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Antibody to Caspase-Cleaved Actin Detects Apoptosis in Differentiated Neuroblastoma and Plaque-Associated Neurons and Microglia in Alzheimer's Disease

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During apoptosis, activation of a family of cysteine proteases related to interleukin-1β-converting enzyme (ICE)-related proteases or "caspases" results in endoproteolytic cleavage of multiple substrates at specific aspartate residues. We have sought to develop new antibody probes for the neoepitopes in protein fragments produced by ICE-related proteolytic cleavage as specific markers of events tightly linked to apoptotic mechanisms. Here, we demonstrate that an antibody probe specific for the C terminus of a 32-kd actin fragment produced by ICE-like activity specifically labels apoptotic but not necrotic, differentiated human neuroblastoma cells in culture. Unlike probes for nonspecific DNA strand breaks confined to the nucleus or cell body, this method allows the detection of cytoskeletal fragments in cell processes as well as the perikaryon long before DNA fragmentation and cell death and therefore serves as a novel marker of apoptosis-related events in distal parts of cells such as axons and dendrites. To illustrate this new tool, we show that the antibody detects the processes and cell bodies of degenerating neurons and plaque-associated microglia in Alzheimer's disease. *In situ* detection of caspase-cleaved actin provides a new means to evaluate the role of caspase activation in pathological and physiological processes. *(Am J Pathol 1998, 152:379–389)*

At the molecular level, activation of one or more aspartate-specific, cysteine proteases (caspases) is the critical signal required to carry out apoptotic cell death. The first of these proteases to be identified is *Caenorhabditis elegans* death gene 3 (CED-3) and has the mammalian homologue interleukin-1β-converting enzyme (ICE), which is made as a proenzyme constitutively and is activated by cleavage. A family of ICE homologues has been identified, which include ICE, CPP-32, Nedd-2/Ich-1, TX/Ich-2, and Mch-2.[1] Overexpression of these proteases in transfected cells leads to apoptotic cell death, and inhibition of ICE-related protease activity protects against apoptotic cell death. Target substrates for caspases include other ICE-related proteases[2] such as poly(ADP-ribose) polymerase,[3,4] actin,[5] and lamins.[6] Recently, CPP-32 or caspase 3 has been identified as the protease that cleaves poly(ADP-ribose) polymerase,[3,4] whereas ICE itself has been shown to cleave and activate CPP-32 in one model for apoptotic cell death.[7] Both poly(ADP-ribose) polymerase and actin regulate DNase I nuclease activity, and their cleavage is hypothesized to contribute to degradation of DNA to 180-bp intranucleosomal bands by activated DNase I.[8]

The ICE-related proteolytic cleavage sites for its most abundant substrate, actin, have been identified.[5,8] In extracts of apoptotic cells, the 45-kd actin is cleaved between Asp11 and Asn12 and between Asp244 and Gly245 to produce N-terminal, ~32-kd fragments and C-terminal, ~15-kd fragments. However, a recent report states that actin cleavage by caspases does not occur *in vivo* (cell lines).[9] To develop an assay to detect caspase activity *in situ*, we have produced an antibody that specifically detects fragments of actin generated by caspase cleavage but not intact actin. This antibody recognized the ~32-kd fragment generated by apoptotic cleavage of endogenous actin in human neuroblastoma cells as they bleb and undergo apoptosis.

The extent and significance of apoptotic cell death in pathological conditions remains controversial in part because of the dearth of specific methods for its detection in tissue sections. Currently, the most widely used method is the terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) technique for

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Table 1.

Diagnosis	Age (yrs.)	Postmortem (hrs.)	Fractin label
Normal	46	23.5	–
High plaque normal	64	10.5	–
Normal	64	24	–
Pick's	71	24	–
Normal	77	4	–
Normal	93	6	–
AD, familial	48 (onset 38)	4	++
AD, familial	68 (onset 59)	15.5	++
AD	80	3.5	+
AD	81	14.5	+/–
AD	84	6	+
AD	88	3	+

+/–, occasional positive cells.
+, 1 to 5 cells per 10× field of gray matter.
++, 6 to 10 cells per 10× field of gray matter.

detection of DNA fragments by labeling free 3′-OH ends. For example, in Alzheimer's disease (AD), several reports indicate the occurrence of scattered TUNEL positive neurons,[10–12] astrocytes,[11] and plaque-associated microglia.[10] Some of these cells are clearly apoptotic by morphological criteria, but others, particularly lightly labeled cells, may not necessarily contain 180-bp DNA fragments but simply an accumulation of DNA strand breaks. Using alkaline elution on control and AD brain DNA, strand breaks were elevated in AD and found to increase with postmortem time so that the number of breaks per cell doubled from approximately 4500 at 3 hours to 9000 at 6 hours postmortem time for AD.[13] As nonspecific DNA strand breaks also occur in necrosis and can accumulate as postmortem phenomena, additional probes related to specific protein cleavages relevant to the apoptotic phenotype in postmortem tissue would be useful.

To illustrate the use of our actin fragment probe in autopsied tissue, we show that it labels a subset of neurons and microglia and their processes in early onset Alzheimer's brain but not in control brain. The prominent labeling of microglia is discussed in relationship to inflammation in AD brain.

Materials and Methods

Materials

Unless otherwise specified, chemicals were from Sigma (St. Louis, MO). DEVD-CHO and Ac-YVAD-CHO inhibitors were from Bachem (Torrance, CA). Skeletal muscle actin was the generous gift of Dr. E. Reisler (UCLA). SY5Y human neuroblastoma cells were kindly supplied by Dr. June Biedler (Sloan-Kettering, NY). Alzheimer and control brains were from UCLA's Alzheimer's Disease Clinical Center. Additional brains were obtained from Dr. Carole Miller at the USC Alzheimer's Disease Research Center. Details of the cases are shown in Table 1. Ages for the controls averaged 69 ± 14 hours and for AD, 74.8 ± 13.5 hours. Postmortem times for the controls averaged 15.3 ± 9.5 hours and for the AD cases, 8.7 ± 6.6 hours.

Antibodies

A synthetic peptide (K-YELPD) representing the last five amino acids of the C terminus of the 32-kd actin fragment produced during apoptosis, β-actin residues 240–245 (YELPD), was coupled via the K residue to carrier protein (purified protein derivative of tuberculin) and injected into rabbits as previously described.[14] A monoclonal antibody to native Aβ1–40, 10G4, was used to detect β-amyloid.[15] Mac-1 antibody to the C3bi complement/integrin receptor was from Chemicon (Temecula, CA). Two commercial monoclonal antibodies to β-actin were used: clone C4, anti-actin from Boehringer Mannheim (Indianapolis, IN), which recognizes an epitope near residues 50–70, and clone AC-74 from Sigma, which is to a synthetic peptide representing the N-terminal 15 amino acids. CPP-32 antibody was from Signal Transduction Laboratories (Lexington, KY).

Immunocytochemistry

Ten-μm cryostat sections were immunolabeled with primary or peptide preabsorbed primary antibody using peroxidase-coupled secondary antibody and metal-enhanced diaminobenzidine essentially as previously described.[14] Sections were counterstained with hematoxylin. For Aβ double labeling, sections were stained with fractin and diaminobenzidine as above, formic acid pretreated, and then labeled for Aβ using 10G4 monoclonal and a species-specific anti-mouse alkaline phosphatase secondary antibody to label Aβ with Vector Blue substrate (Vector Laboratories, Burlingame, CA).

