Identification of a Caspase-9 Substrate and Detection of Its Cleavage in Programmed Cell Death during Mouse Development*

Received for publication, June 19, 2001, and in revised form, August 10, 2001 Published, JBC Papers in Press, August 20, 2001, DOI 10.1074/jbc.M10648200

Keiko Nakanishi**, Masumi Maruyama†, Takehiko Shibata‡§, and Nobuhiro Morishima§**
From the Bioarchitect Research Group, Cellular and Molecular Biology Laboratory, RIKEN (Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, the Department of Molecular Biology, Faculty of Science and Engineering, Saitama University, 255 Shimo-Okubo, Saitama, Saitama 338-8570, and NEDO (New Energy and Industrial Technology Development Organization), 3-1-1 Higashi-Ikebukuro, Toshima, Tokyo 170-6028, Japan

The caspase family of proteases represents the main machinery by which apoptosis occurs. In vitro studies have revealed that upstream caspases are activated in response to apoptotic stimuli, and the active caspases in turn process downstream effector caspases that are involved in the destruction of cellular structure. Caspase-9 is an upstream caspase that can become active in response to cellular damage, including deprivation of growth factors, exposure to oxidative stress, and death receptors of Fas and TNF-α. This result is consistent with observations that gene knockouts of caspase-9 and its activator, Apaf-1, result in embryonic lethal phenotypes. Immunohistochemical analysis, using a specific antibody against the vimentin fragments generated by caspase-9, showed that caspase-9 cleaves vimentin in apoptotic cells in the embryonic nervous system and the interdigital regions. This result is consistent with observations that gene knockouts of caspase-9 and its activator, Apaf-1, result in developmental defects in these tissues. Our results show that the specific antibody is useful for in situ detection of caspase-9 activation in programmed cell death.

The caspase protease family plays a central role in the implementation of apoptosis in vertebrates. All members of the caspase family are synthesized in a precursor form that requires specific processing for activation (1, 2). These enzymes can be broadly divided into two groups: initiator caspases (such as caspase-8, -9, and -12) and effector caspases (caspase-3, -6, and -7). The main function of initiator caspases is to activate effector caspases, which are responsible for dismantling cellular structures. Cell biological studies have identified three main apoptotic pathways (the death receptor, endoplasmic reticulum (ER) stress, and mitochondrial pathways) that are activated by caspase-8, -12, and -9, respectively. Caspase-8 is recruited for activation to a death-inducing signaling complex only when death receptors such as Fas or the tumor necrosis factor receptor are oligomerized after the binding of specific ligands (3). Caspase-12 is specifically activated by ER stress (4), although the mechanism of its activation remains to be elucidated. In response to various death stimuli, mitochondria release cytochrome c, which forms a complex with Apaf-1 to induce the aggregation and processing of procaspase-9 (5–7). The importance of mitochondria in the apoptotic signaling pathway has increasingly been recognized, and recent studies suggest that signals from both the death receptor and the ER pathways may converge onto the mitochondrial pathway (8). These three pathways have mainly been studied in vitro using cultured cells (5–8), so that little is known about how and when activation of the caspase cascade is triggered in vivo, e.g., during development.

We have recently found that one of the major components of intermediate filaments, vimentin, can be a substrate for an initiator caspase (9). Vimentin is cleaved at the Asp259, and Asp259 residues by caspases during apoptosis of human T cells (9). Several lines of evidence, including the order of the multiple cleavages on the vimentin polypeptide, indicate that the initial cut is made at Asp259 by an initiator caspase, possibly either caspase-8 or caspase-9. The Asp259 cleavage occurs in the absence of the activities of effector caspases (9). The sequence around the cleavage site (Ile-Asp-Val-Asp259-Val) matches the sequence preferences of both caspases-8 and -9 in having an aliphatic amino acid residue at the P4 position (10). Caspase-3 and caspase-6 likely cleave vimentin at Asp85 and Asp129, respectively, at a later stage in the cascade.

