Dual Role of Caspase-11 in Mediating Activation of Caspase-1 and Caspase-3 Under Pathological Conditions

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Abstract. Caspase-11, a member of the murine caspase family, has been shown to be an upstream activator of caspase-1 in regulating cytokine maturation. We demonstrate here that in addition to its defect in cytokine maturation, caspase-11-deficient mice have a reduced number of apoptotic cells and a defect in caspase-3 activation after middle cerebral artery occlusion (MCAO), a mouse model of stroke. Recombinant procaspase-11 can autoprocess itself in vitro. Purified active recombinant caspase-11 cleaves and activates procaspase-3 very efficiently. Using a positional scanning combinatorial library method, we found that the optimal cleavage site of caspase-11 was (I/L/V/P)EHD, similar to that of upstream caspses such as caspase-8 and -9. Our results suggest that caspase-11 is a critical initiator caspase responsible for the activation of caspase-3, as well as caspase-1 under certain pathological conditions.

Key words: caspase-11 • initiator caspase • stroke • middle cerebral artery occlusion • apoptosis

Introduction

Mammalian caspases are a family of cysteine proteases involved in regulating cytokine maturation and apoptosis (Cryns and Yuan, 1998). Caspases can be classified according to their amino acid sequence homology into caspase-1, -3, and -9 subfamilies. The caspase-1 subfamily includes caspase-1, -4, -5, and -11. Since the predominant defect of caspase-1 knockout mice is the inability to process pro–interleukin-1β (pro–IL-1β)1, the major function of caspase-1 is believed to be regulating cytokine maturation (Kuida et al., 1995; Li et al., 1995). Caspase-11 is a murine caspase that shares the highest homology with human caspase-1 (60% identity). The expression of caspase-11 is undetectable in healthy mice and highly inducible upon injection of lipopolysaccharide (LPS; Wang et al., 1996). Caspase-11 does not process pro–IL-1β directly. Nevertheless, caspase-11 mutant mice are deficient in the processing and secretion of IL-1β (Wang et al., 1998). We showed that caspase-11 can physically interact with caspase-1 to promote its activation. Based upon these data, we proposed that elevated expression of caspase-11 under pathological condition is directly responsible for activation of caspase-1, and thus, caspase-11 is an upstream regulator of caspase-1 (Wang et al., 1998).

The caspase-3 subfamily includes caspase-3, -6, -7, -8, -9, and -10 (Cryns and Yuan, 1998). Among this family, caspase-3 shares highest homology with caspase-7 and both have short prodomains; whereas caspase-6, -8, and -10 have long prodomains. Caspase-3 has been shown to be a major execution caspase that acts downstream in the apoptosis pathway and is involved in cleaving important substrates such as ICAD (inhibitor of caspase activated DNase), which activates the apoptotic DNA ladder-forming activity of CAD (caspase activated DNase); E nari et al., 1998; Sakahira et al., 1998). The major route of activating short prodomain caspses is through direct proteolytic processing. Two known pathways that can activate procaspase-3 are through proteolytic cleavage by caspase-8 and -9. In the Fas apoptosis pathway, the activation of Fas receptor induces the formation of death inducing signaling complex (DISC) which recruits and activates caspase-8 (Kischkel et al., 1995; Muzio et al., 1996). In Fas type I cells, activated caspase-8 directly activates procaspase-3 by cleavage (Scaffidi et al., 1998). In apoptosis, where mitochondria play a critical role, the release of cytochrome c from damaged mitochondria induces the formation of cytochrome c/Apaf-1/caspase-9 complex (Li et al., 1997).
A paf-1 is a mammalian homologue of Caeinhadonis elegans cell death gene product Ced-4 (Zou et al., 1997). The interaction of cytochrome c, A paf-1, and caspase-9 triggers the activation of caspase-9 that in turn activates procaspase-3 through direct proteolytic processing (Li et al., 1997). Thus, caspase-8 and -9 have been known as the two major upstream activators of caspase-3. Caspase-8 and -9 mutant mice exhibit severe defects in developmental apoptosis, suggesting that they are critical for activation of apoptosis and caspase-3 during development (Kuida et al., 1998; V arfolomeev et al., 1998).

