Intermediate Filaments Control the Intracellular Distribution of Caspases During Apoptosis

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Caspases are responsible for a cascade of events controlling the disassembly of apoptotic cells. We now demonstrate that caspase-9 is activated at an early stage of apoptosis in epithelial cells and all its detectable, catalytically active large subunits (both the p35 and p37) are concentrated on cytokeratin fibrils. Immunolabeling of distinctive neoepitopes, exposed by cleavage of procaspase-9 at either Asp315 or Asp330, was co-localized on these fibrils with active caspase-3, caspase-cleaved cytokeratin-18, death-effector-domain containing DNA-binding protein and ubiquitin. Cytokeratin filaments may thus provide a scaffold whereby active subunits of caspase-9 can activate caspase-3 which, in turn, can activate more caspase-9 so forming an amplification loop to facilitate cleavage of cytokeratin-18, disruption of the cytoskeleton and the ensuing formation of cytoplasmic inclusions. These inclusions, formed from the collapse of fibrils, together with their associated components, also contain ubiquitinated proteins, vimentin, heat-shock protein 72, and tumor necrosis factor receptor type-1-associated death domain protein. Many of their constituents, including active caspases, remain sequestered within these inclusions, even after detergent treatment and isolation. Thus, such inclusions do not merely accumulate disrupted cytokeratins but also sequester potentially noxious proteins that could injure healthy neighboring cells. (*Am J Pathol* 2004, 164:395–407)

Apoptosis facilitates the homeostatic regulation of cell populations by removing damaged or potentially harmful cells.¹ Induction of apoptosis involves either the triggering of death receptors in the plasma membrane or the perturbation of mitochondria.² Both these mechanisms lead to the activation of caspases, a family of aspartate-specific cysteine proteases, often by autoprocessing or processing by other caspases, generating a large and small subunit that together form the active enzyme.^{3,4} Initiator caspases, with long prodomains, such as caspase-8 and caspase-9, activate, either directly or in-

directly, the effector caspases, such as caspases-3, -6, and -7.^{3–5} In certain cell types, mitochondria may also amplify the apoptotic response,^{6,7} by recruitment of multiple procaspase-9 molecules to the Apaf-1 apoptosome where activation results from autocatalytic cleavage, at Asp315, to its p35/p12 form.⁸ The activity of caspase-9 is, however, inhibited as the ATPF motif at the N-terminus of the small p12 subunit binds to a surface groove on the BIR3 domain of XIAP.^{9,10} In contrast, this inhibitory mechanism does not affect the activity of subunits generated by cleavage at Asp330, by activated caspase-3, thus enabling activation of further caspase-3 in a feedback amplification loop.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) binds to the receptors DR4/TRAIL-R1 (TRAIL-receptor-1) and DR5/TRAIL-R2 (TRAIL-receptor-2), resulting in receptor trimerization and recruitment of FADD/MORT1, which in turn binds to the death effector domains of caspase-8. This results in the activation of caspase-8 which can then activate effector caspases, so resulting in the execution phase of apoptosis.^{11,12} Active effector caspases cleave numerous intracellular substrates including various cytoskeletal components,^{13,14} resulting in cytoplasmic budding, nuclear condensation, and the formation of apoptotic bodies.

Approximately 5% of the cytokeratin 8 (K8) and cytokeratin 18 (K18), the major constituents of intermediate filaments in epithelial cells, are in dynamic equilibrium with the insoluble, filamentous component. Remodeling of keratin fibrils, such as that occurring during mitosis, requires phosphorylation. This destabilizes filaments and drives the equilibrium toward depolymerization;¹⁵ phosphorylation also modulates filament ubiquitination and thus their turnover.¹⁶ Hyperphosphorylation of keratins occurs early in apoptosis, it has no apparent effect on caspase susceptibility,¹⁷ but it may render oligomers unsuitable for reincorporation into filaments¹⁸ and thus promote the formation of the cytoplasmic inclusions that also contain both activated caspase-3 and caspase-cleaved K18.^{19,20} Unlike K18, K8 is largely untouched by caspases and the resulting imbalance between intact K8/K18 may contribute to the formation of these inclusions.^{21,22} The reorganization of intermediate filaments during apoptosis thus involves both phosphorylation and

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caspase cleavage^{23,24} and often results in cytoke-
rich cytoplasmic inclusions.¹⁹ Recent studies, in cells
treated with apoptotic stimuli that signal through both the
death-receptor and mitochondrial pathways, have indi-
cated that the death-effector-domain containing DNA-
binding protein (DEDD) acts as a scaffold protein that
can direct procaspase-3 to filaments of cytokeratin.²⁵
These studies showed that procaspase-3-GFP, tran-
siently transfected into MCF-7 cells, was distributed
throughout the cytoplasm until the onset of apoptosis,
when it became associated with cytokeratin fibers.