In this study, we have further analyzed vimentin cleavage in apoptosis to identify the caspase responsible for the cleavage at Asp259. We prepared antibodies that specifically recognize the cleaved ends at Asp259, but not the intact form of vimentin. The antibodies detected the specific cleavage products of vimentin in cultured cells at an early stage of apoptosis. Treatment of cells with a specific inhibitor for caspase-9 suppressed such specific cleavage, whereas caspase-8 was active, cleaving Bid, a Bcl-2 family member (11, 12) in the presence of the inhibitor. Null mutation of caspase-8 did not affect the vimentin cleavage at Asp259. These results strongly suggest that caspase-9 is responsible for the cleavage of vimentin at Asp259. The major role of caspase-9 has been considered to be the transmission of the apoptotic signal from mitochondria to the downstream effector caspases. Our results suggest that caspase-9 can also function as an effector caspase that is involved in dismantling the cytoskeleton.
Since vimentin is almost ubiquitously expressed in cells of developing tissues (13–15), including in neurons at their early developmental stages (16), we expected that caspase-9 activation in many developing tissues could be detected through the cleavage of vimentin by the protease. We have taken advantage of the specificity of the antibodies for in situ detection of caspase-9 activation. Binding of the specific antibodies was detected in the embryonic nervous system and the interdigital regions, consistent with previous observations that gene knockouts of either caspase-9 (17, 18) or Apaf-1 (19, 20) show defects in these tissues.

EXPERIMENTAL PROCEDURES

Creation of Antibodies against Vimentin Fragments—Cleavage site-directed antibodies were prepared based on the protocol for the preparation of a cleavage site-directed antibody against calpain (21). Briefly, an anti-V1 antibody and an anti-V2 antibody were generated by immunization of rabbits with synthetic peptides, V1 (CQIDVD) and V2 (VSKPDC), which were obtained from Bio-Synthesis Inc. (Lewisville, TX). In order to enhance antigenicity, the peptides were covalently attached to activated hemocyanin (Pierce) prior to immunization. The same peptides were immobilized on an FMP-Cellulofine resin (Seikagaku Kogyo, Tokyo, Japan) for affinity purification of the peptide-specific antibodies. Immunoglobulins adsorbed onto the peptide resin were eluted with 0.1 M glycine (pH 2.6) and then neutralized by adding 1 M Tris(hydroxymethyl)aminomethane.

Cell Culture—Jurkat and SKW6.4 cells (ATCC) were cultured at 37°C with 5% CO2 in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (BioWhittaker), 50 units/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). A caspase-8-deficient mutant of Jurkat cells (I9-2) and its parental cells (A3) were kindly provided by Dr. John Blenis (Harvard Medical School, Boston, MA). B-2-2-overexpressing Jurkat cells, Jurkat/HB2–8 and Jurkat/HB2–10, were gifts from Drs. Y. Eguchi and Y. Tsujimoto (Osaka University, Osaka, Japan). Apoptosis induction was performed as described previously (9). Briefly, cells (5 × 105 cells/ml) were treated with 200 ng/ml anti-Fas monoclonal antibody (clone CH-11, Medical and Biological Laboratories, Nagoya, Japan) and 20 μg/ml cycloheximide (Calbiochem) or 1 M Tris(hydroxymethyl)aminomethane (Sigma-Aldrich). For the inhibitor experiment, cells were pretreated with 10 μM aprotinin (Sigma-Aldrich), and 100 μM cycloheximide (Calbiochem) or 1 M Tris(hydroxymethyl)aminomethane.