Fluorescence double labeling of TUNEL positive cells used affinity purified fractin antibody at 1.5 μg/ml detected by goat anti-rabbit rhodamine Red-X (Molecular Probes, Eugene, OR), which has less fluorescent overlap with fluorescein isothiocyanate than lissamine-linked rhodamine. Fluorescent images in 15 consecutive 0.5-μm optical sections were reconstructed using a Scanalytics pseudoconfocal image processing system and a pseudocolor overlap image generated in Adobe Photoshop 3.0.

Cell Culture

SY5Y human neuroblastoma cells were grown and differentiated in 10^{-5} mol/L retinoic acid as previously described.[16] Delayed cell death with DNA laddering was produced by addition of 1 μmol/L colchicine to 5-day retinoic acid-differentiated cells according to the method of Nakagawa-Yagi.[17] Necrosis was induced by the addition of 0.003% or more hydrogen peroxide. DNA fragmentation was assayed by lysing 5×10^6 cells in sodium dodecyl sulfate, proteinase K, and RNase A, loading the lysate on a 1.5% agarose gel, running at low voltage, and staining with ethidium bromide.

CPP-32 Extracts and Western Analysis

100 K × *g* supernatant (CPP-32 extract) and pellet fractions of SY5Y culture homogenates were made in CPP-32

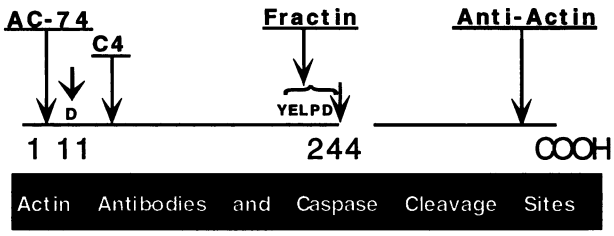

Figure 1. A schematic diagram of β-actin indicates the location of the antibody epitopes (**large arrows**) and the major putative ICE (caspase 1) cleavage sites (**small arrows**).

extract buffer containing 50 mmol/L Tris, pH 7.2, 2 mmol/L EDTA, 0.1% (w/v) CHAPS (3-[(chlamidopropyl)-dimethylammonio]-1-propanesulfonate), 5 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and pepstatin A, and 20 μg/ml leupeptin.[4] Pellet and supernatant or CPP-32 extracts were assayed for total protein, and pellet fractions were run on gels while CPP-32 containing supernatant fractions were incubated with or without exogenous actin substrate before Western analysis on blots after electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels. Blots were developed with actin, fractin, or CPP-32 antibodies using enhanced chemiluminescence (Amersham, Arlington Heights, IL) and autoradiography to detect. Nonsaturating exposures of autoradiographs were scanned on a Biorad densitometer (Bio Rad, Richmond, CA) to generate relative band densities.

TUNEL

The Apoptag kit (Oncor, Gaithersburg, MD) for end-labeling DNA fragments with fluorescein-tagged nucleotides was used per the manufacturer's instructions except the proteinase K incubation was reduced from 30 minutes to 5 minutes. Under these conditions double-labeling with fractin antibody used as a second step gave the same staining pattern as non-proteinase K treated, single-labeled adjacent sections.

Results

We produced an end-specific, polyclonal rabbit antiserum directed to actin residues 240–244 (YELPD), which represents the C terminus of the ~32-kd N-terminal actin fragment generated by apoptotic cleavage at Asp 244 (Figure 1). We have named this antibody "fractin" for "fragment of actin." The 5-amino acid peptide antigen was used so that antibody recognition required the free C-terminal carboxyl group. Because our goal is to develop probes for human neurodegenerative disease, we validated our fractin antibody using a simple model for apoptotic cell death, the differentiated human SY5Y neuroblastoma. Treatment with 1 μmol/L colchicine for 36 to 72 hours caused 40 to 50% of the SY5Y cells to round up, bleb, and show other morphological changes characteristic of apoptosis, confirming previous observations of colchicine-induced apoptotic cell death in these cells.[17] In the CPP-32 extract from cells undergoing colchicine-induced apoptosis, but not in control cells, the fractin antibody labeled a ladder of bands beginning at approximately 32 kd (Figure 2A). The labeling was blocked by preabsorption with the free YELPD peptide (Figure 2B). To verify that the fractin antiserum recognizes the expected actin fragment but not intact actin, we incubated purified muscle actin with CPP-32 extracts from either untreated or colchicine-treated cultures, stopped the reaction with Laemmli sample buffer, electrophoresed the products, and Western blotted using fractin antibody or a commercial monoclonal anti-actin (C4) that recognizes a region somewhere in the first 100 N-terminal amino acids of actin. Figure 2C demonstrates that the fractin antibody failed to detect large amounts of exogenous full-length actin (lane 1) or endogenous full-length actin in the apoptotic cell extract without exogenous actin (lane 2) but readily identified the ladder of bands beginning at ~32-kd band generated by incubation of actin with the CPP-32 extract from apoptotic cells (lane 3). It also labeled a much weaker band derived from extract of untreated cells, which demonstrates a baseline level of actin cleaving activity in the retinoic acid differentiated neuroblastoma (lane 4). This activity is not surprising because retinoic acid treatment does not affect all SY5Y cells equally. Some cells continue to proliferate and there is some cell loss during differentiation of SY5Y. The actin monoclonal (C4) detected the exogenous and endogenous actin and the major ~32-kd band produced by extract of apoptotic cells (Figure 2D). The lower molecular weight bands detected by fractin presumably result from additional N-terminal cleavages, which clip the N-terminal epitope recognized by the C4 monoclonal to actin, leaving fragments recognized only by the fractin antibody, which targets the C terminus of the actin frag-

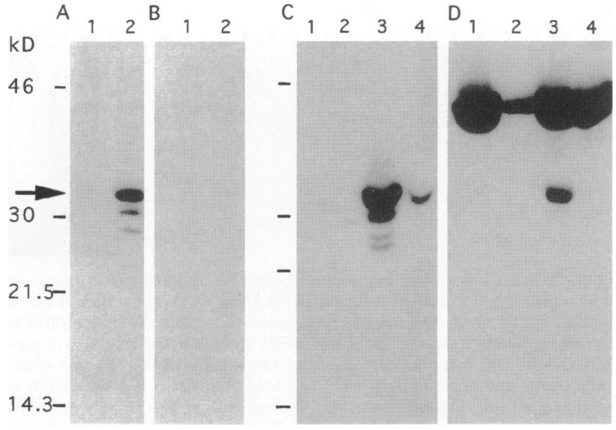

Figure 2. Fractin antibody staining of cleaved actin. **A** to **B:** Retinoic acid-differentiated SY5Y neuroblastoma were incubated without (**lane 1**) or with (**lane 2**) 1 μmol/L colchicine for 24 hours to induce apoptosis, and 20 μg of whole cell lysate were run on 10% sodium dodecyl sulfate page gels for Western analysis. The fractin antibody stained a ladder of bands beginning at ~32 kd (**arrow**) only in the apoptotic cells (**A**), and the bands were abolished by preabsorption with 20 μg/ml of the free peptide antigen (**B**). To confirm that the antibody detected cleaved actin, 10 μg of CPP-32 extract from SY5Y cells with or without colchicine was incubated with or without 18 μg of exogenous actin for 1 hour and Western blotted with the fractin antibody (**C**) or the anti-actin monoclonal (C4) (**D**). **Lane 1**, Actin; **lane 2**, extract from apoptotic SY5Y; **lane 3**, actin + extract from apoptotic SY5Y; and **lane 4**, actin + extract from control cells.

