase.

Assay for cytochrome c/dATP-dependent caspase-activating activity An appropriate amount of tissue extracts (10 to 150 μg protein) or a combination of purified factors (Apaf-1, procaspase 9, and pro-DEVDase) was incubated at 30℃ for 30 min in a reaction mixture containing 1 mM dATP, 40 μg/ml cytochrome c, 5 mg/ml BSA, and 1 mM DTT in a final volume of 10 μl of 1/2 Med-A (activation of proDEVDase). DEVDase activity was measured as described above. DEVDase-activating activity was tentatively expressed by the DEVDase activity attained under the condition.

Supplementation assay for the activity of the respective factors involved in caspase-activation The activity assay for Apaf-1, procaspase 9, or proDEVDase was carried out by supplementing 125 units/10 μl of other of these factors in the reaction mixture for caspase activation as described above. The amount of a factor required for the activation of 50 units of DEVDase under the condition was tentatively defined as 50 units. The assay was carried out in a range of the tested sample between 30 to 80 units/10 μl since the changes in DEVDase activity were approximately linear in this range.

Purification of Apaf-1, procaspase 9, and proDEVDase Fifty g of bovine thymus were minced and homogenized in 2.5 volumes of the extraction medium for 40 sec with a home mixer. The homogenates were centrifuged at 17,000 g for 30 min. The supernatants were

fractionated by a successive precipitation with 45 and 70% ammonium sulfate. The obtained fractions were separately dissolved in appropriate volumes of 1/2Med-A and dialyzed against the same medium. Each sample was applied to a Q-Sepharose column (100ml-bed volume), and, after washing with 150ml of 1/2Med-A, the samples were eluted with a linear gradient of increasing concentration of NaCl in Med-A. The fractions were subjected to supplementation assays to detect Apaf-1, procaspase 9, and proDEVDase as described above. Active fractions were separately collected and further purified by hydroxylapatite column (30ml-bed volume). The samples were eluted with a linear gradient of increasing concentration of potassium phosphate buffer (pH7.2) in Med-A. Active fractions were separately collected and further purified by a Phenyl Sepharose column (5 ml-bed) with a linear gradient of decreasing concentration of ammonium sulfate in Med-A. In some experiments, a stepwise elution was used and the elution medium was modified as indicated. Purification of the factors of bovine brain and liver was also carried out as above unless otherwise indicated.

Immunoblot analysis SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transblot onto a polyvinylidene fluoride (PVDF) membrane (Pall Biosupport, USA) were carried out as described previously[28]. The membrane was double-blocked with 50% and 10% SuperBlock (PIERCE, Illinois, USA) in TTBS (Tris-buffered saline containing 0.05% Tween), and then probed with primary and secondary antibodies, successively. The immuno-complex was detected using ECL+ Western blot detection reagents (Pharmacia-Amersham).

Other procedures Protein concentrations were measured by using the Bio-Rad protein assay kit.

RESULTS

Purification of bovine thymus procaspase 9, Apaf-1, and proDEVDase Preliminary analysis of DEVDase activity in protein extracts from bovine thymus demonstrated low background levels of this activity (data not shown). After incubation of the extracts with 40 μg/ml of cytochrome c and 1 mM dATP, DEVDase activity was markedly increased in a time-dependent manner and reached an approximately 100-fold increase of the initial activity after 30 min. Both cytochrome c and dATP were essentially required for DEVDase activation (data not shown). These results confirmed that the extracts isolated from bovine thymus contained all components necessary for the mitochondria-mediated (type II) pathway of caspase activation as revealed by the group of Wang[17-19]. Thus, we attempted to purify procaspase 9, Apaf-1, and proDEVDase, which are all essential for type II apoptotic pathway.

Since we did not possess purified factors at the initial stage of the study, we detected the three components in the chromatographic fractions by supplementing a limited amount of the thymus extracts in the DEVDase-activation assay. The three factors thus obtained were further purified, characterized, and used for the present study. As shown in Fig. 1A, bovine thymus procaspase 9 and Apaf-1 were recovered in 45% ammonium sulfate precipitates and, upon Q-Sepharose column chromatography, they were eluted at 140 and 250 mM NaCl, respectively. The molecular size of the native procaspase 9 was approximately 100 kDa as estimated by Sephacryl-S200 column chromatography (data not shown). The procaspase 9

fraction was further purified by hydroxylapatite column chromatography as described in "Materials and Methods". The procaspase 9-activity was eluted at 90 mM of potassium phosphate as a single peak. The active fractions were combined and further purified by Phenyl Sepharose column chromatography. The activity was eluted at 370 mM of ammonium sulfate. The active fractions were combined and concentrated by ammonium sulfate precipitation. The precipitates were dissolved in 1/2 Med-A and dialyzed against the same medium and stored at −85℃.