Cleavage of a Caspase-9 Substrate during Development—For analysis of vimentin cleavage in apoptotic cells, human B cells (SKW6.4) were induced to die by treatment with an anti-Fas antibody, and then both intact and cleaved vimentin were extracted from the cells. A commercially available anti-vimentin monoclonal antibody (V9) detects both intact vimentin (58 kDa) and its cleavage products (47, 41, 28, and 22 kDa) (Fig. 1A). This result is consistent with our previous observation that vimentin was cleaved at Asp85, Asp259, and Asp529 in a human T cell line, Jurkat (9). To raise antibodies that specifically recognize the cleaved ends at Asp85, but not the intact form of vimentin, we immunized rabbits with oligopeptides whose sequences corresponded to either Gin–Val–Asp–Val–Asp (peptide V1) or Val–Ser–Lys–Pro–Val (peptide V2). Since these sequences are conserved in the vimentin of several mammals (e.g. human, mouse, and rat), the specific antibodies raised against these peptides could be versatile tools for the detection of vimentin cleavage.

The antibodies were purified by affinity chromatography from the antisera. To determine the specificity of these antibodies, they were used to probe Western blots of extracts from apoptotic cultured cells. The anti-V1 antibody specifically recognized 31- and 21-kDa fragments, whereas the anti-V2 antibody detected 28- and 22-kDa fragments in extracts of dying cells (Fig. 1B). Neither antibody recognized the 58-kDa intact form, indicating that these antibodies are specific to the cleaved ends. The 31-kDa (V1-positive) and 28-kDa (V2-positive) fragments transiently appeared during early apoptosis whereas the smaller fragments (21-kDa fragment for V1, 22-kDa fragment for V2) appeared no earlier than the larger fragments and persisted afterward. According to the proposed model of cell lysates, caspases are activated as early as 10.5, 11.5, or 12.5 h after treatment is followed by activation of downstream caspases including caspase-3 and caspase-6 (reviewed in Ref. 22). Caspase-3 is predicted to efficiently cleave vimentin at Asp85, within the 31-kDa V1-positive fragment, whereas caspase-6 is expected to cleave at Asp429 in the V2-positive fragment of 28 kDa (Fig. 1C and Ref. 9).

The Asp259 Site Is Cleaved by Caspase-9, but Not by Caspase-8, in Jurkat Cells—Among caspase family members, caspase-8 (3) and caspase-10 (23) are the cell surface death receptor-associated proteases whose activation has been observed in response to stimulation of the death receptors. To
Vimentin is probably cleaved at Asp85, Asp259, and Asp429 by caspases C, schematic representation of the possible cleavage sites in vimentin. After induc-
examine the possible involvement of these receptor-associated caspases in the vimentin cleavage at Asp259, we used a caspase-8-deficient mutant of Jurkat cells (I9-2, Ref. 24). Fig. 2A shows that vimentin cleavage at Asp259 did not occur in I9-2 cells that were treated with an anti-Fas antibody. Since the caspase-8-dependent apoptotic pathway from the death receptor to the mitochondria is impaired in this cell line, activation of caspase-9 is also expected to be suppressed. In this cell line, however, caspase-10 is expressed at wild-type levels (24). The absence of the V1-positive signal thus suggests that caspase-10 is not involved in the vimentin cleavage.

We next examined vimentin cleavage in Jurkat cells, either wild type or the caspase-8-deficient mutant, after apoptotic induction of cells with staurosporine, a general kinase inhibitor which can induce apoptosis regardless of death receptor stimulation. Fig. 2B shows that the V1-specific signals (31 and 21 kDa) were detected in both wild-type and mutant cells when treated with staurosporine. This result indicates that vimentin can be cleaved in cells without caspase-8, leaving caspase-9 as the best candidate for the Asp259 cleavage.

We have previously detected the Asp259 cleavage of vimentin in a Bcl-2-overexpressing cell line, Jurkat/HB2–8, after induction of apoptosis through Fas stimulation (9). Bcl-2 is expected to inhibit the caspase cascade at the mitochondrial step. Activation of caspase-3 and caspase-6 was almost completely suppressed in the cell line, supporting the idea that caspase-9 upstream of these effector caspases is responsible for the Asp259 cleavage. The cleavage was much less prominent than that in the parental cell line (9), probably due to marginal activation of caspase-9. To confirm that caspase-9, located downstream of the mitochondrial step in apoptotic signaling, was responsible for the cleavage, we examined vimentin cleavage in another Bcl-2-overexpressing cell line, Jurkat/HB2–10. This cell line showed higher resistance to Fas stimulation than did Jurkat/HB2–8. Fas stimulation did not induce apoptosis, and we detected no vimentin cleavage (Fig. 2C), consistent with the idea that the Asp259 cleavage is an event downstream of the mitochondrial step.