Caspases may be involved in both acute and chronic neurodegenerative diseases (Friedlander and Y ,an, 1998). Irreversible caspase inhibitors, zV A D.fmk and zDEVD.fmk, protected brains from ischemic injury and improved neurological deficits in both mouse and rat (H ara et al., 1997b). Caspase-3–like enzyme activity and the activated caspase-3 subunit are detected in ischemic brain samples (Namura et al., 1998). Intraventricular injection of DEVD.fmk significantly reduced middle cerebral artery occlusion (MCAO)-induced apoptosis, suggesting that caspase-3 may play a critical role in ischemic brain injury (H ara et al., 1997b). A s caspase-3 is a short-prodomain caspase, it is usually activated by an upstream caspase through direct proteolytic cleavage. Although caspase-3 has been implicated in a number of pathological conditions, including brain ischemia, the identity of the upstream caspase(s) is not known. We show here that caspase-11 fits all the criteria to be this upstream caspase in activation of caspase-3 under pathological conditions. Caspase-11 mutant mice are defective in apoptosis and caspase-3 activation induced by brain ischemic injury. A c tivated caspase-11 is an efficient activator of caspase-3 in vitro. Combinatorial library analysis showed that the preferred cleavage sequence of caspase-11 is (I/L/V/P)E HD, similar to that of caspase-8 and -9 (Thornberry et al., 1997). In addition, procaspase-11 can process itself in vitro. We propose that caspase-11 is an upstream caspase responsible for the activation of caspase-3, as well as caspase-1, under pathological conditions.

Materials and Methods

Animals

Caspase-11 knockout mice have been described previously (W ang et al., 1998). In brief, mutant ES cell clones carrying a null mutant caspase-11 allele were injected into C57BL/6J blastocysts. The resulting chimeric males were then mated with C57BL/6J × DBA 2 F1 females to obtain germline transmission of the mutant allele. Chimaera of clone 444 produced germ-line transmitted mutant mice. The offspring from clone 444 were backtransmission of the mutant allele. Chimera of clone 444 produced germ-
caspase-1, under pathological conditions.

In Vitro Cleavage Assay

In vitro translation of 35S-labeled proteins was made by using TNT-coupled transcription/translation kit (Promega) in the presence of [35S]me-thionine. [35S]-labeled proteins were incubated with 0.5 μg of purified caspase-11 in the presence of 50 μM Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.5 mM sucrose, and 5% glycerol. Protease inhibitors (1 mM PM SF, 5 μg/ml pepstatin, and 10 μg/ml leupeptin) were also added. A fter centrifugation at 10,000 g the supernatant was mixed with 2 ml of nickel-charged resin (Qiagen) and incubated for 2 h at 4°C with rotation. The beads were then washed serially with 20 ml of buffer plus 1 M NaCl and lysozyme buffer alone. The bound caspase-11 was eluted with 5 ml lysis buffer containing 125 mM imidazole and verified by SDS-PAGE and autoradiography.

Cell Culture

L929 (mouse fibrosarcoma cell line) cells were maintained in DM E supplemented with 10% FCS.

Immunoblot and Immunoprecipitation Assay

Generation of mAb against caspase-11 (17D9, hybridoma culture supernatant) was described previously (W ang et al., 1996). For immunoblotting, tissue samples were ground in liquid N2 and the resulting tissue powder was solubilized in 0.7 ml of IP lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% NP-40) with protease inhibitors (1 mM PM SF, 10 μg/ml leupeptin, and 5 μg/ml pepstatin). A fter incubating on ice for 10 min, samples were centrifuged at 13,000 rpm in a microfuge at 4°C for 20 min. Protein concentration was measured using Bior ad protein as say reagent. 60 μg of protein was subjected to SDS-PAGE on a 15% gel. Proteins were then transferred onto immobilon-P membrane (Millipore) and incubated with blocking solution containing 5% skim milk in T BST (50 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature. The membrane was then incubated with primary antibody in blocking solution at 4°C overnight. A fter washing three times with T BST for 10 min each, the membrane was incubated with HRP-conjugated anti-rat or anti-rabbit IgG (Jackson ImmunoResearch) for 40 min at room temperature. A fter washing three times, the bound antibody was revealed using the ECL Western blotting reagent (A msersham Pharmacia Biotech).