Apaf-1 fraction was purified further also by hydroxylapatite column chromatography as described above. Apaf-1 activity was eluted at 260 mM potassium phosphate as a single peak (data not shown). At this purification stage, Apaf-1 became very unstable: incubation of this factor at 30℃ for 30 min in 1/2Med-A led to loss of more than 90% of its initial activity. Addition of 0.1 mM ATP to the sample markedly increased stability of this factor (Table 1). Thus, for further purification, 0.1 mM ATP was added to the combined sample. Ammonium sulfate was added to the Apaf-1 sample to a final concentration of 0.2 M and the sample was applied to a phenyl-Sepharose column. Stepwise elution of proteins was carried out with 1/2 Med-A containing 0.1 mM ATP. The active fractions were combined, concentrated as above, and stored at −85℃. Molecular size of native bovine thymus Apaf-1, estimated by Sephacryl-S200 column chromatography, was approximately 130 kDa (data not shown).

A major part of the activity of proDEVDase was recovered in 70% ammonium sulfate fraction. Upon Q-Sepharose column chromatography of the fraction, a major proDEVDase activity was eluted at 170 mM NaCl. Two minor peaks were eluted at flow-through fractions and at 50 mM NaCl, suggesting that the tissue contains multiple terminal proDEVDases (Fig. 1B). The major proDEVDase fraction was further purified by hydroxylapatite column

Table 1. Effect of nucleotides on the stability of Apaf-1

nucleotides added	activity remained %*
1. none (no incubation)	100
2. none (30 min at 30℃)	1
3. dATP	48
4. dGTP	1
5. dCTP	1
6. dTTP	1
7. ATP	86
8. ADP	89
9. AMP	3
10. AdR	1
11. GTP	1
12. CTP	4
13. UTP	2

* Apaf-1 (200 units, hydroxylapatite column chromatography step) was incubated for 30 min at 30℃ in the absence and the presence of 0.1 mM of various nucleotides in 1/2 Med-A and the remaining activity was assayed by supplementation asay as described in "Materials and Methods". The activity of the sample without incubation was set at 100 %.

chromatography. The proDEVDase activity was eluted from the column as two splitting peaks eluted at 180 (BT proDEVDase-1) and 240 mM (BT proDEVDase-2) potassium phosphate, respectively. The proDEVDase-1 and -2 were separately purified by Phenyl Sepharose column chromatography. In some experiments we combined the proDEVDase-1 and -2 fraction from hydroxylapatite column and the sample was further purified by phenyl-Sepharose column chromatography. The combined fraction is referred to as purified bovine thymus (BT) pro-DEVDase in the present study.

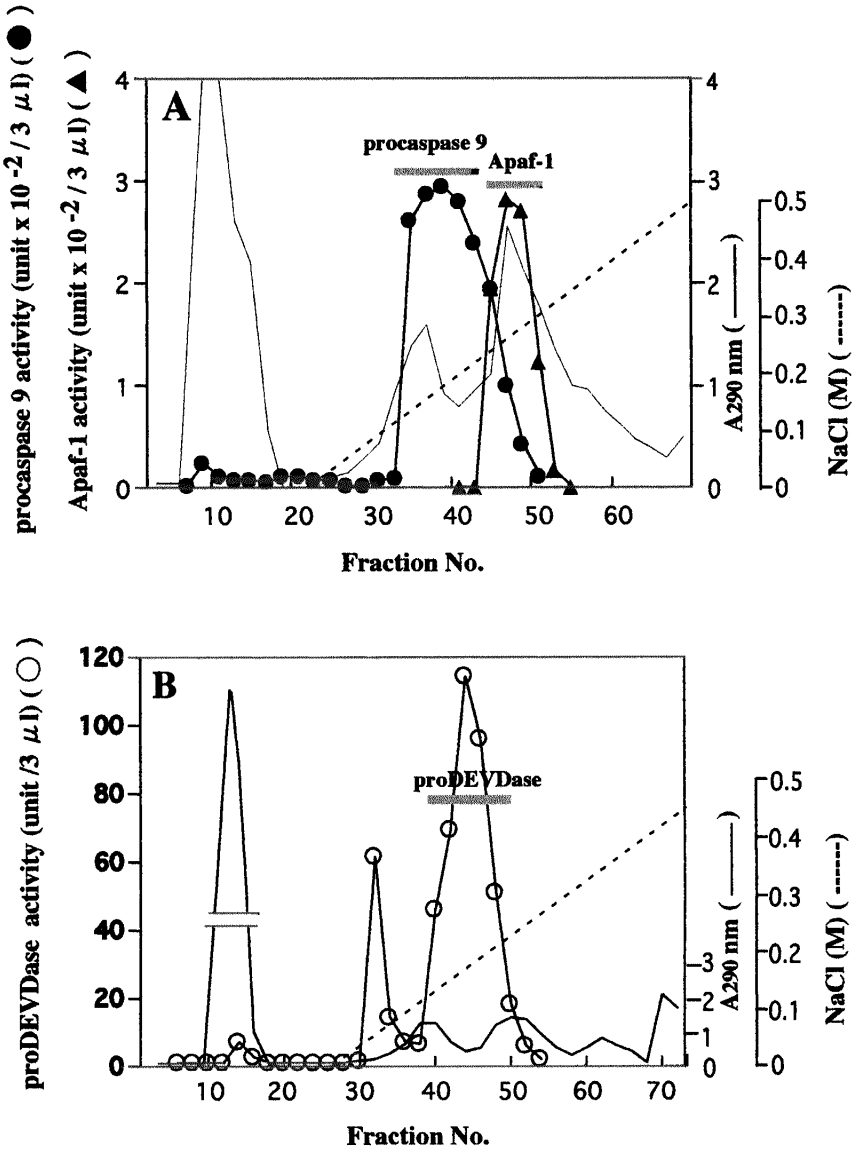

Fig. 1. Q-Sepharose column chromatography of bovine thymus extracts. Bovine thymus extracts were fractionated by differential precipitation with ammonium sulfate. The 0-45% (A) and 45-70% (B) ammonium sulfate fractions were separately fractionated by Q-Sepharose column chromatography. The activities of procaspase 9, Apaf-1, and proDEVDase were assayed as described in "Materials and Methods".