To examine if the vimentin cleavage at Asp259 is dependent on caspase-9, we induced apoptosis of wild-type Jurkat cells in the presence of a peptide inhibitor specific for caspase-9 (LEHD-FMK, Ref. 25). Fig. 2D shows that LEHD-FMK significantly suppressed generation of the V1-specific fragments (31 and 21 kDa) of vimentin in Jurkat cells. Control experiments showed that a caspase-3-specific inhibitor (DEVd-CHO) did not reduce the cleavage at Asp259, whereas a general inhibitor for caspases (Z-VAD, Ref. 26) did. The 31-kDa fragment was stable in the presence of the caspase-3-specific inhibitor, because the fragment was not further digested due to lack of the caspase-3 activity. Caspase-8 was still active in the presence of LEHD-FMK, as shown by cleavage of a caspase-8 substrate, Bid (Fig. 2E). These results indicate that the vimentin cleavage at Asp259 is dependent on caspase-9, whereas active caspase-8 does not cleave vimentin in apoptotic cells. The suppression of the V1-specific vimentin cleavage by LEHD-FMK, the absence of vimentin cleavage in Jurkat/HB2–10 cells, and the independence of the Asp259 cleavage from caspase-8 strongly suggest that caspase-9, and not caspase-8 or caspase-10, cleaves vimentin at Asp259 in apoptotic cells.

To examine whether caspase-9 can, in fact, cleave the Asp259 site, we performed a cleavage assay of vimentin with purified caspase-9. Preliminary experiments showed that vimentin had multiple cleavage sites for caspase-9 in vitro, the major one being Asp-Ser-Val-Asp (data not shown). Fig. 3 shows that caspase-9 cleavage generated a 21-kDa fragment that was detected by the V1 antibody, whereas LEHD-FMK suppressed the generation of the V1-specific fragment. Mutation at Asp259 (D259N) in the vimentin polypeptide abolished the cleavage, indicating that the caspase-9 cleavage occurred at the Asp259 site within wild type vimentin. Taken together with the results of vimentin cleavage in cultured cells, we conclude that caspase-9 cleaves vimentin at Asp259 during the early stage of apoptosis. This is the first example of a caspase-9 substrate that is not a precursor form of a caspase family member.

**Immunocytochemical Analysis of Vimentin Cleavage by Caspase-9 in Jurkat Cells**—The cleavage site-directed antibodies were used to stain apoptotic Jurkat T cells. After 6 h of incubation with the anti-Fas antibody, over 80% of Jurkat cells in culture showed either condensed or fragmented nuclei with DAPI staining, a morphology typical of dying cells (Fig. 4A, arrowheads). These apoptotic cells were intensely labeled by the anti-V1 antibody (Fig. 4A, arrow). A similar staining pattern was also obtained from the anti-V2 antibody (data not shown). Control experiments, in which the anti-Fas antibody was omitted, showed that growing Jurkat cells were stained by anti-V1 only at a background level (Fig. 4C). These results indicate that the cleavage site-directed antibodies can be used for the staining
and detection of apoptotic cells in which vimentin is cleaved by caspase-9.