Construction of Plasmid

The construction of expression plasmid of caspase-11 was made by PCR amplification using pgi 667 as a template (W ang et al., 1998). The primers used are: SF 6, GA GA T CCCAT GG CTT GA A A C A A C C C T; and mNO/BH2, T TGG A T CGC TA GTT TGG C AA GA A A A G T. The PCR fragment was cloned into the BamHI site of pET-15b and named pET-15b.
For immunoprecipitation, half of the mouse brain was ground in liquid N₂ and solubilized in 700 µl of lysis buffer. The resulting lysate supernatant (total 1 mg of protein) was incubated with 50 µl 17D9 overnight at 4°C with rotation, followed by incubation with protein G-coupled agarose beads for 40 min. The immune complexes were resolved by SDS-PAGE under reducing conditions with 10% sucrose, 0.1% CHAPS, 10 mM DTT, pH 7.5, in a total volume of 100 µl.

Immunohistochemistry

After MCAO or LPS challenge, mice were anesthetized and transcardially perfused with cold PBS and then with 4% paraformaldehyde in PBS. Brains were dissected, embedded in OCT medium, and were then frozen on top of dry ice. The frozen blocks were kept in −80°C until use. 5-µm sections were made in a Leica CM3000 cryostat. Sections were then postfixed in ice cold ethanol/acetic acid (2:1) solution for 5 min. After brief washing with PBS, endogenous peroxidase activity was quenched by incubating sections with 0.3% hydrogen peroxide in PBS for 30 min when TSA signal amplification kit (NEL EN Life Science Products) was used for visualization. Sections were then incubated with 10% normal goat serum in PBS containing 0.1% Triton X-100 for 1 h at room temperature. When secondary antibody is biotin/avidin system, samples were first blocked with Vector Laboratories' Biotin/avidin blocking kit before incubating with normal goat serum. After blocking, sections were incubated with drops of anticaspase-11 monoclonal (undiluted 17D9), anticaspsase 3p20 (CM1) rabbit polyclonal (12,000), anti-NeuN mouse monoclonal (11,000; Chemicon International), or anti-GFAP (giall fibrillary acidic protein) rabbit polyclonal (1,500; DAKO) antibody in PBS containing 1% normal goat serum and 0.1% Triton X-100 overnight in a humid chamber. For detection of microglial cells, biotin-conjugated B₈ isolectin (5 µg/ml) was used. Samples were then washed four times with PBS containing 0.1% Triton X-100 for 10 min each, followed by incubation with secondary antibodies in PBS containing 1% normal goat serum and 0.1% Triton X-100 for 30 min at room temperature. For the detection of caspase-11, HRP-conjugated anti-rat goat IgG (Jackson ImmunoResearch) and TSA signal amplification kit were used following manufacturer's protocol. For the detection of caspase-3, NeuN, and GFAP, matching secondary antibodies conjugated with biotin were used. After washing, biotin-decorated samples were incubated with Texas red- or FITC-conjugated streptavidin (Jackson ImmunoResearch) for 30 min at room temperature. Tissue sections were then counterstained with Hoechst 33258 dye (1 µg/ml in PBS; Sigma Chemical Co.) for 10 min at room temperature. After washing, slide glass samples were mounted with mounting medium (1 mg/ml p-phenylenediamine in PBS containing 90% glycerol). Samples were examined using an Axiovert 135 microscope (Carl Zeiss, Inc.) and images were recorded using Northern Exposure software.

For TUNEL assay, ethanol/acetic acid postfixed samples were briefly washed and then treated with proteinase K (20 µg/ml in PBS) for 15 min at room temperature. After washing with PBS, samples were processed using ApopTag kit (Intergen) according to the manufacturer's manual. Samples were also counterstained with Hoechst 33258.