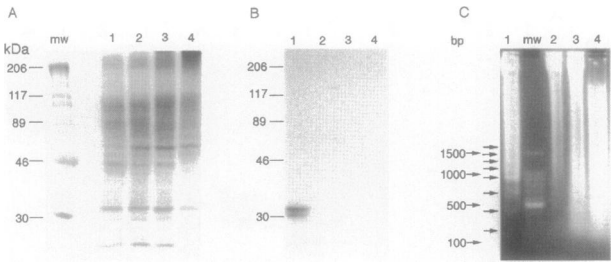

Figure 3. Specificity of fractin immunolabeling for apoptosis (**A**) Coomassie blue stain of gel of 100 K × *g* cell pellet from SY5Y cells after Western blotting for **B**. **Lane 1**, 1 μmol/L colchicine treatment, 24 hours; **lane 2**, untreated; **lanes 3** and **4**, 0.03 and 0.3% hydrogen peroxide, 3 hours, respectively. The lower dose hydrogen peroxide cells (**lane 3**) were swollen, rounded up, and beginning to detach at 3 hours but showed similar bands as the untreated cells in **lane 2**, whereas the higher dose had already detached and had protein aggregates (**lane 4**). **B:** A Western blot from the gel in **A** with fractin antibody showing marked fractin-labeling is detected only in the colchicine-treated cells. **C:** An agarose gel of DNA extracts from cells treated as in **A**. **mw**, 100-bp DNA ladder standards. The colchicine-treated cells (**lane 1**) did not show DNA laddering at earlier time points, whereas the necrotic cells already showed DNA smearing at 3 hours (**lane 3**), which was increased at the higher dose (**lane 4**).

ment. The polyclonal anti-C-terminal actin (anti-actin) and the monoclonal antibody to the N-terminal 15 amino acids of actin (AC74) labeled intact actin but did not recognize the 32-kd actin fragment (not shown). These results are consistent with the initial removal of the 11 N-terminal actin amino acids described by Kayalar et al,[8] followed by additional N-terminal degradation of the C4 epitope of the 32-kd actin fragment that begins at actin 12 and ending in YELPD as shown in Figure 1. The three lower, endogenous 25- to 30-kd bands are also likely actin-derived as a ladder of three lower bands between 25 and 30 kd is produced by adding exogenous actin to the CPP-32 supernatant extract as shown in Figure 2C. The intensity of the smallest endogenous band produced in the cells in the absence of protease inhibitors (Figure 2A) is less than the intensity of the smallest band produced from exogenous actin and the CPP-32 extract with protease inhibitors (Figure 2C). This may be because in the absence of protease inhibitors in the living cells, additional proteases further degrade the smallest band.

The specificity of fractin antibody labeling for apoptosis as opposed to necrosis was tested by comparing untreated SY5Y cells with cells treated with 1 μmol/L colchicine or with 0.03 or 0.3% hydrogen peroxide. These relatively high doses of hydrogen peroxide were chosen to induce necrosis because 50- to 100-fold lower doses induced apoptosis (not shown). As shown in Figure 3B, fractin labeling in the 100 K × *g* cell pellet was seen only with colchicine treatment (lane 1), whereas no fractin labeling was found with hydrogen peroxide (lanes 3 and 4). Colchicine treatment resulted in the DNA laddering characteristic of apoptosis (Figure 3C, lane 1, compared with untreated lane 2), whereas hydrogen peroxide treatment resulted in DNA smearing (Figure 3C, lanes 3 and 4) as well as rapid swelling, detachment, and lactate dehydrogenase release beginning at 3 hours and consistent with necrosis (not shown). Although it can be detected at earlier time points, we have illustrated the fractin staining at 24 hours to match the DNA laddering that was first evident at 24 hours.

To show fractin staining *in situ*, retinoic acid differentiated SY5Y cells were treated with colchicine, methanol fixed at varying time points, and immunolabeled with fractin antibody and goat-anti-rabbit fluorescein isothiocyanate (Figure 4). Although untreated control cells (Figure 4A) showed weak or no labeling, punctate fractin immunostaining could be seen as early as 30 minutes after colchicine treatment (Figure 4B, arrow). By 3 hours after colchicine exposure (Figure 4C), the cells showed trails of punctate labeling (arrow) or extensive process and perikaryal labeling that was fully developed by 6 hours (Figure 4D). This immunostaining was blocked by preabsorption with free peptide antigen (Figure 4E). After 72 hours, only some of the detached apoptotic cells

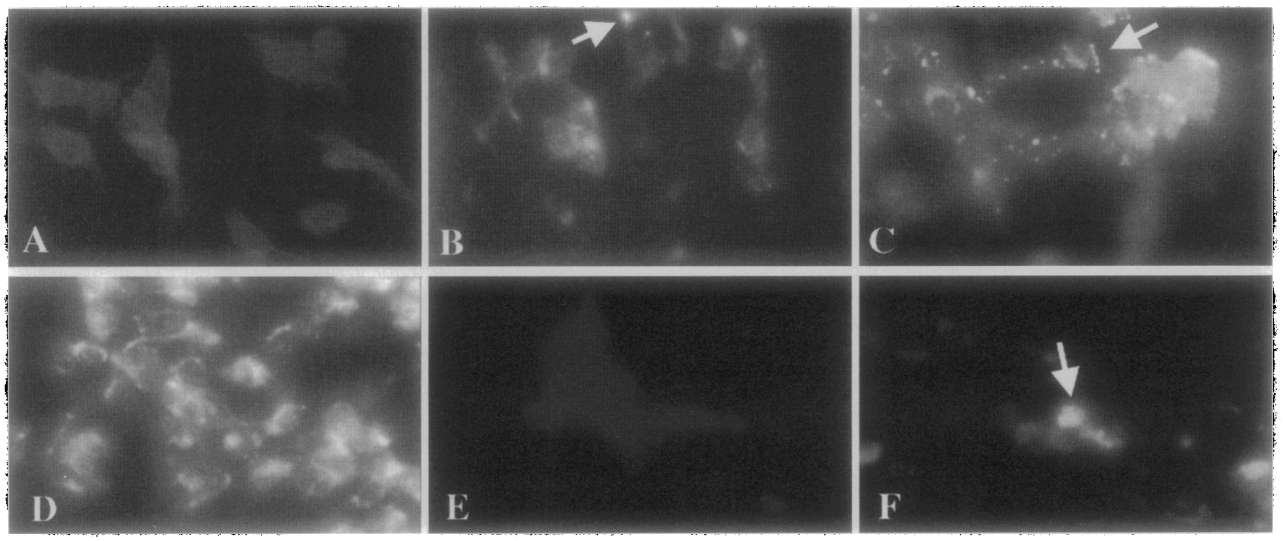

Figure 4. Fractin immunostaining of retinoic acid differentiated SY5Y cells. **A:** Untreated; **B** to **F:** treatment with 1 μmol/L colchicine; **B**, 30-minute treatment shows punctate labeling (**arrow**); **C**, 3-hour treatment shows rows of dots (**arrow**); **D**, 6-hour treatment had extensive cytoskeletal staining; **E**, 6-hour treatment, immunolabeled with peptide absorbed antibody; **F:** 72-hour treatment, detached cells fixed on slides showed condensed staining (**arrow**). Original magnification, ×600