The present study has established a comparable as-
sociation of activated caspase-9, cleaved at both Asp315
and Asp330, with cytokeratin fibrils. These fibrils may
provide a scaffold for the proximity-induced autocleav-
age and activation of procaspase-9 in close association
with caspase-3. The resulting activation of caspase-3
may then provide an amplification loop to cleave yet more
procaspase-9. Activated caspase-3 also cleaves the K18
component of the fibrils that, together with the associated
proteins, becomes incorporated into cytoplasmic inclu-
sions. These inclusions, which exhibited the same immu-
nocytochemical and morphological features as Mallory
bodies, but not aggresomes, were virtually detergent-
insoluble and thus sequester potentially noxious pro-
teins from the cytoplasm. Thus cytokeratin filaments may
both control key stages of the caspase cascade, to fa-
cilitate an ordered cellular demolition during apoptosis,
and minimize the effects of this process on adjacent
cells.

Materials and Methods

Materials

Media and serum were purchased from Life Technol-
ogies, Inc. (Paisley, UK). All other chemicals, unless
stated otherwise, were from Sigma-Aldrich (Poole, Dor-
set, UK).

Cell Culture and Quantification of Apoptosis

The A549 human type II pneumocyte-derived cell line was
obtained from the European Collection of Animal Cell Cul-
tures (Salisbury, Wiltshire, UK) and MCF7-C3, caspase-3-
repleted human breast epithelial cells were a gift from Dr.
Alan Porter (Institute of Molecular and Cell Biology, The
National University of Singapore, Singapore). They were
grown as described previously^{19,25} and treated with 500
ng/ml of recombinant soluble TRAIL²⁶ or 1 to 2.5 μ mol/L of
staurosporine (STS). 293T human embryonic kidney cells
were grown and transfected as described previously.²⁷

Gel Electrophoresis, Immunoblotting, and Immunoprecipitation

Adherent cells were removed by gentle scraping,
washed once with ice-cold phosphate-buffered saline
(PBS), and snap-frozen. Equal numbers of cells were

lysed in sodium dodecyl sulfate-polyacrylamide gel elec-
trophoresis buffer and separated on 13% sodium dodecyl
sulfate-polyacrylamide gels followed by electro-
phoretic transfer onto nitrocellulose (Hybond-C extra;
Amersham, Bucks, UK) as previously described.²⁶ Equal
protein loading per lane was verified by staining the
membranes with 0.1% Ponceau S. Immunodetection was
performed using the enhanced chemiluminescence de-
tection system (Amersham). Keratins were solubilized by
incubating cells in 2% Empigen (Calbiochem, Notting-
ham, UK) at 4°C for 45 minutes or 2 hours²⁸ and the
pellets were retained for electron microscopy. Proteins
were immunoprecipitated from the supernatant using
protein G-Sepharose beads.

In Vitro Ubiquitin-Binding Assays

Ubiquitin binding was demonstrated by binding of ubi-
quitinated proteins to GST-DEDD as well as binding of *in
vitro*-translated DEDD to ubiquitin-Sepharose beads. For
the first method, GST or GST-DEDD beads were pre-
pared as previously described.²⁹ 293T cells were tran-
sfected with or without HA-ubiquitin.³⁰ Twenty-four hours
after transfection, the cells were scraped and resus-
pended in 25 mmol/L of Tris-HCl, pH 7.6, lysed via three
cycles of freeze/thaw, and debris was spun down. The
supernatant was then transferred to a new tube and an
equal volume of 2 $\times$ ubiquitin binding buffer (25 mmol/L
Tris-HCl, pH 7.6, 10 mmol/L MgCl₂, 200 mmol/L NaCl, 2
 μ mol/L dithiothreitol, 4 μ mol/L ATP) and GST or GST-
DEDD beads was added. The reaction was incubated at
30°C for 90 minutes, washed four times in 1 $\times$ ubiquitin
binding buffer, and resuspended in sample buffer for
Western blot analysis. For the second method, wild-type
DEDD and dominant-negative FADD was *in vitro*-tran-
slated using the TnT System (Promega, Madison, WI) for 1
hour at 30°C. Five μ l of the *in vitro*-translated products
were then diluted in 1 $\times$ ubiquitin binding buffer, supple-
mented with 1% Triton X-100, and incubated with 20 μ l of
resuspended Sepharose (Sigma) or ubiquitin-Sepharose
(Affiniti Research Products Ltd., Exeter, UK) at 4°C for 2
hours rotating end to end. After incubation, the beads
were washed four times with 1 $\times$ ubiquitin binding buffer
supplemented with 1% Triton X-100.