Enzyme Activity Assay

To see if caspase-11 can activate procaspase-3 or -1, 15 µg of purified recombinant caspase-11 (pS15) and/or 10 µg of recombinant procaspases were added into Heps buffer (50 mM, pH 7.4) containing 2 mM DTT and 12 µM fluorogenic peptide substrates (AcDEVD-amc or AcVAD-amc) in a total volume of 300 µl. Fluorescence as a result of caspase activation was measured at 10-s intervals using a micro Bowman Series 2 Luminescence spectrophotometer (excitation wavelength = 380 nm, emission wavelength = 460 nm).

Substrate Specificity Screening

The design, synthesis, and validation of the PS-SCL have been described elsewhere (Rano et al., 1997). Each of the 60 samples (20 amino acids × 3 sublibraries) of the PS-SCL was prepared as a stock of c~10 mM in DMSO. To determine protease specificity, 2.3 nM caspase-11 was added to reaction mixtures containing 100 µM substrate mix, 100 mM Heps, 10% sucrose, 0.1% CHAPS, 10 mM DTT, pH 7.5, in a total volume of 100 µl. Under these conditions, the final concentration of each individual compound is c~0.25 µM. Production of AMC was monitored continuously at room temperature in a Tecan fluoroscan 96-well plate reader using an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

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Results

Protection of Apoptotic Cell Death in Caspase-11-deficient Mice

Previous studies suggesting a role of caspase-11 as an essential activator of caspase-1 (Wang et al., 1996, 1998) and a potential role of caspase-1 in ischemic brain injury (Friedlander et al., 1997; Hara et al., 1997a,b; Schielke et al., 1998) led us to investigate the involvement of caspase-11 in apoptosis induced by MCAO, a mouse model of stroke. Caspase-1 knockout mice showed resistance to ischemic brain injury (Schielke et al., 1998). However, since we failed to detect the LPS stimulated caspase-11 induction in caspase-1 knockout mice generated both by Kuida et al. (1995) and Li et al. (1995; Fig. 1 A), we cannot distinguish whether loss of caspase-1 or caspase-11 expression is directly responsible for the protection against ischemia-induced apoptosis. The expression of caspase-1 in caspase-11 mutant mice, on the other hand, is completely normal (Wang et al., 1998). To determine whether caspase-11 is critical for ischemic brain injury, we subjected caspase-11 mutant mice and their littermate wild-type controls to MCAO. Blood flow was blocked in the territory of the middle cerebral artery of the left hemisphere by inserting a nylon filament into the artery and 12 h later, the control and injured brain samples were processed for TUNEL staining as a marker of apoptotic cell death. Caspase-11 knockout mice exhibited markedly reduced population of apoptotic cells induced by permanent occlusion of middle cerebral artery at the level of striatum and hippocampus (Fig. 1, B and C). This reduction was not due to the genetic background effect in the knockout mice since wild-type C57L/J and 129SvJ mice showed no significant difference in the number of TUNEL-positive cells after MCAO (data not shown).

Induction of Caspase-11 by Pathological Stimuli

Caspase-11 expression in normal healthy mice is undetectable by Western blot (Wang et al., 1996). We examined if caspase-11 is upregulated by MCAO and immunocytochemistry methods. Caspase-11 mutant and wild-type control littermates were subjected to the permanent MCAO for 12 h and the expression of caspase-11 was examined (Fig. 2 A). As the majority of the cells was not dying in these ischemic brains, and therefore only a small portion of cells was expressing caspase-11, we had to use immunoprecipitation to see caspase-11. We have shown that the caspase-11 locus encodes two proteins of 38 and 43 kD (Wang et al., 1996). Interestingly, the 43-kD species was induced only in the ischemic side of the brain, whereas the 38-kD species was upregulated both in ipsilateral and contralateral sides of the brain; in contrast, both were absent in control brains. In the ischemic brain samples, immunoreactivity was strong in the cortical area (Fig. 2 B, a and b) and localized in the cytoplasm with occasional filamentous or patchy condensation of the immunoactivity (Fig. 2 B, e–g) and such staining was absent in caspase-11 mutant mice (Fig. 2 B, c and d). This result showed that caspase-11 is upregulated in a subpopulation of cells in brain after ischemic injury.