Vimentin cleavage by caspase-9 starts at an early stage of apoptosis, preceding the changes in nuclear morphology that can be visualized with DAPI staining (9). We next examined the time course of the appearance of V1 positive signals, in comparison with that of TUNEL (27). It has been established that digestion of chromosomal DNA occurs at a late stage of apoptosis, after the DNase responsible for the fragmentation of DNA, CAD, becomes active through proteolysis of its specific inhibitor (ICAD) by a downstream caspase, caspase-3 (28). One can thus expect that the reactivity of dying cells to the V1 antibody and TUNEL would change according to the following order: (i) V1-negative, TUNEL-negative; (ii) V1-positive, TUNEL-negative; (iii) V1 and TUNEL double-positive. Phase I would occur before and at the earliest onset of apoptosis. Following a 3-h treatment of cells with the anti-Fas antibody to induce apoptosis, more than 50% of the cells were V1-positive, about half of which were also TUNEL-positive (Fig. 4D); this corresponds to an overlap of phase II and phase III. After a 6-h treatment, almost all of the V1-positive cells (over 80% of cells in the culture) were also stained by TUNEL (phase III; Fig. 4E). These results suggest that cells first become V1-positive due to the cleavage of vimentin by caspase-9 and then suffer chromosomal DNA digestion.

It should be noted that the percentage of V1-positive cells in the culture significantly decreased after a longer treatment with the anti-Fas antibody, probably because of the disintegration of the filamentous structure of vimentin (9). After an 18-h incubation with anti-Fas, most TUNEL-positive cells showed only faint signals with V1 staining (Fig. 4F), whereas fewer than 10% of apoptotic cells still exhibited intense labeling with the V1 antibody. These results indicate that the cleavage-specific antibody can recognize the vimentin fragments during early stages of apoptosis, after which apoptotic cells lose the immunoreactivity. This suggests the presence of a fourth phase (phase IV) in which the cells are V1-negative and TUNEL-positive. We have detected these staining patterns in mouse embryos, which will be described in the following sections. We did not further use the V2 antibody for the staining of embryos because it did not label cells in paraffin-embedded sections (data not shown).

Detection of Caspase-9 Activation in Neurons at E10.5—Neuronal cell death in both the central and peripheral nervous
systems is a paradigm of developmental apoptosis, which involves the loss of a significant number of neurons in a given population (e.g. 20–80%; Ref. 29). We immunostained E10.5 mouse embryos with the anti-V1 antibody and detected clusters of V1-positive signals in the lamina terminalis of the forebrain (Fig. 5A). The lamina terminalis was also stained by TUNEL (Fig. 5B), consistent with previous observations by others (30, 31). Although some apoptotic cells in the lamina terminalis were stained by both the V1 antibody and TUNEL (Fig. 5C, arrowheads), most apoptotic cells were either V1-positive (phase II) or TUNEL-positive (phase IV). This result could be explained if phase III persists for only a short time, thereby making the transition from phase II to phase IV too short to detect. Alternatively, chromosomal DNA digestion may start after the V1 immunoreactivity of dying cells is lost, so that apoptotic cells are never in phase III (see “Discussion”). Intense staining of V1-positive cells was also detected in the vestibular ganglion where TUNEL signals were seen (Fig. 5D).

The regions where V1-positive signals were detected also contained significant numbers of TUNEL-positive cells. However, the reverse was not true, because we detected TUNEL-positive cells without V1 signals in regions of the brain other than the lamina terminalis and the vestibular ganglion. TUNEL-positive cells were found in the optic stalk (Fig. 5E), the telencephalon (Fig. 5F), and the trigeminal ganglion (Fig. 5G) where no V1 signals were detected. One possible explanation for this observation is that caspase-9 is not activated for apoptosis in these regions, and that vimentin stayed intact, perhaps because the caspase cascade in these regions depends on a different initiation mechanism from that which occurs in the lamina terminalis. It is also possible that the expression level of either caspase-9 or vimentin in these regions at E10.5 may be too low for detection.

In the peripheral nervous system, the dorsal root ganglia were also stained by both anti-V1 and TUNEL (Fig. 5H), although these two signals did not overlap. Therefore, at E10.5, caspase-9 activation occurs in both the central and the peripheral nervous systems of mice.