Primary Antibodies

Antibodies against neopeptides on the large, 17- to 20-
kd, fragment of activated caspase-3 (Asp 175); the small,
12-kd, fragment of activated caspase-8 (Asp 384); the
large, 35-kd, fragment of activated caspase-9 (Asp315)
and the large, 37-kd, fragment of activated caspase-9
(Asp330) were from Cell Signaling Technology (Beverly,
MA). Further antibodies to the same neopeptides of ac-
tivated caspase-9 were generously provided by Dr. D.
Nicholson (Merck Frosst, Quebec, Canada) and a rat
monoclonal antibody (clone2E12) to Apaf-1 was donated
by Dr. D. Huang (The Walter and Eliza Hall Institute for
Medical Research, Melbourne, Australia). A fluorescein
isothiocyanate-conjugated rabbit anti-active caspase-3

monoclonal antibody was from BD Biosciences Clontech (Palo Alto, CA). Rabbit polyclonal antibodies specific for tumor necrosis factor receptor type-1-associated death domain protein (TRADD) (SC-7868) and for phosphorylated K18 (SC-17032R) as well as a mouse monoclonal antibody against K18 (SC-6259) were from Santa Cruz Biotechnology, Santa Cruz, CA. Mouse monoclonal antibodies directed against β -actin (AC15), the FLAG epitope (M2), cytokeratin K8 (M20), cytokeratin K18 (CY-90), and vimentin (V9) were from Sigma. A mouse monoclonal (RPN 1197) against heat-shock protein 72 (HSP72) was supplied by Amersham. The mouse monoclonal antibodies (MAB1510) and (P4D1), which detect ubiquitin, polyubiquitin, and ubiquitinated proteins, were supplied by Zymed (San Francisco, CA) and Cell Signaling Technology, respectively. A mouse monoclonal antibody directed against conjugated ubiquitin (clone FK2) was from Affiniti Research Products Ltd. Mouse monoclonal antibodies directed against the caspase cleavage site in K18 at DALD³⁹⁷ ↓ S (M30) and against the 85-kd cleavage fragment of PARP were from Roche Diagnostics Ltd. (Lewes, UK) and Pharmingen (Oxford, UK), respectively. Rabbit polyclonal antibodies that recognized Bid(44-433) and ubiquitin (AHB0231) were from Biosource (Camarillo, CA). A rabbit polyclonal antibody (DED3) was raised against a peptide in the death effector domain of DEDD²⁵ and three mouse monoclonal antibodies (C5, C15, and N2) were raised against caspase-8.³¹ All were used at a 1:10,000 dilution for Western blotting, at 1:100 to 1:250 for confocal immunocytochemistry, and at 1:5 to 1:100 for ultrastructural immunocytochemistry. Immunoglobulins, for use as negative controls, were from DakoCytomation (Ely, UK).

Electron Microscopy and Immunogold Cytochemistry

Adherent and nonadherent cells were processed and embedded in epoxy resin.¹⁹ Duplicate pellets were fixed with 4% formaldehyde (freshly made up from paraformaldehyde) or a mixture of 4% formaldehyde and 0.1% glutaraldehyde in PBS, pH 7.4, for 1 hour at room temperature and rinsed in PBS. Other pellets were fixed in absolute ethanol. All were embedded in LR-White resin (Agar Scientific, Stansted, UK)³² and labeled with immunogold (British Biocell International, Cardiff, UK).¹⁹ Double-labeling involved incubation with mixtures of mouse and rabbit antibodies and subsequent treatment with a mixture of anti-mouse antibody conjugated with 10-nm gold and anti-rabbit antibody conjugated with 5-nm gold, respectively. This procedure was repeated using different pairs of primary and secondary antibodies, on the reverse of the grid, to achieve quadruple labeling. Ultrathin sections were examined unstained or after staining with lead citrate and/or uranyl acetate. Control incubations involved the replacement of primary antibody with an equivalent concentration of the appropriate immunoglobulin.

Light Microscopy and Immunocytochemistry

Cells were grown on polyprep slides (Sigma-Aldrich, St. Louis, MO) and fixed with 2% formaldehyde for 20 minutes at room temperature. After the fixation the slides were washed three times with 50 mmol/L of NH₄Cl (in PBS) for 3 minutes, permeabilized in ice-cold PBS with 0.3% Triton X-100 for 1 minute, washed with PBS + 1 mmol/L of MgCl₂ (PBS-Mg), and incubated for 10 minutes in blocking solution (PBS, 0.01% saponin, 0.25% bovine serum albumin, and 0.02% NaN₃). The slides were incubated for 2 hours at room temperature or overnight at 4°C with the primary antibodies. After washing with PBS-Mg, slides were incubated with secondary antibodies for 1 hour at room temperature, washed, and dehydrated in 100% ethanol. Coverslips were mounted onto the slides with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and analyzed with an Axiovert S100 immunofluorescence microscope equipped with an AxioCam digital camera and software (Carl Zeiss Microimaging, Thornwood, NY). Confocal images were taken and analyzed with a confocal microscope LSM 510 (Zeiss).