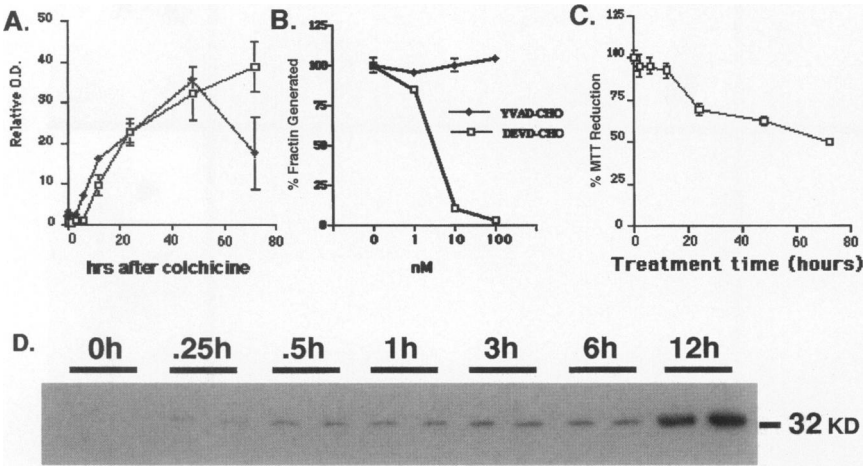

Figure 5. Parallel development of actin-cleaving activity and CPP-32. Retinoic acid-differentiated SY5Y cells were treated with 1 μmol/L colchicine and harvested at the indicated time points. **A:** The CPP-32 extracts were either Western blotted with an antibody to CPP-32 or incubated with 5 μg of purified muscle actin for 1 hour and then Western blotted with fractin antibody. Quantitative densitometry of the autoradiograms was used to generate a time course for fractin-generating activity (**open squares, solid line**) and full-length CPP-32 protein induction (**closed diamonds, dotted line**). $n = 3$ lanes/time point. **Error bars** indicate SD (some SD bars were too small to show). **B:** After the 72-hour colchicine treatment, relative O.D. of fractin band generation from exogenously added actin was markedly reduced by addition of 1 and 10 nm of Ac-DEVD-CHO, a relatively specific inhibitor of CPP-32, but not by Ac-YVAD-CHO, a relatively specific ICE inhibitor ($n = 4$ lanes per treatment, error bars often too small to show up). **C:** Cell viability data with MTT reduction did not show a significant loss of viability over the first 12 hours of 1 μmol/L colchicine treatment. **D:** In contrast, a Western blot analysis of the initial time course for fractin-generating activity using exogenous actin (quantified in **A**) shows a clear elevation within 15 minutes and a dramatic increase after 12 hours of 1 μmol/L colchicine treatment of retinoic-acid-differentiated SY5Y before any significant drop in viability.

retained fractin staining that were typically collapsed (Figure 4F, arrow).

To identify the actin-cleaving activity induced by colchicine treatment, we conducted a time course for the colchicine-induced actin cleaving activity and CPP-32 immunostaining on Western blots. Figure 5A shows that, overall, the induction of actin cleaving activity closely parallels the induction of CPP-32 protein. Actin cleaving activity was inhibited by low nanometer doses of the specific inhibitor of CPP-32-like proteases, DEVD-CHO, but not YVAD-CHO, an ICE-like inhibitor (Figure 5B). Other protease inhibitors (TPCK and leupeptin) had no effect (not shown). The initial part of the time course for fractin generating activity showed that an increase occurred within the first hour of exposure to colchicine and that there was a very marked increase at 12 hours (Figure 5D). It seems likely that the initial increase in fractin-generating activity corresponded to activation of preformed enzyme, whereas the major increase seen at 12 hours (which followed the increase in full-length CPP-32 at 6 hours) was due to the activation of induced CPP-32. Whatever, the explanation for increased fractin-generating activity following colchicine exposure, both the activity increase and the increased cellular immunostaining shown in Figure 4 are clearly detected before significantly decreased viability. Loss of viability occurs after 12 hours as shown in Figure 5C.

Having proven that the fractin antibody could detect actin fragment generated by a CPP-32-like caspase induced during apoptotic cell death in differentiated human neuroblastoma cells, we tested the antibody on postmortem human brain from six cases of AD, one high plaque, four low-plaque, age-matched, normal controls, and one Pick's case (Table 1). Little to no blockable fractin immunostaining was seen in low-plaque normal controls, the Pick's case, or one AD brain (14.5 hours postmortem time), which did not show significant TUNEL labeling. In some cases with long postmortem times TUNEL labeling was artifactually high and labeled large numbers of cells (>100 per 10× field) lacking apoptotic morphology (not shown). Whether this marked TUNEL labeling represented unusual antemortem or postmortem conditions, necrosis or persistent artifact remains unclear. However, these densely TUNEL positive areas were not identified by fractin labeling.

To illustrate the usefulness of the fractin antibody, we focused on three cases of Alzheimer's brain with short postmortem times in which TUNEL labeling was prominent in amyloid plaque rich frontal cortex. As shown in Figure 6, the fractin antibody labeled scattered cells and their processes in Alzheimer's frontal cortex (Figure 6A). On adjacent sections, this labeling was markedly diminished by preabsorption with the free peptide antigen (Figure 6B) and no staining was seen with second antibody alone (Figure 6C). Aβ immunolabeling demonstrated that the fractin label was concentrated in a plaque-rich region of cortex (Figure 6D). Cortex from control brains double labeled for Aβ, and fractin bands were negative (Figure 6E). However, one high plaque but clinically normal brain showed some limited, apparent microglial fractin-staining associated with plaques (not shown). Higher magnification of the fractin-positive cells is shown in Figure 6 (F to K). Double-labeling (fractin, anti-Aβ) showed cells with microglial morphology and small basophilic nuclei (arrows) scattered around the plaque-forming region and showed clusters of intensely labeled cells resembling microglia (Figure 6, F and G) sometimes adjacent to fractin stained neurons (Figure 6G, arrowhead). At even higher magnification, single labeling with fractin revealed clusters of many cells with densely labeled ramified microglia-like cells (Figure 6H, arrows). Whereas many of the fractin labeled structures