Detection of Caspase-9 Activation in Neurons in the Peripheral Nervous System at E13.5 and E18.5—Neuronal apoptosis within developing mouse ganglia has been observed from E11.5 to E16.5 (29, 32). At E13.5, we detected V1-positive cells in the trigeminal, superior cervical, and dorsal root ganglia in the peripheral nervous system. In these ganglia, V1-positive cells contained condensed nuclei, indicating that the antibody specifically stained dying cells in these regions (Fig. 6, A–C). These ganglia were also stained by TUNEL, which identified both phase III cells (Fig. 6D, arrows) and phase IV cells (Fig. 6D, arrowheads) in all three ganglion types (data not shown). It is interesting to note that V1 signals were detected in the trigeminal ganglion at E13.5, but not at E10.5. This result suggests that the initiation mechanism for the caspase cascade is independent of caspase-9 in the trigeminal ganglion at E10.5, whereas the caspase-9-mediated apoptotic pathway may be activated at a later developmental stage.

After E14.5, the peripheral nervous system contains a smaller number of apoptotic cells (29, 32), so we examined whether the number of V1-positive cells decreases at a late embryonic stage. In E18.5 embryos, the V1 antibody detected a few (2–5 positive cells in a single section) V1-positive cells in the superior cervical ganglion (Fig. 6E) and the trigeminal ganglion (Fig. 6F). This result suggests that caspase-9 activation diminishes when developmental apoptosis in the peripheral nervous system has decreased to an almost undetectable level after E16.5.

Detection of Caspase-9 Activation in the Interdigital Regions of Mouse Embryos at E13.5—Loss of interdigital cells, which is a well known example of developmental apoptosis (33, 34). We performed double staining of the hindlimb buds with the V1 antibody and TUNEL. Fig. 7A shows that both the V1 signal and TUNEL were restricted to the interdigital regions of the limb buds at
E13.5. The signals obtained with these two staining methods were almost mutually exclusive, so that only phase II cells (V1-positive) and phase IV cells (TUNEL-positive) were seen (Fig. 7B). The numbers of the two types of cells were comparable to each other. Similar results were obtained for forelimb buds at E13.5 (data not shown), although the numbers of both V1-positive and TUNEL-positive cells were smaller than those observed in the hindlimb at E13.5, probably due to the time lag of limb formation between the forelimbs and the hindlimbs (35).

To examine whether vimentin fragments are produced in limb buds with a cleavage pattern similar to that observed in apoptotic T cells, we performed Western blot analysis of limb buds at E13.5. Fig. 7C shows that the anti-V1 antibody detected a 21-kDa fragment of vimentin. As was the case with apoptosis in cultured cells (Fig. 1B), the anti-V1 antibody also detected a cleavage product that was slightly smaller than the 21-kDa fragment (Fig. 7C). These results support the conclusion that active caspase-9 is cleaving vimentin at Asp259 in dying cells within the interdigital regions.

**DISCUSSION**

Previously, we obtained results suggesting that caspase-9 might be the caspase responsible for the cleavage of vimentin at Asp259. In the present study, we have further examined the role of caspase-9 in this cleavage. The following data support the conclusion that caspase-9, alone among the initiator caspases, cleaves vimentin at Asp259, (i) the Asp259 cleavage was sensitive to a caspase-9-specific inhibitor (LEHD-FMK), (ii) the cleavage was suppressed by Bcl-2 overexpression, (iii) the cleavage was observed in caspase-8-deficient cells, (iv) active caspase-9 cleaves the Asp259 site in vitro, and (v) the cleavage was not detected in the presence of active caspase-8 and caspase-10 unless caspase-9 was activated. Together, these
results indicate that caspase-9 is the predominant caspase cleaving vimentin at Asp<sup>259</sup>. The critical test of this concept would be the evaluation of the Asp<sup>259</sup> cleavage in caspase-9 null mutant cells, which remains to be conducted.

Caspase-9 is the initiator caspase of the mitochondrial cascade that is activated by Apaf-1 in the presence of cytochrome c and dATP (5–7). The main function of the initiator caspases has been considered to be the activation of the effector caspases downstream of the initiator caspases, and in vitro studies have shown that caspase-9 processes and activates effector caspases. Vimentin is the first example of a caspase-9 substrate that is not a procaspase, suggesting that this initiator does more than merely mediate apoptotic signals, it also dismantles intracellular structures.