The purification of bovine thymus procaspase 9, Apaf-1, and proDEVDase is summarized in Table 2. Procaspase 9, Apaf-1, and pro-DEVDase-1 and -2 were enriched from thymus extracts by 575, 1,552, 1,475, and 4,375-fold, respectively.

Immunoblot analysis of bovine thymus procaspase 9, Apaf-1, and pro-DEVDase-1 and -2 The purified factors were examined by immunoblot analysis with the specific antibodies. As shown in Fig. 2A and B, procaspase 9 and Apaf-1 were specifically recognized by the respective specific antibodies and were located at 50 and 130 kDa as a single band, respectively. BT (bovine thymus)-proDEVDase-1 was located at 30 kDa by both anti-caspase 7- and anti-caspase 3-antibodies, while BT-proDEVDase-2 was reactive only to anti-caspase 3-antibody and was located at 29.5 kDa (Fig. 2C).

Stability of bovine thymus Apaf-1 Apaf-1 was very unstable especially at a later stage of purification. As shown in Table 1, incubation at 30 ℃ for 30 min almost completely abolished Apaf-1 activity: addition of either ATP or ADP in the incubation mixture, however, markedly stabilized Apaf-1 activity. The Apaf-1-stabilizing ability of these nucleotides seem to be higher than dATP whereas dATP shows higher activity than ATP in proDEVDase-activation assay[29]. Other nucleotides tested were essentially ineffective. These results suggest that ATP may have a role to stabilize Apaf-1 in the cell.

Supplementation assay for the activity of procaspase 9, Apaf-1, and proDEVDase Activation of proDEVDase was carried out in the presence of a constant amount of proDEVDase and procaspase 9 (125 units each) and varying amounts of Apaf-1 as described in "Materials and Methods". As shown in Fig. 3A, activation of proDEVDase was completely dependent on Apaf-1 and increased with increasing concentration of the factor. The curve was sigmoid but was approximately linear in a range of 40 to 80 units/10 μl. Thus, we defined the activity of Apaf-1 required for the activation of 50 units of proDEVDase under the conditions as 50 units. And Apaf-1 activity of various samples was determined using this curve as a standard.

The dose-response curve for procaspase 9, which was obtained in the presence of 125 units of Apaf-1 and proDEVDase, was also sigmoid but was approximately linear in a range of 30 to 80 units/10 μl (Fig. 3B). Thus, we defined the activity of procaspase 9 required for the

Table 2. Summary of purification of bovine thymus caspase-activating factor

factors	protein (mg)	activity (unit x 10^{-6})	specific activity (unit x 10^{-6}/mg)	purification (folds)	yields (%)
procaspase 9	1.03	7.1	6.9	575	46
Apaf-1	0.153	1.8	11.8	1,552	19
BT-proDEVDase-1	0.24	1.7	7.1	1,475	26
BT-proDEVDase-2	0.04	0.84	21	4,375	11

Purification from 100 g of thymus is shown. Yield was calculated by setting the activity of these factors in ammonium sulfate fraction as 100 %, except that the yields of TF-IV-1 and TF-IV-2 were calculated by setting the activity of the respective fraction of hydroxylapatite column chromatography step at 100 %.