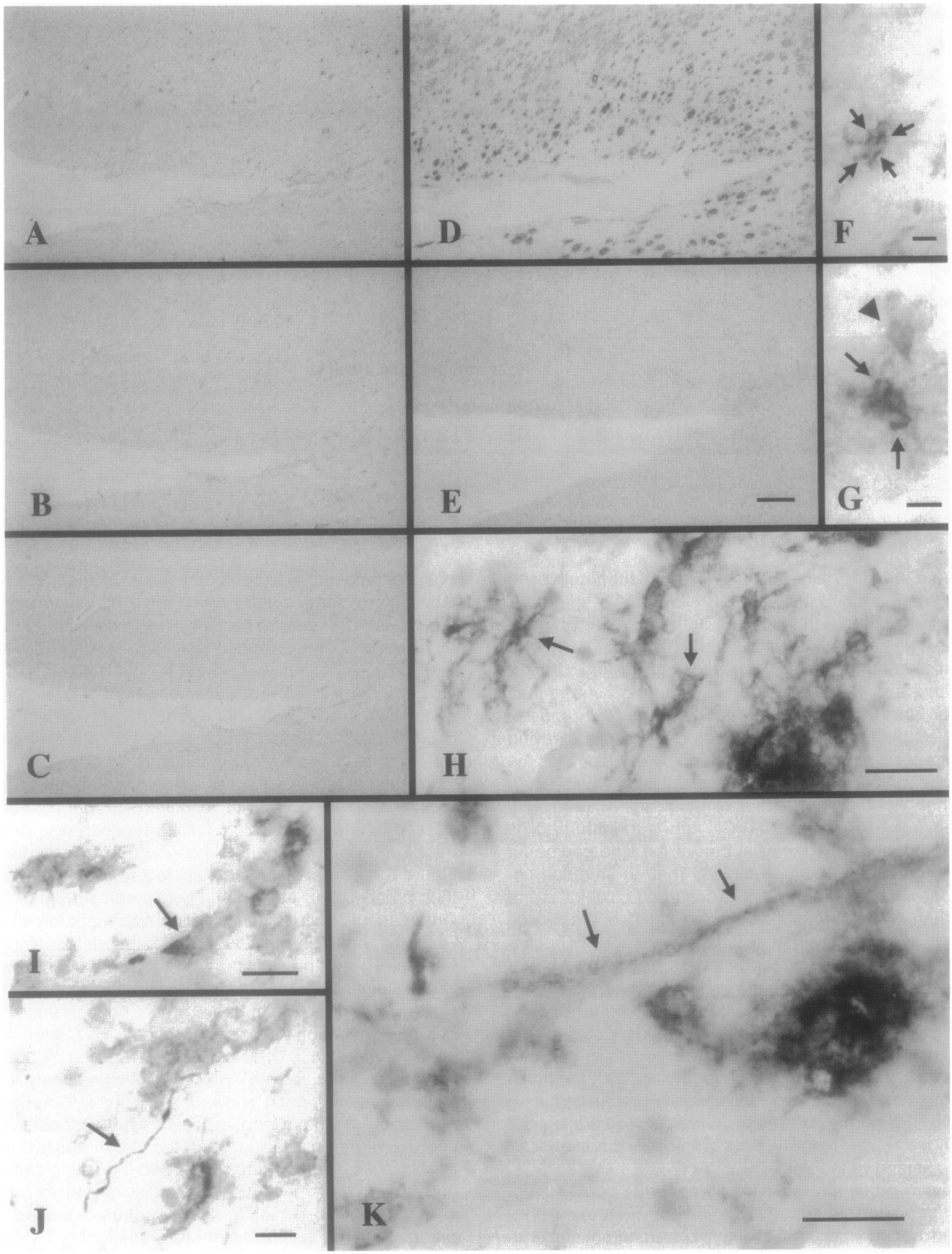

Figure 6. Fractin labeling in Alzheimer's disease. Adjacent sections from frontal cortex from an early onset AD case were labeled with affinity purified fractin antibody (**A**), fractin antibody preabsorbed with peptide antigen (**B**), and secondary antibody alone (**C**). Aβ (10G4) immunostaining on a neighboring section shows this area to be plaque-rich (**D**). Similar areas of control brain cortex showed minimal or no fractin immunostaining (**E**). Double-labeling with fractin antibody and anti-Aβ antibody (**F** and **G**). Fractin-stained cells resembling ameboid microglia (**arrows**) clustered around amyloid cores (**F**). Fractin staining of apparent microglia (**arrow**) were frequently found within Aβ stained plaques and adjacent to a fractin stained cell with neuron morphology (**G, arrowhead**). At high magnification, a plaque-rich area of AD brain showed fractin staining of apparent microglia (**H, arrows**). Fractin staining of neurons and their processes (**I** to **K, arrows**). The fractin-immunostaining of neuronal processes is punctate (**K**). Bar, 100 μm (**A** to **E**) and bar, 10 μm (**F** to **K**).

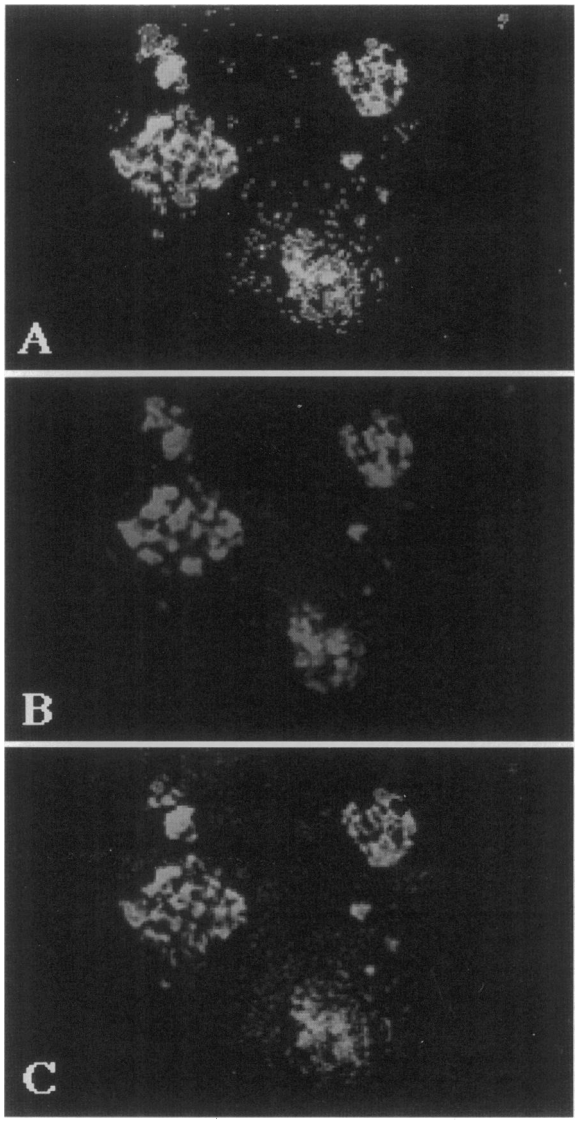

Figure 7. Confocal double immunofluorescence overlap image (**A**) of plaque-rich AD brain using TUNEL to end label DNA fragments with fluorescein-tagged nucleotides followed by fractin immunostaining with goat anti-rabbit rhodamine Red-X. The fractin alone is shown in **B** and TUNEL alone in **C**. Original magnification, ×600.

appeared to be degenerating and difficult to identify, clear examples of neuronal and aberrant process labeling could be found at higher power (Figure 6, I and J, arrows). Under oil immersion, examples of what appeared to be cytoskeletal breakdown in fragmenting processes was occasionally observed (Figure 6K, arrows). Labeling of neurons and their processes was completely blocked by preabsorption (not shown).

Confocal fluorescent double labeling for TUNEL (fluorescein, green) and fractin (rhodamine Red-X) showed substantial overlap of clusters of plaque-associated cells (Figure 7). The fractin staining extended more into the processes of cells. Similar double labeling with TUNEL/peroxidase (diaminobenzidine) and fractin/anti-rabbit alkaline phosphatase (vector blue) also revealed consistent overlap in TUNEL and fractin labeling patterns in rodent models of ischemic and kindling-induced cell death (in collaboration with C. Wasterlain, manuscript in preparation). In AD brain, TUNEL was usually closely associated with fractin immunostaining, except in the cases with apparently artifactually high TUNEL as described above.

To positively identify the fractin-stained cells in AD brain, double-labeling studies were undertaken. Cells were examined at 100× under oil immersion to reveal areas with incomplete overlap and so that cells were only in focus over a depth of 0.6 μm. As shown in Figure 8 A, fractin positive cells were often as large as neurons and positive for AT8 (a marker for a phosphorylated tau found in neurofibrillary tangles), dystrophic neurites, and curly fibers of neurons in AD. Plaque-associated microglia were identified by Mac-1 antibody to receptor for complement and integrin (blue), and many of these were also fractin-positive (brown). (Figure 8B). A number of the cells are doubly-stained and markedly darker than the singly labeled cells and show patches or halos of brown or blue. As an additional marker, antibody to the 150-kd neurofilament protein (the gift of Dr. D. Dahl) was used to label neurons (brown), and fractin was labeled in blue. Examples of neurons doubly-labeled for fractin and neurofilament or phosphotau are shown in Figure 8C. Reversing the order of labeling led to the same pattern of staining. Double-labeling for fractin and for astrocytes with glial fibrillary acidic protein antibody revealed only very rare cells (not shown).