To determine the cell types that express caspase-11 un-
Under ischemic conditions, we carried out double-immuno-staining on sections of ischemic brain samples using mAb against caspase-11 and cell type marker antibodies. Caspase-11 staining was found in cells positive for neuN (Fig. 2 C, a and b), a neuronal marker (Mullen et al., 1992), as well as B4-isoelectin (Fig. 2 C, d and e), a microglial marker (Streit and Kreutzberg, 1987). Double-positive cells for GFAP and caspase-11 were not detected (data not shown). From these results, we concluded that caspase-11 is upregulated in subpopulations of neurons and microglial cells after ischemic brain injury.

Inhibition of Caspase-3 Activation in Caspase-11 Mutant Mice

Since caspase-3 has been shown to be a major downstream caspase in apoptosis, we set out to test if resistance of caspase-11 knockout mice to apoptosis induced by brain ischemia is correlated with a deficiency in the activation of caspase-3. Mice were challenged by MCAO and brains were processed for immunohistochemistry. An antibody to activated caspase-3, CM1, clearly labels a subpopulation of cells in wild-type cortex after ischemic injury (Fig. 3, a and b). This staining was significantly reduced in caspase-11−/− cortex that has been subjected to the same ischemic treatment (Fig. 3, c and d). Untreated control wild-type brains showed little staining with CM1 (Fig. 3, e and f). These results suggest that caspase-11 is essential for the activation of caspase-3 in a subpopulation of cortical neurons and microglial cells after ischemic brain injury.

Since caspase-11 regulates the activation of both caspase-1 and caspase-3, in ischemic tissues caspase-11 may activate caspase-3 through direct proteolytic processing or indirectly through regulating cytokines. We reasoned if caspase-11 acts directly to activate caspase-3, we should be able to detect the upregulated caspase-11 and activated caspase-3 in the same cells. To test this possibility, we immunostained ischemic brain samples with anticaspase-11 and CM1, and we found that a significant portion of caspase-11-positive cells are also positive for CM1 (Fig. 3, g–i). Among 400 cells counted in the ischemic area, 27% of the cells were positive for both caspase-11 and CM1, 7.3% were positive for caspase-11 only, and 2.3% were positive for CM1 only. Importantly, we did not find any caspase-11 single-positive cells that were right next to CM1 single-positive cells (data not shown), thereby making it highly unlikely that activated caspase-3 was mediated by cytokines released from neighboring cells with activated caspase-11. These results suggest that in the cells that are positive for both caspase-11 and activated caspase-3, the activation of caspase-3 is most likely mediated through direct processing of procaspase-3 by caspase-11.

Activation of Caspase-3 by Caspase-11 In Vitro

Since caspase-11 appears to be able to activate caspase-3 in vivo independent of its ability to regulate cytokine processing, we tested if caspase-11 can cleave procaspase-3 directly. Purified recombinant caspase-11 was incubated with in vitro translated 35S-labeled caspase-3 and the resulting products were visualized by autoradiography. As shown in the Fig. 4 A, caspase-3 was found to be an excel-