Vimentin is a major component of the intermediate filament, being composed of a central α-helical rod domain flanked by the non-α-helical sequences of the N-terminal head and C-terminal tail domains (for a review, see Ref. 36). Asp<sup>259</sup> is located between the second and third helices within the rod domain, which constitutes the main body of the intermediate filament. Cleavage at Asp<sup>259</sup> would break the central domain, likely affecting the integrity of the intermediate filament. In Jurkat cells stably transfected with a caspase-resistant vimentin, the onset of condensation and fragmentation of nuclei was delayed during apoptosis induced by Fas activation (9). Recently, we also observed that this stable cell line shows partial resistance to apoptosis induced by oxidative stress (37). These results suggest that cleavage of vimentin may play a role in the apoptotic process as a prerequisite for morphological change.

In a cell culture model of death receptor-mediated apoptosis, we observed three different types of dying cells in terms of immunoreactivity and TUNEL: V1-positive cells (phase II), V1- and TUNEL-double positive cells (phase III), and TUNEL-positive cells (phase IV). In developing tissues, however, we rarely detected phase III cells. This may be because there are parallel pathways for apoptosis that involve either vimentin cleavage or DNA digestion, which would yield phase II and phase IV cells, respectively. It is more likely, however, that DNA digestion usually follows vimentin cleavage or DNA digestion, which would yield phase II and phase IV cells, respectively. It is more likely, however, that DNA digestion usually follows vimentin cleavage by caspase-9, because caspase-9 launches the caspase cascade, thereby activating CAD through the cleavage of ICAD by caspase-3 (28).

Therefore, it is reasonable to expect that the phase II cells are transformed into phase IV via phase III in vivo, although phase III likely persists for only a short time, making its detection less likely. Alternatively, chromosomal DNA digestion may not start until the V1 immunoreactivity is lost in the apoptotic cells of developing tissues. Vimentin cleavage by caspase-9 starts at an early stage of apoptosis before full activation of the caspase family can be detected (9). We observed the loss of V1 reactivity when the filamentous structure of vimentin was further dismantled by the action of other caspases in cultured cells (Fig. 4F and Ref. 9). If the time lag between the initiation of vimentin cleavage and DNA digestion is long enough in vivo, the V1 reactivity could disappear before apoptotic DNA digestion gives TUNEL-positive signals; thus, phase III would be skipped.

In mouse embryos, V1-positive signals were detected in the lamina terminalis, in several ganglia in the peripheral nervous system, and in the interdigital regions, suggesting the involvement of caspase-9 in apoptosis of these regions. Consistent with our results, gene knockouts of caspase-9 in mice result in significant reductions in programmed cell death in the lamina terminalis at E10.5 (17) and the trigeminal ganglion at E12.5 (18). The decreased apoptosis causes brain malformations and perinatal lethality. A specific antibody that can recognize the mature form of caspase-9 in situ has recently been generated (38). With this antibody, processing of caspase-9 has been detected in the lamina terminalis, dorsal root ganglia, and ventral hindbrain of mouse embryos at E11.5 (38). These studies support our data suggesting that the V1-specific signals we have observed in the developing nervous system represent apoptotic cells in which caspase-9 plays a key role for normal development.

The involvement of caspase-9 in developmental apoptosis in the interdigital regions has not previously been reported. Caspase-9 null mice show brain-specific malformations, whereas the retraction of the interdigital webbing in the fetal hand plate is completed in the mutant embryos according to the normal developmental schedule (18). Therefore, caspase-9 is dispensable for the development of limb formation. Mutation of Apaf-1, however, results in defects including brain overgrowth as well as delay of limb formation (19, 20). Redundancy of apoptosis systems could explain the apparent discrepancy in the involvement of Apaf-1 and caspase-9 in the interdigital regions. It is possible that Apaf-1 induces not only the activation of caspase-9 but that of other caspases in these regions.