Results

Caspase Processing and Cleavage of Caspase Substrates during Apoptosis

Preconfluent monolayers of A549 cells consisted of clusters of flattened polygonal cells. The cytoplasm of these cells contained an open network of intermediate filaments that were rarely bundled together to form fibrils, except in cells undergoing mitosis. Caspases-3, -6, -8, and -9 were all present as their unprocessed zymogens in these control cells. Treatment of these cells with TRAIL caused a time-dependent processing of initiator and effector caspases. Reductions were detected in the levels of procaspase-3 and procaspase-8 (Figure 1A). No change was detected in the levels of procaspase-9 (data not shown) but the formation of cleaved fragments was clearly evident. Signs of caspase-8 being processed to both its p43 and p41 forms, after removal of the small subunit from caspase-8a and -8b, were evident after 30 minutes. These bands were much more marked after 60 minutes, when the p18 large subunit, formed by cleavage of the death effector domains from the p43 and p41 form, also became evident. Caspase-3 was processed to its p20 and p17 forms and caspase-9 was cleaved, at Asp315, to its catalytically active p35 form. Cleavage of Bid, after 60 minutes, was indicated by loss of full-length Bid and concomitant appearance of the 15-kd cleavage fragment of Bid (Figure 1B). PARP cleavage was only detectable after 120 minutes of TRAIL treatment, consistent with its relatively late occurrence in the apoptotic program and its dependence on the activation of effector caspase-3. These results are consistent with the classical death-receptor pathway in type II cells where caspase-8, the apical caspase in these cells, is activated at the death-inducing signaling complex (DISC) before triggering mitochondrial amplification and thus caspase-3 acti-

Figure 1. Immunoblots from A549 cells incubated with TRAIL (500 ng/ml) showing: **A:** processing and activation of caspase-3, -8, and -9. **B:** Formation of the p15 cleavage product of Bid (arrowhead) at 60 minutes and the 85-kd fragment of PARP (arrowhead) at 120 minutes. **C:** Cleavage products (arrowheads) of K18 and vimentin, 120 minutes after treatment with 500 ng/ml of TRAIL (T), the positive control, 0.5 μ mol/L staurosporine (S) but not in control (C) cells.

vation.³³ Cytokeratin (K18) and vimentin were both cleaved during TRAIL- and STS-induced apoptosis (Figure 1C) but cytokeratin 8 was relatively resistant to cleavage (data not shown). Treatment of cells with STS also resulted in apoptosis and a similar activation of caspases-3, -8, and -9 together with cleavage of both K18 and vimentin (data not shown).

Morphological Changes during Apoptosis

Morphological effects were first clearly observed 2 hours after treatment of A549 cells with TRAIL, when a small proportion (<5%) of cells began to round-up, develop cy-

toplasmic buds, and then detach from the substrate (data not shown). Most (>90%) of the detached cells contained cytokeratin-rich inclusions that were totally absent from untreated cells. The proportion of detached cells increased with time and they were readily collected, from the overlying medium, as a pure population of apoptotic cells. A few (<5%) of the cells still adhering to the substrate showed signs of apoptosis but most were indistinguishable from untreated cells, no intermediate stage being recognized. Thus, the transition to an apoptotic morphology was very rapid and could occur within 2 hours.

Active Caspase-3, Active Caspase-9, DEDD, Ubiquitin, and Cleaved Cytokeratins Co-Localize on Fibrils of Apoptotic Cells

In apoptotic cells, most of the neopeptide antibody to caspase-3 recognizing the N-terminus of the catalytically active large subunit formed after cleavage at Asp175 into the large and small subunits, was clearly localized on cytoplasmic fibrils by both confocal and ultrastructural immunocytochemistry (Figure 2, A and B; Figure 3A).

Caspase-8 is the key initiator caspase in the death-receptor pathway and thus the most likely candidate for the activation of procaspase-3. Activation of caspase-8 was shown to precede cleavage of procaspase-3 (Figure 1A) but immunolabeling by three antibodies (C5, C15, and N2), all of which detect the intact proenzyme and various cleavage products,³¹ was only detected in small indistinct foci, 150 to 250 nm in diameter, within the cytoplasm (Figure 3B and data not shown). These foci were devoid of detectable cytokeratin or other caspases and reminiscent of the distinct punctate distribution seen in apoptotic cells transfected with a green fluorescent protein construct of caspase-8.¹⁹ Caspase-8 labeling was not associated with any cytokeratin fibrils.