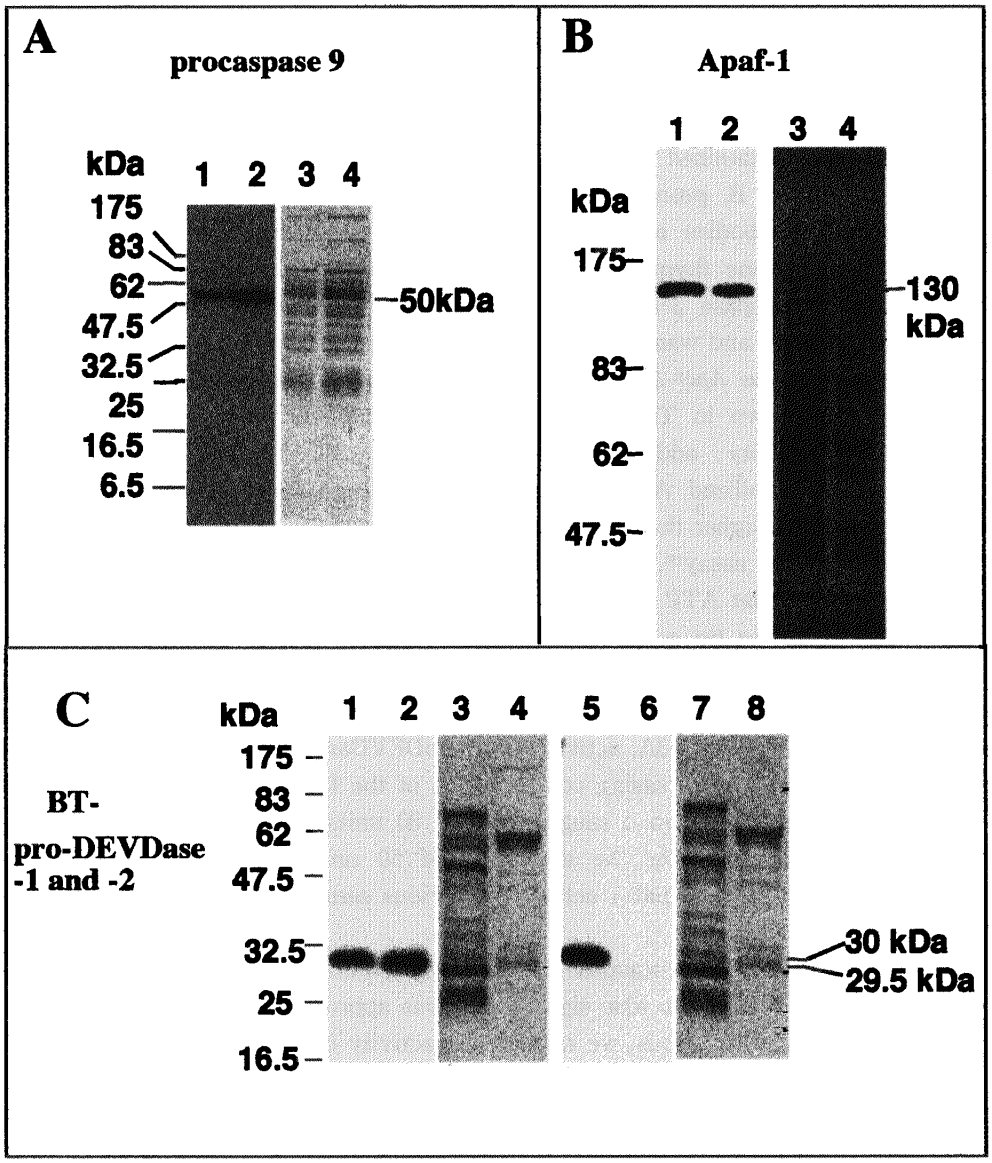

Fig. 2. Immunoblot of purified bovine thymus procaspase 9, Apaf-1, and proDEVDase-1 and -2. Purified bovine thymus factors were analyzed by immunoblot following SDS-PAGE. A: 1,250 units (lanes 1) and 2,500 units (lanes 2) of procaspase 9 were analyzed by SDS-PAGE (12% gel) followed by immunoblot with anti-caspase 9 antibody. Coomassie brilliant blue (CBB)-staining of the blot is shown in lanes 3 and 4. B: 2,000 (lanes 1) and 1,000 units (lanes 2) of Apaf-1 were analyzed by SDS-PAGE (7.5% gel) followed by immunoblot with anti-Apaf-1 antibody (goat polyclonal; Santa Cruz Biotech. Inc.). CBB-staining of the blot is shown in lanes 3 and 4. C: 4,000 units of BT-proDEVDase-1 (lanes 1 and 5) and BT-pro-DEVDase-2 (lanes 2 and 6) were analyzed by SDS-PAGE (10 % gel) followed by immunoblot with anti-caspase 3 antibody (Pharmingen; lanes 1 and 2) or anti-caspase 7 antibody (Transduction Laboratories; lanes 5 and 6). CBB-staining of lanes (1 and 2) and (5 and 6) are shown in lanes (3 and 4) and (7 and 8), respectively.

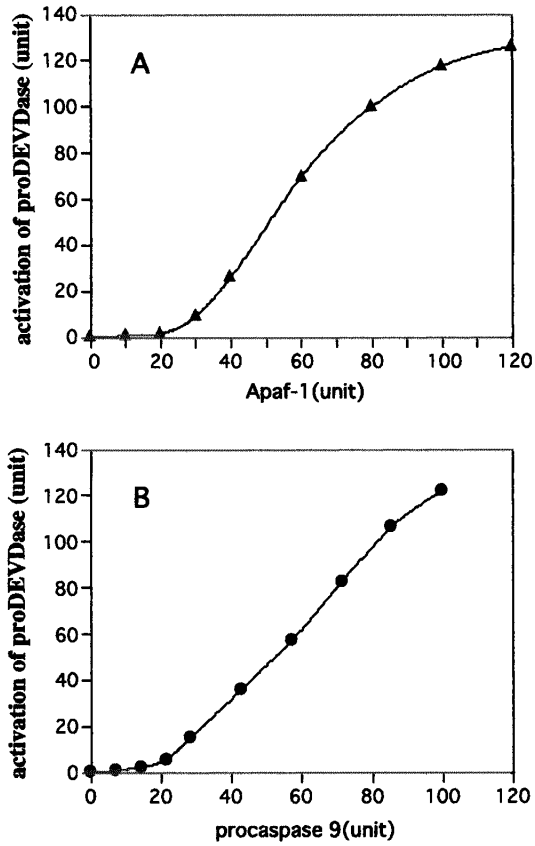

Fig. 3. Apaf-1- and procaspase 9-dose dependent curve of proDEVDase activation. Supplementation assay for proDEVDase-activation was examined with varying concentration of either Apaf-1 (A) or procaspase 9 (B) and 125 units of other factors as described in "Materials and Methods".

activation of 50 units of proDEVDase under the conditions as 50 units, and the activity of various procaspase 9 samples was determined using this curve as a standard.