This study shows that cleavage-site-directed antibodies against caspase substrates can be potent tools for the detection of caspase activation in vivo. Apoptosis during development takes place in a less synchronized manner than it does in cell culture systems, where massive apoptosis can be induced by various apoptotic stimuli. Therefore, detection of caspase activation in embryos is more difficult than it is in cultured cells. A higher sensitivity for detection would be expected for cleaved substrates than for the active caspases responsible for their cleavage, as the number of substrate molecules is higher than the number of protease molecules. The V1 antibody also has an advantage over other methods for studying the role of caspase during development. It detects vimentin cleavage in situ, thereby showing when and in which cells caspase-9 is working. Detection of active caspase per se (i.e. caspase cleaved from its inactive pro-caspase precursor) does not necessarily indicate that the action of the caspase on its substrate has begun. Recent studies have shown that inhibitors of apoptosis proteins can physically interact with activated caspases (caspase-3, -7, and -9) and block their activity (reviewed in Ref. 39). Furthermore, the caspases’ substrates are not always in close proximity to the active caspases, and a caspase may have to move from the intracellular location where it becomes active to another location where it will cleave its substrate (40). Therefore, detection of the processed caspase-9 may not be enough to conclude that caspase-9 has started to cleave its substrates. Another advantage of the use of the V1 antibody is that it recognizes vimentin cleavage at an early stage of apoptosis, before the degradation of chromosomal DNA gives TUNEL-positive signals. Therefore, one can discriminate dying cells in which the apoptotic events are ongoing from dead cells in which the caspase family has already finished its role in dismantling intracellular structures.

The neurotrophic theory has explained the induction mechanism for programmed cell death in the developing nervous system (reviewed in Ref. 29). The theory suggests that synaptic contact between neurons and their target is necessary for cell survival, and whether a cell survives or dies is decided through competition for the trophic interaction. However, this theory cannot be applicable to all forms of programmed cell death in the developing nervous system, for instance, to those where cell death occurs before the neuronal population has contacted its target (41, 42). According to studies using mainly in vitro systems, a specific combination of an extracel lular ligand and a cell surface receptor constitutes a strong signaling system for apoptosis, and causes cells to die in a quite efficient manner (43). Such a combination of a ligand and its specific receptor
may actively mediate developmental apoptosis, because developmental apoptosis needs to occur efficiently at the right time for normal development. Recent studies suggest that apoptotic signaling mediated by death receptors also operates in vivo (reviewed in Refs. 44 and 45). In the developing chick embryo, the distribution of tumor necrosis factor-like proteins correlates with some regions of apoptosis (46). Nerve growth factor may play a pro-apoptotic role in the nervous system through p75NTR (47, 48). In the interdigital region, a bone morphogenic protein has been found to positively regulate interdigital apoptosis (33, 49). Dependence of caspase-9 activation on these receptors and their ligands can now be examined through the application of the V1 antibody to its ligand, or its associated proteins.

Acknowledgments—We thank the members of the Animal Facility in RIKEN for generation of the anti-V1 and anti-V2 polyclonal antibodies. We thank John Blenis (Harvard Medical School, Boston, MA) for Jurkat I9-2 and Jurkat A3, and Yutaka Eguchi and Yoshihide Tsujimoto (Osaka University, Osaka, Japan) for Jurkat/HB2. We also thank Tatsuhiko Sudo (RIKEN, Saitama, Japan) for critical reading of the manuscript, and Kawashima (University of Tokyo, Tokyo, Japan) for helpful discussions.

REFERENCES

Identification of a Caspase-9 Substrate and Detection of Its Cleavage in Programmed Cell Death during Mouse Development

Keiko Nakanishi, Masumi Maruyama, Takehiko Shibata and Nobuhiro Morishima

doi: 10.1074/jbc.M105648200 originally published online August 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105648200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 16 of which can be accessed free at http://www.jbc.org/content/276/44/41237.full.html#ref-list-1