Caspase-9 is also capable of activating caspase-3 and, in contrast to caspase-8, active caspase-9 was clearly localized on the cytokeratin fibrils of apoptotic but not untreated cells. Using two different antibodies to a neopeptide exposed by cleavage of procaspase-9 at Asp315, labeling was evident by both confocal (Figure 2C) and electron microscopy (data not shown). Western blot analysis and densitometry demonstrated that at this time point (3.5 hours of STS treatment) ~45% of procaspase-9 was cleaved resulting in the appearance of p37 and p35 fragments (data not shown). Although formation of this p35 subunit has generally been attributed to the Apaf-1-mediated self-cleavage of procaspase-9 in the cytoplasm, no cytoplasmic labeling was detected. The active subunit could have migrated to the cytokeratin fibrils, after its formation by the Apaf-1 apoptosome or, alternatively, procaspase-9 may have been activated directly on the fibrils. The antibody to Apaf-1 provided only low levels of cytoplasmic labeling, with no detectable concentration on cytokeratin fibrils (data not shown). Similar results have been reported previously and attributed to the low abundance of this molecule in epithelial cells.³⁴

Further evidence for the accumulation of caspase-9 on cytokeratin filaments of apoptotic cells is provided by

Figure 2. Confocal and immunofluorescent images of MCF-7/C3 cells treated with STS for 3.5 hours. Cytokeratin fibrils are labeled intensely by an antibody to a neoepitope on the large (17 to 20 kD) fragment of activated caspase-3 (**A** and **B**). Similar labeling resulted from antibodies to neoepitopes on the large (35 kD) fragment of activated caspase-9 (**C**) and the large (37 kD/17 kD) fragment of activated caspase-9 (**D**). The co-localization of these enzymes is shown in the merged images (**E** and **F**). Merged images also show the co-localization of K18 (red) with activated caspase-3 (green) (**G**) and activated caspase-9 (green) (**H**). Scale bars, 5 μ m.

their labeling with two different antibodies to another neoepitope, exposed by cleavage at Asp330 (Figure 2D and Figure 3C). These antibodies, which did not result in any labeling of untreated cells, label the large active (37 kD) fragment, a result of cleavage by caspase-3. Localization of this subunit on cytokeratin fibrils is thus consistent with fibril-associated caspase-3 cleaving a significant proportion of any procaspase-9 that is in close association with them. The 37-kD subunit, like the 35 kD formed by cleavage at Asp315, is capable of activating

additional caspase-3 and thus may amplify both this activity and the cleavage of the closely associated cytokeratin filaments. Co-localization of the neoepitope formed during activation of caspase-3 with neoepitopes formed by cleavage of caspase-9 at both Asp315 and Asp330 is evident in image overlays (Figure 2, E and F). Similarly, overlays of images showing the localization of K18 with those of neoepitopes characterizing active caspase-3 (Figure 2G) and active caspase-9 (Figure 2H) also showed co-localization on cytokeratin fibers. Consistent with our previous analysis of the localization of DEDD²¹ the neoepitopes showed a strong perinuclear but a weak peripheral co-localization with K18. Cytokeratin cleavage was confirmed by the labeling of fibrils with the antibody to a neoepitope exposed, on K18, by caspase-dependent cleavage at DALD³⁹⁷ ↓ S (see Figure 8A). The fibrils were also labeled by antibodies to native K8 (data not shown), native K18 (Figure 3, A and D), hyperphosphorylated K18 (data not shown), and DEDD (Figure 3D). They were also labeled by the antibodies to ubiquitin, polyubiquitin and ubiquitinated proteins (Figure 4A). In contrast, the fibrils of nonapoptotic cells were only labeled by antibodies to native K8, native K18 and, in some cells, hyperphosphorylated K18 (data not shown).

The localization of ubiquitin on the fibrils of apoptotic, but not untreated cells may result, in part, from the association of DEDD with these fibrils. Ubiquitination is necessary for DEDD to perform its role as a scaffold protein and it was found to have a high affinity for ubiquitin. This affinity was particularly evident in studies with 293T cells, which normally show a diffuse cytoplasmic labeling for ubiquitinated proteins, like the other cells in our study. In cells transfected with DEDD-FLAG, but not in adjacent nontransfected cells, this diffuse cytoplasmic labeling is lost and the ubiquitinated proteins become concentrated, with DEDD, in the nucleoli (Figure 4, B and C). Thus the transfected DEDD, which accumulates in the nucleoli,²⁷ can attract most of the endogenous ubiquitinated proteins from the cytoplasm. The affinity of DEDD for ubiquitin has also been confirmed by an *in vitro* ubiquitin-binding assay from cell lysates of 293T cells transfected with a HA-tagged ubiquitin plasmid (Figure 4D). The binding of DEDD to ubiquitin was confirmed using a direct binding assay of FLAG-tagged *in vitro* translated DEDD to ubiquitin-Sepharose (Figure 4E). Immunocytochemical studies, using the FK2 antibody, also showed that ubiquitinated proteins, but not free ubiquitin, co-localize with FLAG-labeled N-DEDD (Figure 5, A and B). This localization occurs within nucleoli (Figure 5C), just as efficiently as full-length DEDD (see Figure 4B). Unlike the full-length form, however, N-DEDD cannot be ubiquitinated (Figure 5D) and so it must bind to ubiquitinated proteins other than itself.