The dose-response curve for proDEVDase, which was obtained in the presence of 125 units of Apaf-1 and procaspase 9, was approximately linear in a range between 0 to 100 units (data not shown). One unit of proDEVDase was defined as one unit of DEVDase activity attained under the conditions.

Substrate specificity of bovine thymus DEVDase-1 and -2 Incubating with purified procaspase 9 and Apaf-1 in the mixture for caspase-activation as described in "Methods" activated bovine thymus (BT) proDEVDase-1 and -2. Thus activated BT-DEVDase-1 and -2 were examined for their substrate specificity. As shown in Table 3, both caspases showed a similar tendency to prefer some of the synthetic peptide substrates: the order of the preference was DEVD > DQTD > VDVAD >> VEID > and others. Judging from the substrate specificity BT-DEVDase-1 and -2 are considered to be bovine homologues of caspase 3- or 7-like enzyme[30].

Inhibitors of caspase-activation As shown in Table 4, the caspase-activation reaction in vitro by the combination of purified factors was markedly inhibited by 1 μM Z-VAD-fmk, a

Table 3. Substrate specificity of activated terminal caspases from bovine thymus and brain

	relative activity (%)		
	thymus		brain
substrate	BT-DEVDase-1	BT-DEVDase-2	BB-DEVDase
1. Ac-DEVD-MCA (cas-3)	**100**	**100**	**100**
2. Ac-YVAD- MCA (cas-1)	0.6	0.5	0.2
3. Ac-VEID- MCA (cas-6)	3.6	3.4	2.7
4. Ac-IETD- MCA (cas-8)	1.6	1.4	1.1
5. Ac-WEHD- MCA (cas-4, 5)	2.0	1.9	0.7
6. Ac-LEHD- MCA (cas-9)	2.0	1.4	2.1
7. Ac-DQTD- MCA (cas-7/3)	45.2	45.4	45.9
8. Ac-VDVAD- MCA (cas-2)	17.8	18.7	20.1

The caspase activity was assayed, after activation, with the indicated substrate as described in "Methods". The activity (approximately 100 units) attained with Ac-DEVD-MCA, was set at 100 %.

The putative hydrolyzing caspase of the respective synthetic substrate is shown in parenthesis.

Table 4. Inhibitors of the caspase activation

inhibitor	concentration*	inhibition (%)
1. none		0
2. Z-VAD-fmk	1 μM	69
	10 μM	99
3. Z-FA-fmk	1 μM	0
	10 μM	4
4. Ac-YVAD-CHO	1 μM	0
	10 μM	18
5. TPCK	1 μM	0
	10 μM	0
6. TLCK	1 μM	0
	10 μM	0
7. NaCl	50 mM	44
	100 mM	95

***The indicated concentration of inhibitors was added to the mixture for caspase-activation. Caspase-activation assay was carried out with 125 units each of purified Apaf-1, procaspase 9, and proDEVDase as described in " Methods".**

known pan-caspase inhibitor[31-33]. Since the concentration of Z-VAD-fmk did not affect so significantly the catalytic activity of the activated DEVDase (data not shown), the inhibitor may inhibit either the formation or the activity of apoptosome. Other oligopeptide inhibitors tested were ineffective or slightly inhibitory. The caspase activation in vitro was significantly inhibited also by a relatively low concentration of NaCl (Table 5).

Bovine brain extracts contain a proDEVDase and procaspase 9 but lack Apaf-1 Assessment of caspase activation in protein extracts from bovine brain by added cytochrome c and dATP did not detect increase in DEVDase activity, suggesting that the brain extracts lack one of the factors that are essential for caspase activation. Alternatively, brain extracts might contain an unidentified endogenous inhibitor of caspase activation. To clarify either of these possibilities, we supplemented bovine brain extracts individually with purified thymus factors in the assay of proDEVDase-activation. As shown in Table 5, supplementation of

Table 5. Bovine brain extracts lack Apaf-1 activity

	factors supplemented	DEVDase activation (unit)
1.	none	0.3
2.	proDEVDase; 125 u	0.4
3.	procaspase 9; 125 u	0.2
4.	Apaf-1; 125 u	33.1

Bovine brain extracts (4 μl, 29.4 μg protein) was activated with or without supplementation of the indicated amounts of purified bovine thymus factors and, then, the DEVDase activity was measured as described in "Materials and Methods".

extracts with purified thymus Apaf-1 caused a marked DEVDase-activation whereas supplementation with proDEVDase and procaspase 9 did not. This suggested that the brain extracts contain a pro-DEVDase and a caspase 9-replacing activity but lack Apaf-1. To develop this hypothesis in more detail, we fractionated brain extracts by ammonium sulfate precipitation followed by Q-Sepharose column chromatography essentially as we performed this procedure for extracts from thymus. As shown in Fig. 4A, two procaspase 9-replacing activities (BB-procas-9-I and -II) were fractionated from the 45% ammonium sulfate precipitates. The BB-procas-9-I and -II were separately purified essentially according to the procedures for the purification of thymus factors. Although they showed a slightly different elution pattern upon hydroxylapatite and Phenyl-Sepharose column chromatography, both fractions contained a protein of 50 kDa stained specifically with anti-caspase 9 antibody (data not shown). Thus the differential elution from the column may be due to association of brain procaspase 9, at least partly, with some dissimilar factors within the brain.