Figure 1. Reduction of apoptotic cell numbers after ischemic brain injury in caspase-11−/− mice. A, Western blot of spleen lysate from 3 wild-type (+/+ ) and 4 caspase-1 (−/− ) mice, probed by anticaspase-11 (top), anticaspase-1 (middle) and antitubulin (bottom). L929 cell lysate (L929) was used as a positive control. B, TUNEL (a, c, and e) and Hoechst dye (b, d, and f) staining of wild-type (a, b, e, and f) and caspase-11−/− (c and d) brains. a–d are from ischemic brains and e and f are from untreated control brain. Permanent ischemia was induced by occlusion of the left middle cerebral artery using monofilament as described by Hara et al. (1997a). 12 h after the occlusion, brains were processed for TUNEL. C, Quantification of TUNEL-positive cells in the wild-type and caspase-11−/− ischemic brain. The percentages of TUNEL-positive cells were determined by the number of TUNEL-positive cells divided by the number of Hoechst dye-positive cells. TUNEL- and Hoechst dye-positive cells were quantified using the Zeiss Axiovert microscope equipped with Northern exposure software. Mean of the reading from five brains is shown. Error bar represents standard error.
Figure 2. Induction of caspase-11 by ischemic brain injury. A, Permanent ischemia was induced by 12-h occlusion of the left middle cerebral artery. Wild-type ischemic (Isch), ipsilateral (L), and contralateral (R), or sham-operated (con), ipsilateral (L), and contralateral (R) sides of brain lysates were immunoprecipitated with monoclonal anti-caspase-11 antibody and Western-blotted with anti-caspase-11 mAb. IgG H and IgG L indicate IgG heavy and light chain, respectively. Anticaspase-11 stains a weak background band slightly smaller than 38 kD sometimes in brain samples. B, Wild-type and caspase-11−/− ischemic brains were immunostained with anticaspase-11 (a, c, and e) and counterstained with Hoechst dye (b, d, and f) 12 h after MCAO. e and f are overlaid in g. C, Wild-type ischemic brain 12 h after the occlusion was double-stained for caspase-11 (a) and anti-NeuN as a neuronal marker (b); caspase-11 (d) and B4-isolectin as a microglial cell marker (e). Counterstainings with Hoechst dye are shown in c and f.
taining all the amino acid residue combinations at P2, P3, and P4 positions (P4 P3 P2 D A M C). Interestingly, when the cleavage specificity of caspase-11 was tested in this library, we found that although caspase-11 is a member of the caspase-1 subfamily based upon its sequence homology, it prefers to cleave (I/L/V/P)EHD, which is similar to the specificity of upstream caspases, such as caspase-8 and -9 (Fig. 4 C). Since it has been well established that caspase-8 or -9 can directly activate caspase-3 through proteolytic cleavage (Muzio et al., 1996; Li et al., 1997), these results further argue that caspase-11 can directly activate caspase-3 under pathological conditions.

Processing of 43-kD Procaspase-11 by Active Caspase-11 In Vitro

The caspase-11 locus encodes two polypeptide of 43- and 38-kD (Wang et al., 1996). Since caspase-11 cDNA under the control of a heterologous promoter is transcribed and translated into the same 43- and 38-kD in vitro and in cells (Fig. 5, and data not shown), it is reasonable to assume that they are the products of alternative starts of translation. In addition, only the 43-kD product would carry the NH$_\text{\textsubscript{2}}$-terminal tag (data not shown), which provides a further support that the 38-kD product is initiated downstream from the Met for 43 kD, and therefore is missing most of the prodomain of caspase-11. To elucidate the functional difference of 43- and 38-kD products of caspase-11, we incubated the purified bacterially expressed procaspase-11 with in vitro translated, $^{35}$S-labeled full-length caspase-11. Like all the other caspases, procaspase-11 is autoprocessed when expressed in bacteria and therefore is proteolytically active (Fig. 4 and data not shown). As shown in Fig. 5 A, purified bacterially expressed procaspase-11 can process in vitro translated full-length caspase-11 into a protein fragment of 30 kD (p30) and 10 kD (p10). Interestingly, the cleavage appears to target preferentially at the 43-kD species, whereas the 38-kD species is stable during the incubation. This result suggests that the 43-kD caspase-11 protein product may be activated early in the apoptosis pathway.

To study if caspase-11 can be activated and processed during apoptosis in culture, we chose the L929 cell line because it expresses high levels of caspase-11. Most of the cell lines express undetectable amount of caspase-11 and have to be induced with LPS to express caspase-11. The L929 cells were treated with different amounts of etoposide and incubated overnight. The cell lysates made from etoposide-treated L929 were Western blotted with anti-caspase-11 mAb made against p10 of the caspase-11 (17D9). As shown in Fig. 5 B, the 43-kD product of caspase-11 was processed into p30 during etoposide-induced cell death, whereas the 38-kD species is stable during the incubation. We predict that the p30 is the caspase-11 fragment removed of its N-prodomain because it still can be recognized by the mAb against the p10 subunit. This result is consistent with our in vitro cleavage analysis and supports the conclusion that the 43-kD product of caspase-11 is activated more easily and earlier in apoptosis than that of the 38-kD product.