Intermediate Filaments and Their Associated Proteins Accumulate in Cytoplasmic Inclusions

The formation of numerous cytoplasmic inclusions, which varied in diameter from 0.1 to 5.0 μ m, was one of the most striking features of apoptosis in all of the cell lines examined. These inclusions were first detected at 1 to 2 hours after treatment with TRAIL (Figure 5E) and labeled

Figure 3. Immunogold cytochemistry of A549 cells, 3 hours after treatment with 500 ng/ml of TRAIL, showing: **A:** labeling of cytokeratin fibrils by an antibody to the large (17 to 20 kD) fragment of activated caspase-3 (10-nm gold) and native K18 (5-nm gold). **B:** Cytoplasmic foci labeled with the C5 antibody to caspase-8. **C:** Cytokeratin fibrils labeled by an antibody to the large (37 kD) fragment of activated caspase-9. **D:** Cytokeratin fibrils labeled with an antibody to DEDD (10-nm gold) and to native K18 (5-nm gold). Scale bars, 500 nm.

consistently with antibodies to K8 and K18. Many of the inclusions showed signs of coalescing (Figure 6A) but others were present in the numerous cytoplasmic buds developed during apoptosis (Figure 6B). The inclusions

were also labeled with antibodies to neoepitopes on the activated fragments of caspase-9 (Figure 6A) and caspase-3 (Figure 6, B and C). These neoepitopes were co-localized with the other proteins associated with cyto-

Figure 4. DEDD binds ubiquitin and ubiquitinated proteins. **A:** Immunogold cytochemistry of an A549 cell, 7 hours after treatment with 500 ng/ml of TRAIL, showing labeling of cytokeratin fibrils by an antibody detecting ubiquitin, polyubiquitin, and ubiquitinated proteins. **B:** Fluorescent labeling of FLAG in three 293T cells transfected with DEDD-FLAG (arrows), showing its localization in the nucleoli. **C:** Immunofluorescent labeling of the endogenous, ubiquitinated proteins in the same field shows a diffuse cytosolic distribution in the adjacent, nontransfected cells but co-localization of cell lysates from 293T cells transfected with either control or a HA-ubiquitin plasmid shows that several ubiquitinated proteins were precipitated by GST-DEDD but not by GST alone. **D:** A direct binding assay of FLAG-tagged, *in vitro* translated, DEDD shows binding of DEDD, but not the dominant-negative derivative of FADD (DN-FADD), to ubiquitin Sepharose (Ubi-Seph.). Scale bar, 500 nm.

keratin fibrils, including DEDD, caspase-cleaved K18, and native K18 (Figure 6D). The antibodies detecting ubiquitin, polyubiquitin, and ubiquitinated proteins also labeled many inclusions rich in native K18 (Figure 7A). Immunogold cytochemistry showed remarkably uniform labeling of inclusions with antibodies to neopeptides on activated caspase-9 fragments (Figure 7, B and C). These neopeptides were consistently co-localized with native K18 (Figure 7B) but, surprisingly, there was a marked variation in the relative proportion of phosphorylated K18 to native K18, even between adjacent inclusions within the same cell (Figure 7D). Thus, all of the proteins associated with cytokeratin fibrils were also localized in the cytoplasmic inclusions. Direct evidence for a transition from fibrils to inclusions was seen in favorable sections (Figure 8A). Vimentin was detected over individual fibrils in both A549 and HeLa cells (Figure 8B) and immunoblotting showed that it was cleaved during the early stages of apoptosis (Figure 1C). Vimentin was also localized in the cytoplasmic inclusions of apoptotic cells (data not shown) but no accumulation of vimentin fibrils was found around these, or any other inclusions. Vimentin

Figure 5. **A:** Immunofluorescent labeling with the antibody FK2 shows a concentration of ubiquitinated proteins in discrete foci. **B:** These same foci also accumulate FLAG-tagged N-DEDD. **C:** The foci were identified as nucleoli within DAPI-labeled nuclei. **D:** An immunoblot shows that the N-DEDD (N) form, unlike the wild-type (W-t), is resistant to ubiquitination. The vector control (V) is also devoid of label. **E:** Early cytoplasmic inclusions (arrows) in an A549 cell, 2 hours after treatment with 500 ng/ml of TRAIL, also showing the development of apoptotic cytoplasmic buds. Scale bar, 1 μ m.

cages are a characteristic of aggresomes, which are formed by microtubular transport of misfolded, ubiquitinated proteins into the pericentriolar region of the cell, rarely resulting in more than one inclusion per cell.³⁵ The inclusions in our experiments were thus distinct from aggresomes because several were present throughout the cytoplasm of each cell, their contents were heterogeneous (Figure 8, C and D) and yet all were rich in cytokeratin and all were devoid of a vimentin cage.