Bovine brain proDEVDase (BB-proDEVDase) was eluted as a single peak upon fractionation of 70% ammonium sulfate fraction of the brain extracts by Q-Sepharose column chromatography (Fig. 4B). BB-proDEVDase showed a significantly different elution profile, when compared with that of BT-proDEVDase-1 and -2, upon further purification by hydroxylapatite and Phenyl-Sepharose column chromatography (data not shown). BB-proDEVDase was immuno-stained specifically by anti-caspase 3 antibody but was not reactive to other antibodies tested, including anti-h-caspase 1, 2, 6, 7, and 8(data not shown). BB-proDEVDase, when examined after activation in vitro, showed quite similar substrate specificity as BT-proDEVDase-1 and -2 (Table 3). However, the molecular size of BB-proDEVDase (32.5 kDa, estimated by SDS-PAGE followed by immunoblot with anti-h-caspase-3) was significantly larger than that of BT-proDEVDase-1 and -2 (30 and 29.5 kDa, respectively).

No Apaf-1-replacing activity was detected through the fractions collected (data not shown).

Fractionation of liver procaspase 9, Apaf-1, and proDEVDase The extracts of bovine liver were further centrifuged at 100,000 g for 1 h and the supernatant fraction was directly analyzed by Q-Sepharose column chromatography. As shown in Fig. 4C, Apaf-1, caspase 9, and three proDEVDase fractions were detected in bovine liver extracts by supplementation assay as described in "Materials and Methods".

Distribution of procaspase 9, Apaf-1, and proDEVDase in bovine thymus, brain, and liver In order

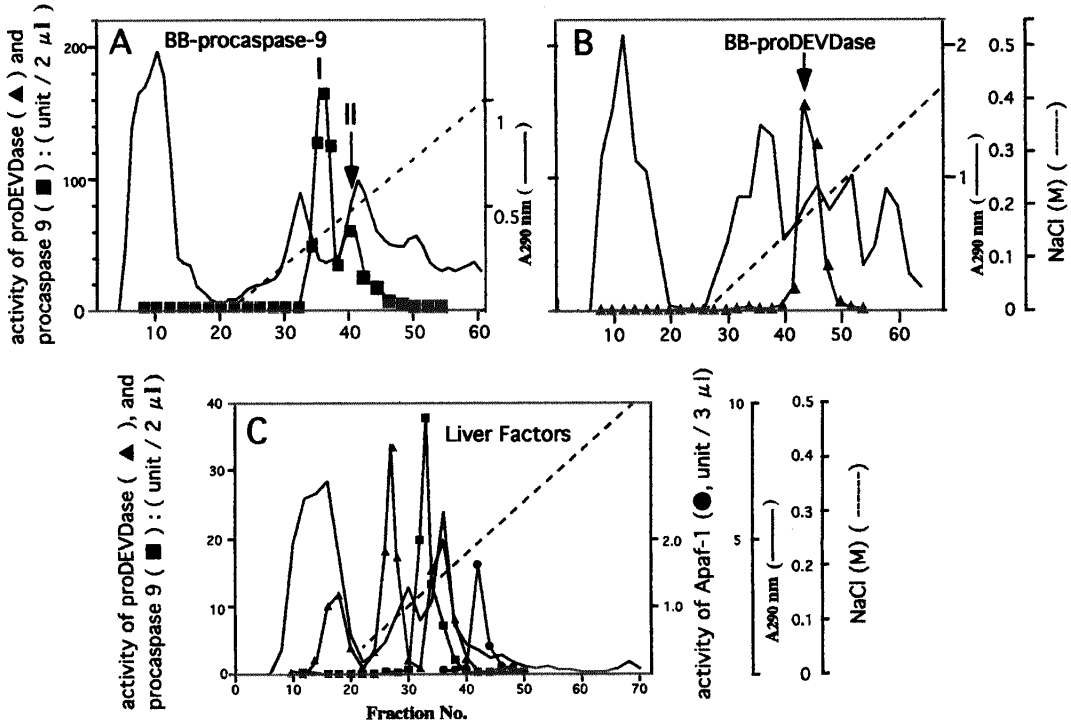

Fig. 4. Q-Sepharose column chromatography of the extracts of bovine brain and liver. A and B: The 0-45 % (A) and the 45-70% (B) ammonium sulfate fractions of bovine brain extracts were separately fractionated by Q-Sepharose column chromatography. Appropriate amounts of fractions were subjected to supplementation assay for Apaf-1, procaspase 9 (■), and proDEVDase (▲) as described in "Materials and Methods". No Apaf-1 activity was detected through the fractions (data not shown). C: Bovine liver extracts (17,000 g supernatant) was further centrifuged at 100,000 g for 1 h and the resultant supernatant was directly analyzed by a Q-Sepharose column chromatography. The activities of Apaf-1 (●), procaspase 9 (■), and proDEVDase (▲) were assayed as described above.

to examine the significance of type II apoptotic pathway in thymus, brain, and liver, we compared the activities of Apaf-1, procaspase 9, and terminal proDEVDase in the extracts of these tissues on a protein basis. As shown in Table 6, bovine liver extracts contained 21, 39, and 45% of Apaf-1, procaspase 9, and terminal proDEVDase activities of the thymus, respectively, suggesting that the type II pathway for caspase-activation is active in the tissue. In contrast, bovine brain contained essentially no Apaf-1 activity although the tissue retained 77 and 42% of procaspase 9, and proDEVDase activities of the thymus, respectively.