Discussion

Caspase activity is central to apoptotic cell death, but there has been no convenient method for the *in situ* detection of the protein fragments generated by this activity. Most studies of neuronal apoptosis have instead centered on nuclear events including transcription changes,[18] chromatin margination, altered patterns of DNA fluorescence, and particularly DNA fragmentation,[19] which is widely used as the definitive assay for apoptotic cell death. However, because DNA strand breaks increase with postmortem time and are a late stage nuclear event that can occur in a variety of situations without apoptosis, TUNEL or ISEL methods should be supplemented by additional criteria for the detection of apoptosis.[20–22] Furthermore, key morphological features observed such as rounding up of the cell body and surface ruffling or blebbing can take place even in the absence of nuclei[23,24] and have been attributed to proteolytic cleavage of cytoskeletal elements. Cytochalasins, which specifically disrupt actin filaments, can block these morphological alterations characteristic of apoptosis indicating a requirement for actin filaments.[25,26] Intact actin filaments are known to bind and safely sequester DNase I, which is the principal endonuclease responsible for generating the DNA fragments observed during the end-stages of apoptotic cell death. As proposed by Kayalar et al,[8] apoptotic cleavage of actin generates actin fragments, which show reduced DNase I binding, and may therefore be part of the mechanism for allowing DNase I activity to

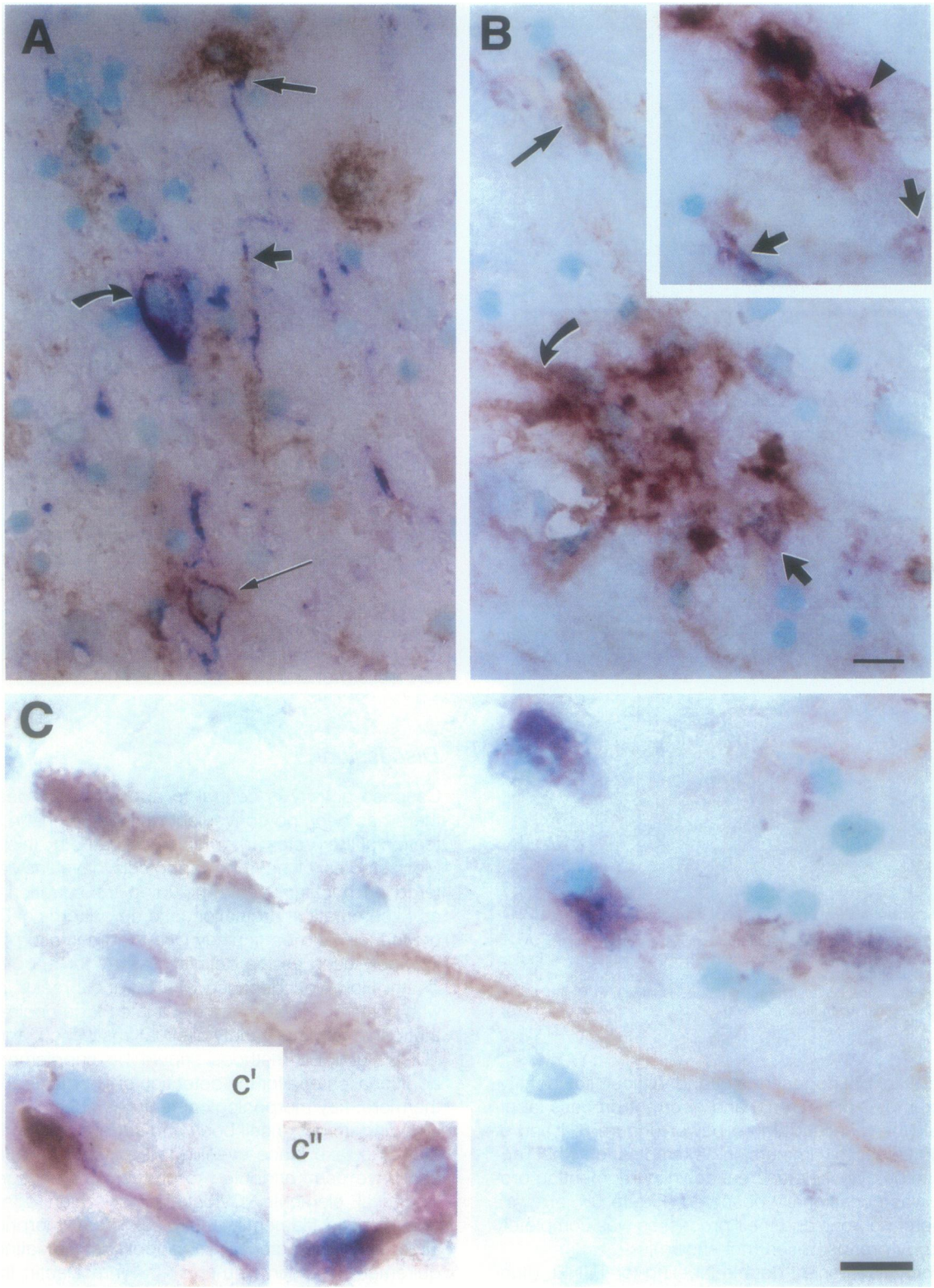

Figure 8. Double-labeling of 8 μmol/L cryostat sections from AD frontal cortex with fractin antibody and cell-specific markers viewed under 100× oil immersion with a depth of field of 0.6 μmol/L and a contrast green counterstain for nuclei. **A:** AT8 antibody to phosphorylated tau (**blue**) detects neurofibrillary tangles (**curved arrow** and **long, thin arrow**) and curly fibers of neurons. Fractin (**brown**) also labels the lower tangle-bearing neuron (**long, thin arrow**) and a neuron with an AT8 positive process (**long, thick arrow**). An AT8 and fractin positive process is indicated by the **short, thick arrow**. **B** and **inset:** Double labeling of plaque-associated microglia with Mac-1 (CD11b C3bi complement receptor) antibody (**blue**) and fractin (**brown**). **Short arrows** indicate fractin-negative microglia (**blue**). The **arrowhead** in the inset shows one example of a strongly double-labeled microglial cell (**purple/black**). A fractin positive, Mac-1 negative cell is indicated at the top of **B** (**arrow**) and a singly labeled, fractin-positive process is marked with the **curved arrow**. **C** and **insets:** Fractin labeling is shown in **blue** in the fragmenting perikaryon of a neurofilament-labeled (**brown**) neuron. **C'** inset shows **brown** fractin labeling in the perikaryon of a neuron with an AT8 positive process. **C''** shows an example of reversing the order of labeling with a **blue** fractin-labeled neuron identified with neurofilament antibody (**brown**). Bar, 10 μm

enter the nucleus. Thus, cleavage of actin could play an important role in both cytoskeletal breakdown and blebbing and events leading to DNA fragmentation.

Whereas a recent study using several cultured cell lines failed to detect actin cleavage in cells induced to apoptose,[9] our results plainly demonstrate actin cleavage can occur with induction of apoptosis *in vitro* and *in vivo*. Our data suggest that some of the initial actin fragmentation in the first hours after colchicine exposure appears to be a biochemical correlate of the blebbing event, which initially appears as punctate labeling near the cell surface. The actin fragment generating activity of CPP-32 cell extracts was inhibited by 0.01 to 0.1 μmol/L DEVD-CHO (but not by an ICE inhibitor, Ac-YVAD-CHO) and closely paralleled CPP-32 immunostaining, which is consistent with other data suggesting that CPP-32 is a major caspase in neurons.[27] More specifically, apoptotic challenge of SY5Y human neuroblastoma cells has been shown to cause a rise in CPP32-like activity with little change in ICE-like activity.[28] As we show that our fractin antibody appears more sensitive for detection of fragments than the two commercial actin antibodies we have tried (Figure 2, C and D), the discrepant results of other investigators might relate to the sensitivity and recognition sites of the antibodies used. Alternatively, the particular cell lines used in the negative study may not produce stable actin fragments when induced to apoptose.