Interestingly all of the detectable DEDD, ubiquitin, cleaved K18, processed caspase-3, and processed caspase-9 within each apoptotic cell was present in either the fibrils or the inclusions. Prolonged (2 hours) treatment of late apoptotic cells with Empigen, a zwitterionic detergent, had no detectable effect on the composition of the inclusions. This treatment resulted in a greatly enriched suspension of inclusions (Figure 9A) that retained antigenicity for all our antibodies (Figure 9, B and C). As with inclusions present within intact cells, this labeling was not found in control incubations (Figure 9D). Thus, most of the phosphorylated and caspase-cleaved cytokeratin together with DEDD and the active subunits of caspase-3 and -9 were intimately associated with the insoluble cytokeratin fraction. The avidity of this association with the insoluble component(s) of cytoplasmic

Figure 6. Immunogold cytochemistry of A549 cells 3 hours after treatment with 500 ng/ml of TRAIL, showing: **A:** numerous cytoplasmic inclusions, showing signs of fusion, are labeled with an antibody to the large (35 kD) fragment of activated caspase-9. **B:** An apoptotic cell with many characteristic cytoplasmic buds and numerous inclusions (arrows) labeled with an antibody to the large (17 to 20 kD) fragment of activated caspase-3. **C:** A detail of the area indicated in **B**. **D:** A cytoplasmic inclusion labeled with antibodies to the large (17 to 20 kD) fragment of activated caspase-3 (5-nm gold), DEDD (10-nm gold), native K18 (15-nm gold), and caspase-cleaved K18 (20-nm gold). Scale bars: 500 nm (**A**, **C**, **D**); 1 μ m (**B**).

inclusions, even after prolonged detergent extraction, may thus account for our failure to immunoprecipitate caspase-9 from the supernatant. The Empigen solution contained a considerable amount of immunoprecipi-

table K18 but no traces of caspase-9 co-precipitated with this protein. Conversely, immunoprecipitation with antibody to caspase-9 did not yield any detectable K18 from this solution.

Figure 7. Immunogold cytochemistry of A549 cells 3 hours after treatment with 500 ng/ml of TRAIL, showing: **A:** a cytoplasmic inclusion labeled with an antibody to native K18 (10-nm gold) and an antibody detecting ubiquitin, polyubiquitin, and ubiquitinated proteins (5-nm gold). **B:** Cytoplasmic inclusions labeled by the antibody to native K18 (10-nm gold) and an antibody to the large (35 kD) fragment of activated caspase-9 (5-nm gold). **C:** A cytoplasmic inclusion labeled with an antibody to the large (37 kD) fragment of activated caspase-9 (5-nm gold). **D:** Adjacent inclusions, both labeled with an antibody to native K18 (5-nm gold) but only one is labeled by the antibody to phosphorylated K18 (10-nm gold). Scale bars: 250 nm (**A**); 500 nm (**B–D**).

HSP72, TRADD, and Caspase-8 Are also Present in Cytoplasmic Inclusions

HSP72 and TRADD, possible modulators of cytotoxicity, were clearly detected in the cytoke-

ratin-containing inclusions (data not shown) but no significant labeling was associated with the cytoke-

Figure 8. **A:** Immunogold labeling of both cytochrome c fibrils and a cytoplasmic inclusion, by the antibody directed against the caspase cleavage site in K18 at DALD³⁹⁷ ↓ S, in an A549 cell, 3 hours after treatment with 500 ng/ml of TRAIL. **B:** Fibrils in a HeLa cell 2 hours after treatment with 500 ng/ml of TRAIL, labeled with the antibody to vimentin. **C:** A549 cell 3 hours after treatment with 500 ng/ml of TRAIL showing the concentration of labeling, with the antibody C5' to caspase-8, within a small zone of a cytoplasmic inclusion. **D:** Detail of **C**. Scale bars, 500 nm.

periphery, particularly in zones that had a slightly greater affinity for uranyl acetate (Figure 8, C and D). These zones probably result from the fusion of the small, cytoplasmic caspase-8-rich foci with cytochrome c inclusions. The con-

tents of these foci may subsequently disperse throughout the inclusion. As many of these inclusions are present in apoptotic buds, this may provide a mechanism for removal of caspase-8, and other enzymes, from the cell.