Comparison of procaspase 9 of the thymus, brain, and liver Tissue extracts obtained from thymus, brain, and liver were examined by SDS-PAGE followed by immunoblotting with anti-procaspase 9-antibody. All these tissues expressed a single class procaspase 9 with a molecular size of 50 kDa (Fig. 5, lane 3, 5, 7). After activation of the crude extracts by incubating with purified thymus Apaf-1, cytochrome c and dATP, the procaspase 9 was processed to a 36 kDa form in all of the tissue extracts examined (Fig. 5, lanes 4, 6, 8). The results suggest that these tissues express a single class of procaspase 9.

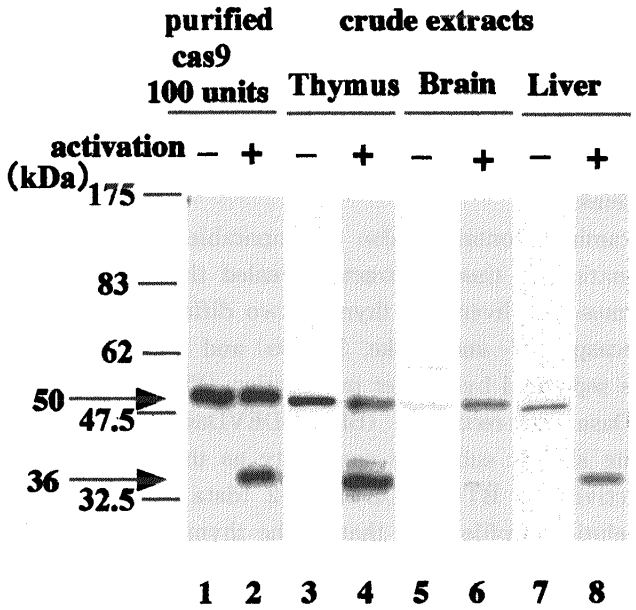

Fig. 5. Immunoblot analysis of procaspase 9 in crude extracts of bovine thymus, brain and liver. Procaspase 9 in the crude extracts from various tissues were analyzed by SDS-PAGE (7.5 % gel) followed by immunoblot with anti-caspase 9 antibody (MBL Medical & Biological Laboratories Co., Ltd.) as described in "Materials and Methods". Purified bovine thymus procaspase 9 (100 units; lane 1, 2) and 30 μg protein of the extracts of bovine thymus (lane 3, 4), brain (lane 5, 6) and liver (lane 7, 8) were analyzed before (lanes 1, 3, 5, and 7) and after activation (lanes 2, 4, 6, and 8) by incubating with purified bovine thymus Apaf-1 (200 units) for 20 min in a mixture for the supplementation assay of cytochrome c/dATP-dependent caspase-activation as described in "Materials and Methods" except that BSA and pro-DEVDase were omitted from the mixture.

DISCUSSION

We fractionated bovine homologues of procaspase 9, Apaf-1, and pro-DEVDase, essential factors for type II apoptotic pathway, from bovine thymus and highly purified through three steps of column chromatography to avoid cross-contamination. With the use of these purified factors, an in vitro assay system for caspase activation was reconstructed. Activation of pro-DEVDase was almost completely dependent on procaspase 9 and Apaf-1 (Fig. 3) as well as dependent on cytochrome c and dATP (data not shown) as reported by the group of Wang[17-19]. The dose-response curves of Apaf-1 and procaspase 9 for activation of pro-DEVDase were sigmoid (Fig. 3), reflecting the complex mechanism of the caspase-activating reaction[19-23]. Utilizing the supplementation assay and the standard curves, we examined tissue distribution of the components for type II apoptotic pathway.

As shown in Table 6, the thymus, brain, and liver contained a comparable level of procaspase 9 activity with a relatively small variation when compared on protein basis. Upon Q-Sepharose column chromatography, procaspase 9 of brain was eluted as a major and a minor peak (Fig. 4A): thymus procaspase 9 fractions also were eluted as a relatively broad peak (Fig. 1), suggesting that these tissues might express two molecular species of procaspase 9. However, analysis of crude extracts by SDS-PAGE followed by immunoblot

with anti-procaspase 9 antibody revealed that all of these tissues express a single class of procaspase 9 with a molecular size of 50 kDa (Fig 5). BB-procaspase 9-I and -II in (Fig. 4A), which were separately purified, also showed an identical molecular size as that of thymus and liver enzyme (data not shown). Thus, the differential elution of procaspase 9 observed during purification may be due to the association of a part of the enzyme with some dissimilar proteins.