Low levels of basal actin cleaving activity were detected in the retinoic acid differentiated neuroblastoma cells when sensitivity was pushed with exogenous actin substrate(Figure 2C, lane 4). Low levels of caspase-3-like activity have recently been detected in healthy cell lines and mouse brain and shown to cleave presenilins 1 and 2.[29] This may not be the only explanation for our baseline because we used retinoic acid treated SY5Y neuroblastoma. Retinoic acid regulates the balance of proliferation, differentiation, and apoptosis in human neuroblastoma lines causing some lines to differentiate and some to apoptose.[30] Neuroblastoma cell lines like SY5Y, which show predominant retinoic acid-induced differentiation rather than apoptosis, show an up-regulation of Bcl-2.[31] It seems likely that the limited fractin staining and DNA degradation seen in control cells after 7 to 10 days of retinoic acid was the result of a small number of cells responding to retinoic acid by apoptosis followed by secondary necrosis. However, we have also detected low levels of fractin generating activity in some but not all cultures of apparently healthy undifferentiated SY5Y. This may mean that a low and variable level of caspase activity can exist in healthy cells, which is a conclusion supported by evidence for limited CPP32 activation in stimulated lymphocytes without concurrent apoptosis.[32]

The likelihood that actin cleavage at Asp244 is physiologically relevant and can be an important event in apoptotic pathways is supported by a series of papers that demonstrated that mutation in β-actin codon 244 found in chemically transformed fibroblasts is linked to transformation.[33] Transfection of β-actin cDNA with a glycine substitution at 244 resulted in aberrant blebbing, a selective advantage for cells with the mutation and increased tumorigenicity *in vivo* by an unknown mechanism. A major factor in the oncogenic activity of actin codon 244 mutations could be the loss of the actin cleavage site, Asp244. If reduced actin cleavage resulted in diminished apoptotic cell death rates, it would be predicted to have a selective advantage and a tumorigenic effect similar to that caused by inhibition of apoptosis by other means, for example Bcl-2 or p53 inactivation.

We found no evidence of fractin labeling in cultured SY5Y cells induced to necrose, which is consistent with the lack of published evidence of caspase activation during necrosis. However, there are numerous studies demonstrating a continuum between apoptosis and necrosis that is largely dependent on the magnitude of the damage.[34] Actin cleavage theoretically occurs prior to the intranucleosomal cleavage by DNase I, and secondary necrosis from additional damage to cell membranes could easily occur after actin cleavage *in vivo*. Therefore, although fractin immunostaining indicates activation of CPP-32-like proteases linked to apoptosis and not associated with necrosis, the presence of actin cleavage cannot be used to rule out an endstage necrotic lysis in any particular cell in a pathological sample.

The evidence for actin fragment formation in TUNEL positive cells in AD brain is consistent with previously published reports of apoptotic cell death in this disease.[10–12] In the small number of cases examined, more fractin staining of neurons was found in the two clearly familial, earlier onset (38 and 59 years) AD cases. This may be attributed to more rapid neuron loss in early onset cases or the possible involvement of a familial AD gene putatively linked to susceptibility to apoptosis such as presenilins 1 and 2.[35,36] Intriguingly, presenilin 1 itself is reported to be a natural CPP32 (caspase 3) substrate.[29]

Double-labeling with TUNEL and fractin antibody confirm that the antibody can pick out cells with a probable apoptotic phenotype. The most common type of cells labeling were plaque-associated glia that invariably surrounded amyloid cores and often clustered in plaques. Based on morphological criteria and the small intensely basophilic nuclei, these cells appeared to be activated microglia consistent with a previous report using TUNEL on AD brain.[10] There are two plausible explanations for this. First, activation of microglia *in vitro* or *in vivo* induces cytoplasmic interleukin converting enzyme and interleukin-1β.[37] Recombinant ICE can cleave actin at Asp244,[8] and ICE can also activate other caspases. If induction of ICE-like proteases in activated microglia results in increased actin cleavage product, the observed increase in fractin-labeled microglia in AD brain would be consistent with the increase in activated, interleukin-1β positive microglia associated with plaques as part of the chronic inflammatory response in AD brain.[38] Cleavage of actin may play a role in the major rearrangement of the actin cytoskeleton involved in the transition from process-bearing to ameboid microglia. Second, although ICE activation is not necessary for apoptosis of monocytic lineage cells,[39] chronically activated microglia eventually apoptose *in vivo*.[40] The observed TUNEL labeling in microglia immunolabeled for actin fragment is consistent with DNA fragmentation and eventual apoptosis. Plaque-associated microglia would be expected to interact with β-amy-

loid fibrils that have recently been shown to bind macrophage scavenger receptor.[41] This should lead to actin fragmentation because stimulation of scavenger receptor, whether by silica, asbestos fibrils, or oxidized lipoproteins, is known to result in ICE activation and macrophage apoptosis.[42] Caspase-mediated actin fragmentation in microglia in and around plaques is also consistent with *in vitro* data showing that aggregated β-amyloid rapidly produces microglial blebbing.[43]

The labeling of swollen or fragmenting neuronal processes demonstrates that actin-244 fragments are not confined to the perikaryon but also involved in process degeneration. One important inference from this result is that ICE-related proteolytic degradation of cytoskeletal elements can take place in the neuronal arbor, far removed from nuclear events, and participate in the degeneration of these processes. If mechanisms exist for the focal and local activation of these proteases in neuronal processes, for example by exposure to toxic substances such as free radicals or β-amyloid or loss of trophic support from target cells, ICE-related proteases could participate in the trimming and removal of processes by a distal activation of caspase activity. This may occur during development, plasticity, or neurodegenerative diseases of aging such as Alzheimer's in which a major loss of arbor occurs. In this scenario, actin cleavage by ICE-family proteases would be analogous to the widely studied calpain-mediated fodrin cleavage that is hypothesized to be involved in synaptic remodeling during learning and memory.[44]

As the pattern of perikaryal labeling with the fractin antibody closely parallels the pattern of TUNEL labeling in the Alzheimer brain, it should provide a useful adjunct to TUNEL labeling and provide a method for identifying or tracing the processes of cells with caspase activation. Together with collaborators, we have found that fractin labeling parallels TUNEL labeling in several other situations (kindling, ischemia) and anticipate that it may be useful in the analysis of other pathological processes.