Discussion

Since our analysis of caspase-11 showed that caspase-11 may be a very important regulator of apoptosis under pathological conditions, it is critical to identify its human ortholog. The obvious candidates for caspase-11 in human
are caspase-4 and -5, since they share the highest homology among all known caspases with caspase-11; however, caspase-4 expression appears to be present in unstimulated cells (Kamada et al., 1997). The preferred cleavage tetrapeptide substrates of caspase-4 and -5 are (W/L)EHD (Thornberry et al., 1997), which differs from that of caspase-11. While caspase-11 prefers LEHD as a substrate, it does not cleave WEHD to a significant degree. Thus, it is possible that either caspase-4 and -5 in human may function like caspase-11 in mouse, but with a somewhat wider specificity; alternatively, the true ortholog of caspase-11 in human remains to be identified. It will be helpful to know whether other characteristics of caspase-11 (such as its inducibility) are preserved in caspase-4 and/or -5 to distinguish these possibilities.

The expression of caspase-11 is the most stringently regulated among all the caspases identified so far. Whereas most of caspases can be detected in healthy, unstimulated cells, caspase-11 expression is below the detection limit of Northern and Western blots and its transcripts are only detectable by reverse transcriptase PCR in most tissues except intestine, where its expression can be detected in unstimulated condition (Wang, S., S. Kang, and J. Yuan, unpublished data). The expression of caspase-11 is highly inducible by a variety of apoptosis stimuli, including ischemic brain injury, systemic inflammation, head trauma, and even transfection of cultured cells (Wang, S., S. Kang, and J. Yuan, unpublished data). Whereas the activation of most caspases is mediated through recruitment into activation complexes, the activation of caspase-11 is regulated at the transcriptional and translational level. We have observed caspase-11 to form filamentous structures in cells with high levels of caspase-11 expression (Fig. 2). Overexpression of caspase-8 has been shown to form filaments that appear to be sufficient for their activation (Siegel et al., 1998). It is possible that elevated caspase-11 protein concentration in stimulated cells may be sufficient for it to form filaments which results in its activation.

The present work, together with our previous study (Wang et al., 1998), demonstrates that caspase-11 is a dual activator of caspase-1 and -3 under pathological conditions, although we cannot rule out a possibility that other caspases are also acting downstream of caspase-11, as caspase-2 and -7 are also cleaved by caspase-11 in vitro (data not shown). Caspase-7 is similar to caspase-3 in its specificity (Thornberry et al., 1997), so it is possible that caspase-7 may contribute to the downstream effect of caspase-11. Caspase-2, however, is unlikely to play a sig-
of brain swelling. Interestingly, intra-injection of YVAD.fmk resulted in both reduction of intracerebral hemorrhage (Hara et al., 1997b). Intraventricular microinjection of both YVAD.cmk and DEVD.fmk have been shown to reduce ischemic brain injury induced by MCAO with intriguing differences (Hara et al., 1997b). Intraventricular injection of YVAD.fmk resulted in both reduction of infarction volume and of brain swelling. Interestingly, intra-

ventricular injection of DEVD.fmk decreased infarction volume, but had no effect on brain swelling. As we have shown that caspase-11 can mediate the activation of both caspase-1 and caspase-3, such damage may be mediated directly through proteolytic activation of caspase-3 and apoptosis, and indirectly, through activating caspase-1 and cytokine release, which causes brain swelling that may in turn initiate additional apoptosis. Thus, the regulation of both downstream caspases, caspase-1 and -3, by caspase-11 may contribute to apoptotic damage under pathological conditions.