All the tissues examined contained also a comparable amount of pro-DEVDase activity (Table 6). Fractionation of tissue extracts revealed the presence of multiple species of proDEVDase in thymus and liver. In thymus, two different species of proDEVDase, BT-proDEVDase-1 (procaspase 7- and 3-like, 30 kDa) and BT-proDEVDase-2 (procaspase 3-like, 29.5 kDa), were separated by further purification (Fig. 2C, Table 2). In brain, a single species of proDEVDase was recovered (BB-proDEVDase, Fig. 4A). Although the brain enzyme showed quite similar substrate specificity as the thymus enzymes (Table 3) and similar immuno-reactivity as BT-pro-DEVDase-2 (data not shown), the BB-proDEVDase showed a different elution profile from that of the thymus enzymes during purification by column chromatography (data not shown). Also the molecular size of BB-pro-DEVDase (32.5 kDa) was significantly larger than the thymus enzymes (data not shown). Liver tissue expressed three major pro-DEVDase activities, at least, judging from the data of Q-Sepharose column chromatography (Fig. 4C), although further characterization of liver pro-DEVDase remains to be examined. Thus, the expression of a terminal proDEVDase seems to be very different among the thymus, brain, and liver, suggesting difference in caspase-activating system in these tissues.

Among the three essential factors of Type II apoptotic pathway the tissue distribution of Apaf-1 showed the most dramatic difference. Although the liver extracts contained approximately 21% of Apaf-1 activity of the thymus extracts, the Apaf-1 activity of the

Table 6. Distribution of Apaf-1, procaspase 9, and proDEVDase in bovine thymus, brain and liver

	Activity (unit / mg protein)		
extracts	Apaf-1 (%)	Procaspase 9 (%)	proDEVDase (%)
thymus	3,900 (100)	5,600 (100)	3,100 (100)
brain	<100* (<3)	4,300 (77)	1,300 (42)
liver	800 (21)	2,200 (39)	1,400 (45)

Bovine thymus, brain, and liver extracts were prepared as described. Activities of Apaf-1, procaspase-9, and pro-DEVDase in the extracts were assayed by supplementation assay for caspase-activation as described in "Materials and Methods". Units of Apaf-1, and procaspase-9 in the extracts were obtained from the respective standard curve shown in Fig. 3. Relative values (%) were calculated by setting the activities of the respective factors in thymus at 100 % and are shown in parentheses.

* Essentially no Apaf-1 activity was detected in the brain extracts and also in the concentrated sample by ammonium sulfate (75 %) precipitation.

brain extracts was less than 3% (Table 6). Essentially no Apaf-activity was detected through purification (data not shown). Combining the results that brain extracts restore the proDEVDase-activation by supplementing purified thymus Apaf-1 (Table 5), all of the present results revealed that, in normal bovine brain, the Type II apoptotic pathway is abrogated mainly by down-regulation of the protein-expression of Apaf-1. In this regard, we observed also that the crude brain extracts lacked Apaf-1 band upon immunoblotting with anti-Apaf-1 antibody following SDS-PAGE, whereas in the thymus sample a band of Apaf-1 was located clearly at 130 kDa by the antibody (data not shown).

It has been established that type II apoptotic pathway is biologically significant in normal development of brain as reported by Cecconi et al.[24] and Yoshida et al.[25]. To reconcile their findings with ours we examined Apaf-1 in the brain of rats at different ages after birth. The results clearly indicated that Apaf-1 in brain rapidly decreased at 1 to 2 weeks after birth and almost disappeared after 4 weeks[34]. In the present study we used mature brain samples obtained from 2- to 3-year-old-oxen and we have observed also that brain extracts of adult mouse also lack Apaf-1 (data not shown). Combining these results, we suggest that the down-regulation of Apaf-1 and thus the down-regulation of type II apoptotic pathway after maturation of the brain tissue are events common to various animal species. The events may have a biological significance in protecting the central nervous system from apoptosis-inducing stimuli from outside.

In spite of the fact that bovine brain tissue is lacking in Apaf-1, the brain expressed a significant level of procaspase 9 and a caspase 3-like pro-DEVDase. In addition, there are many reports indicating that caspase 3 is activated in mature rat or mouse brain upon brain damage caused by trauma[35], ischemia[36, 37] and a chemical[38]. These findings raise the question of how brain caspase 9 is involved in the activation of caspase 3 in mature brain. In this respect, Yakovlev et al.[39] recently found that Apaf-1 was induced in adult rat brain after traumatic brain injury. The finding suggests that type II apoptotic pathway is regulated by the level of Apaf-1 expression, at least in some tissues including adult brain, whereas the factor is known to be broadly expressed among many other tissues and cell lines[18]. Alternatively, brain caspase 9 may have some Apaf-1-independent role in neuronal cell death as Sperandio et al.[40] have recently shown with Apaf-1 null mouse embryonic fibroblasts. Clarification of brain-specific role of caspase 9 or its activation process in mature brain will be very important to understand neuronal cell death in various neurodegenerative human diseases.

The present results, including the properties and tissue specific distribution of Apaf-1, procaspase 9, and terminal proDEVDase, may provide basic information for the study of human brain diseases involving neuronal cell death as well as for the study of bovine diseases such as bovine spongiform encephalopathy (BSE).

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