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References

1. Miller DK: The role of the Caspase family of cysteine proteases in apoptosis. Semin Immunol 1997, 9:35–39
2. Steller H: Mechanisms and genes of cellular suicide. Science 1995, 267:1445–1449
3. Schlegel J, Peters I, Orreniur S, Miller DK, Thornberry NA, Yamin TT, Nicholson DW: CPP32/apopain is a key interleukin 1β converting enzyme-like protease involved in Fas-mediated apoptosis. J Biol Chem 1996, 271:1841–1844
4. Nicholson DW, Ali A, Thornberry NA, Vallancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin T-T, Yu VL, Miller DK: Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995, 376:37–43
5. Mashima T, Naito M, Fujita N, Noguchi K, Tsuruo T: Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. Biochem Biophys Res Commun 1995, 217:1185–1192
6. Lazebnik YA, Takahashi A, Moir RD, Goldman RD, Poirier GG, Kaufmann SH, Earnshaw WC: Studies of the lamin protease reveal multiple parallel biochemical pathways during apoptotic execution. Proc Natl Acad Sci USA 1995, 92:9042–9046
7. Enari M, Talanian RV, Wong WW, Nagata S: Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature 1996, 380:723–726
8. Kayalar C, Örd T, Testa MP, Zhong L-T, Bredesen D: Cleavage of actin by interleukin 1β-converting enzyme to reverse DNase I inhibition. Proc Natl Acad Sci USA 1996, 93:2234–2238
9. Song Q, Wei T, Les-Miller S, Alnemri E, Watters D, Lavin MF: Resistance of actin to cleavage during apoptosis. Proc Natl Acad Sci USA 1997, 94:157–162
10. Lassman H, Bancher C, Breitschopf H, Wegiel J, Bobinski M, Jellinger K, Wisniewski HM: Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ. Acta Neuropathol 1995, 89:35–41
11. Smale G, Nichols NR, Brady DR, Finch CE, Horton WE Jr: Evidence for apoptotic cell death in Alzheimer's disease. Exp Neurol 1995, 133:225–230
12. Su JH, Anderson AJ, Cummings BJ, Cotman CW: Immunohistochemical evidence for apoptosis in Alzheimer's disease. NeuroReport 1994, 5:2529–2533
13. Cotman CW, Anderson AJ: A potential role for apoptosis in neurodegeneration and Alzheimer's disease. Mol Neurobiol 1995, 10:19–45
14. Mak K, Yang F, Vinters HV, Frautschy SA, Cole GM: Polyclonals to β-amyloid (1–42) identify most plaque and vascular deposits in Alzheimer cortex, but not striatum. Brain Res 1994, 667:138–142
15. Yang F, Mak K, Vinters HV, Frautschy SA, Cole GM: Monoclonal antibody to the C-terminus of β-amyloid. NeuroReport 1994, 15:2117–2120
16. Cole GM, Wu K, Timiras PS: A culture model for age-related human neurofibrillary pathology. Int J Dev Neurosci 1985, 3:23–32
17. Nakagawa-Yagi Y: Induction of apoptotic cell death in differentiating neuroblastoma SH-SY5Y cells by colchicine. Biochem Biophys Res Commun 1994, 199:807–817
18. Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R, Johnson EM Jr: Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J Cell Biol 1994, 127:1717–1727
19. Wyllie AH, Kerr JFR, Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 1980, 68:251–306
20. Columbano A: Cell death: current difficulties in discriminating apoptosis from necrosis in the context of pathological processes in vivo. J Cell Biochem 1995, 58:181–190
21. Charriaut-Marlangue C, Ben-Ari Y: A cautionary note on the use of the TUNEL stain to determine apoptosis. NeuroReport 1995, 7:61–64
22. Mundle SD, Raza A: The two in situ techniques do not differentiate between apoptosis and necrosis but rather reveal distinct patterns of DNA fragmentation in apoptosis. Lab Invest 1995, 72:611–613
23. Nakajima H, Golstein P, Henkart PA: The target cell nucleus is not required for cell-mediated granzyme — or Fas-based cytotoxicity. J Exp Med 1995, 181:1905–1909
24. Schulze-Osthoff K, Walczak H, Droge W, Krammer PH: Cell nucleus and DNA fragmentation are not required for apoptosis. J Cell Biol 1994, 127:15–20
25. Cotter TG, Lennon SV, Glynn JM, Green DR: Microfilament-disrupting agents prevent the formation of apoptotic bodies in tumor cells undergoing apoptosis. Cancer Res 1992, 52:997–1005
26. Endresen PC, Fandrem J, Eide TJ, Aarbakke J: Morphological modifications of apoptosis in HL-60 cells: effects of homocysteine and cytochalasins on apoptosis by 3-deazaadenosine. Virchows Arch B Cell Pathol 1995, 426:257–266
27. Kuida K, Zheng TS, Na S, Kuan C-Y, Yang D, Karasuyama H, Rakic P, Flavell RA: Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 1996, 384:368–371
28. Posmantur R, McGinnis K, Nadimpalli R, Gilbertsen RB, Wang KKW: Characterization of CPP32-like protease activity following apoptotic

challenge in SH-SY5Y neuroblastoma cells. J Neurochem 1997, 68: 2328–2337
29. Loetscher H, Deuschle U, Brockhaus M, Reinhardt D, Nelboeck P, Mous J, Grunberg J, Haass C, Jacobsen H: Presenilins are processed by caspase-type proteases. J Biol Chem 1997, 272:20655–20659
30. Melino G, Thiele CJ, Knight RA, Piacentini M: Retinoids and the control of growth/death decisions in human neuroblastoma cell lines. J Neurooncol 1997, 31:65–83
31. Lasorella A, Iavaorne A, Israel MA: Differentiation of neuroblastoma enhances Bcl-2 expression and induces alteration of apoptosis and drug resistance. Cancer Res 1995, 55:4711–4716
32. Miossec C, Dutilleul V, Fassy F, Diu-Hercend A: Evidence for CPP32 activation in the absence of apoptosis during T lymphocyte stimulation. J Biol Chem 1997, 272:13459–13462
33. Leavitt J: Discovery and characterization of two novel human cancer-related proteins using two-dimensional gel electrophoresis. Electrophoresis 1994, 15:345–357
34. Portera-Cailliau C, Price DL: Excitotoxic neuronal death in the immature brain is an apoptosis-necrosis morphological continuum. J Comp Neurol 1997, 378:70–78
35. Vito P, Wolozin B, Ganjei JK, Iwasaki K, Lacana E, D'Adamio L: Requirement of the familial Alzheimer's disease gene PS2 for apoptosis: opposing effect of ALG-3. J Biol Chem 1996, 271:31025–31028
36. Wolozin B, Iwasaki K, Vito P, Ganjei JK, Lacana E, Sunderland T, Zhao B, Kusiak JW, Wasco W, D'Adamio L: Participation of presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer mutation. Science 1996, 274:1710–1713
37. Bhat RV, DiRocco R, Marcy VR, Flood DG, Zhu Y, Dobrzanski P, Siman R, Scott R, Contreras PC, Miller M: Increased expression of IL-1β converting enzyme in hippocampus after ischemia: selective localization in microglia. J Neurosci 1996, 16:4146–4154
38. McGeer PL, McGeer EG: The inflammatory response system of brain: implications for therapy of Alzheimer and other neurodegenerative diseases. Brain Res Rev 1995, 21:195–218
39. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, McDowell J, Paskind M, Rodman L, Salfeld J, Towne E, Tracey D, Wardwell S, Wei F-Y, Wong W, Kamen R, Seshadri T: Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. Cell 1995, 80:401–411
40. Gehrmann J, Banati RB: Microglial turnover in the injured CNS: activated microglia undergo delayed DNA fragmentation following peripheral nerve injury. J Neuropathol Exp Neurol 1995, 54:680–685
41. Paresce DM, Ghosh RN, Maxfield FR: Microglial cells internalize aggregates of the Alzheimer's disease amyloid β-protein via a scavenger receptor. Neuron 1996, 17:553–565
42. Iyer R, Hamilton R, Li L, Holian A: Silica-induced apoptosis mediated via scavenger receptor in human alveolar macrophages. Toxicol Appl Pharmacol 1996, 141:84–92
43. Korotzer AR, Pike CJ, Cotman CW: β-amyloid peptides induce degeneration of cultured rat microglia. Brain Res 1993, 624:121–125
44. Vanderklish P, Saido TC, Gall C, Arai A, Lynch G: Proteolysis of spectrin by calpain accompanies theta-burst stimulation in cultured hippocampal slices. Mol Brain Res 1995, 32:25–35