Caspase-11 locus encodes two proteins of 38 and 43 kD (Wang et al., 1996). We observed that the 43-kD protein is the predominant protein product of caspase-11 cleaved both in vivo during apoptosis and in vitro by autoprocessing. We hypothesize that the 43-kD protein is the full-length caspase-11 that can be autoactivated, whereas the 38-kD protein may be missing most of the C prodomain domain that it may need to be activated by another caspase(s). Interestingly, the 43-kD species was induced only in the ischemic side of the brain, whereas the 38-kD species was upregulated both in the ipsilateral and contralateral side of the brain. Cortical ischemia has been known to modify metabolism and blood flow in nonischemic contralateral brain areas (Feendy and Baron, 1986). It is possible that the concurrent upregulation of 38-kD caspase-11 product in the contralateral hemisphere may reflect activation in homologous brain regions, the consequences of which have not yet been identified.

Whereas caspase-11 can be activated by proteolytic cleavage, the cleavage may not be essential for its activation, as we only observed its cleavage when its expression levels are very high (Fig. 5; and Kang, S., S. Wang, and J. Y. Yuan, unpublished data). In LPS-stimulated mice, although caspase-11 is induced in almost all tissues, its cleavage can only be observed in spleen where its expression is the highest (Wang et al., 1996; Kang, S., and J. Y. Yuan, unpublished results). It has been increasingly accepted that at least long prodomain caspases can be activated without cleavage (Stennicke et al., 1999). Alternatively, it remains possible that the cleavage products of caspase-11 are unstable, and therefore, only when the levels of caspase-11 are very high can we observe its cleavage products. This is certainly possible for caspase-11 in ischemic brain. As the majority of the cells are not dying in these ischemic brains, and therefore only a small portion of cells are expressing caspase-11 that can only be observed through immunoprecipitation, we cannot find the cleavage product of caspase-11 in all cases.

Neither caspase-1 knockout line can be induced to express caspase-11, whereas our caspase-1 knockout mouse line expresses caspase-1 normally (Wang et al., 1998). This may be due to the fact that caspase-1 and -11 are genetically closely linked (Li, P., personal communication) and the disruption of genomic structure in caspase-1 has a negative effect on the expression of caspase-11. Preliminary examination of caspase-11 locus in caspase-1−/− mice by Southern blot and PCR showed that the locus of caspase-11 is intact, suggesting that inability to induce caspase-11 is not due to disruption of caspase-11 gene structure in caspase-1−/− mice (Wang, S., and J. Y. Yuan, unpublished results). Alternatively, the cytokines regulated by caspase-1,
such as IL-1 and IL-18, will have a significant impact on apoptosis. Thus, it remains to be resolved whether the loss of caspase-1 knockout mice are resistant to ischemic brain injury-induced apoptosis. The loss of caspase-11 expression in caspase-1 knockout mice complicated the interpretation as to why caspase-1 knockout mice are resistant to ischemic brain injury (Schielke et al., 1998), because the caspase-11 mutation alone has a strong effect on ischemic brain injury-induced apoptosis. Although our data demonstrated that caspase-11 can directly activate caspase-3, we do not exclude a role for cytokines in the regulation of cell death. Our previous studies showed that IL-1β may promote cell death (Friedlander et al., 1996). One possibility is that cytokines, such as IL-1β and interferon γ, of which processing and secretion of both are controlled by the caspase-11–caspase-1 pathway, may promote the upregulation of caspase-11 and -11. Xu et al. (1998) have shown that interferon γ can up-regulate caspase-1 under certain conditions and our result showed that IL-1β can stimulate the production of caspase-11. Thus, cytokines may contribute to apoptosis by positively regulating the expression of caspases. On the other hand, there is evidence that apoptosis may play a key role in cytokine secretion. IL-1β is a cytokine that lacks an obvious signal peptide and its mechanism of secretion is still under debate. Whereas many cell types have the capacity to produce IL-1, its release has been shown to be restricted predominantly to monocytes/macrophages and associated with apoptosis of producer cells (Hoggart et al., 1991; Zychlinsky et al., 1994). Secretion of IL-1β from U937 cells is partially inhibited by Bcl-2 overexpression (Lizard et al., 1997), suggesting apoptosis may play an important role in regulating IL-1β secretion in mononuclear cells. Perhaps controlling both apoptosis and cytokine secretion through upregulation of a single caspase is advantageous as a way to ensure immediate and complete elimination of damaged cells.

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