Figure 9. **A:** A549 cells, 7 hours after treatment with 500 ng/ml of TRAIL, incubated in 2% Empigen, at 4°C for 2 hours shows a greatly enriched suspension of inclusions. **B:** A detail of the area shows that these inclusions retained antigenicity for an antibody to the large (17 to 20 kD) fragment of activated caspase-3 (10-nm gold). Other inclusions in this sample show immunogold labeling for K8 and K18 (5-nm gold) and for the large (37 kD) fragment of activated caspase-9 (10-nm gold) (**C**) but incubations replacing the primary antibodies with mouse IgG1 were devoid of significant labeling (10-nm gold). Scale bars: 5 μm (**A**); 500 nm (**B–D**).

Discussion

The finding that all of the detectable epitopes characterizing the catalytically active large subunits of caspase-9 are concentrated on cytokeratin fibrils, during the early

stages of apoptosis in epithelial cells, provides a new insight into the cellular mechanisms of caspase activation. Our results are consistent with the classical death-receptor pathway in type II cells where caspase-8, the

apical caspase in these cells, is initially activated at the DISC.³³ The absence of caspase-8 labeling from cyto-keratin fibrils is unlikely to result from problems of epitope accessibility, considering the successful labeling of small cytoplasmic foci with no detectable cytokeratin. The composition of these foci is reminiscent of that reported for death-effector filaments in transfected cells overexpressing a DED region of caspase-8³⁶ but no such filaments were found in any of our, nontransfected, cells. Thus, although activated caspase-8 may be sufficient to trigger the caspase cascade it is probably too dispersed to be detectable by immunocytochemistry, even in a death-receptor-induced model of apoptosis. In contrast the striking concentration of both the p35 and p37, catalytically active large subunits of caspase-9 on the cyto-keratin fibrils is consistent with their having a critical, localized role in the demolition of the apoptotic cell. The generally accepted mechanism for the initial formation of the p35 subunit is by the perturbation of mitochondria, release of cytochrome c, and formation of the Apaf-1 apoptosome. Apaf-1 then recruits and activates procaspase-9 by cleavage at Asp315. This subunit may thus have migrated to the cyto-keratin fibrils, after its formation at the apoptosome. Our data, however, raise the possibility that procaspase-9 could have been activated directly on the filaments of cyto-keratin, which may provide a scaffold for the accumulation and self-cleavage of the proenzyme. This subunit may then activate caspase-3, which is directed to the filaments by DEDD. Endogenous DEDD has previously been shown to have a predominantly cytosolic distribution with little endogenous DEDD found in nucleoli, in contrast with the nucleolar concentration of overexpressed DEDD.^{25,27} Caspase-3 activation and subsequent caspase-9 cleavage at Asp330 may demarcate a critical stage to form the p37 subunit, which is immune to XIAP inhibition. Formation of this subunit is strong evidence for the presence of an amplification loop to activate further caspase-3, accelerate cleavage of the crucial K18 component of the fibrils and thus the formation of cytoplasmic inclusions. This interpretation is consistent with the immunolabeling of fibrils, inclusions, and intermediate structures with antibodies to DEDD, the active subunit of caspase-3 and also caspase-cleaved K18. The co-localization of DEDD with ubiquitin is also consistent with a high affinity of DEDD for ubiquitin and the reported diubiquitination of the DEDD bound to cyto-keratin. DEDD is also thought to have a high affinity for other ubiquitinated proteins, many of which are sequestered in cytoplasmic inclusions or sequestosomes.³⁷ As hyperphosphorylation inhibits the ubiquitination of keratins, differences in the local availability of other ubiquitinated proteins may well account for the marked variation in the level of ubiquitin labeling, between inclusions. In addition to the activation of filament-associated caspases, the resulting close proximity of caspase-3 with a large amount of one of its major substrates will reduce its availability to other normal substrates and so modify the apoptotic program. This may, in part, explain the enhanced susceptibility of epithelial cells, deficient in K8 and K18, to tumor necrosis factor-induced cell death.³⁸ The observed sequestration of TRADD within inclusions

would be a natural progression from its reported removal from the cytosol by binding to K18³⁹ and the incorporation of caspases could indicate a similar fate for these enzymes. Thus, these cytoplasmic inclusions act as true sequestosomes by isolating a wide range of agents involved in the development of apoptosis, possibly including caspase cleavage products such as those from vimentin⁴⁰ and desmin.⁴¹ Mallory bodies, one of the most studied forms of sequestosomes, may also act like the cytoplasmic inclusions in sequestering active enzymes within injured hepatocytes, rather than merely acting as accumulations of modified cyto-keratin.

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