Apoptotic and Necrotic Death Mechanisms Are Concomitantly Activated in the Same Cell After Cerebral Ischemia

Isın Ünal-Çevik, MD, PhD; Munire Kılınç, MD, PhD; Alp Can, MD; Yasemin Gürsoy-Özdemir, MD, PhD; Turgay Dalkara, MD, PhD

Background and Purpose—Both necrotic and apoptotic cell death mechanisms are activated after cerebral ischemia. However, whether they are concomitantly active in the same cell or in discrete cell populations is not known.

Methods—We investigated activation of both pathways at the cellular level in mice brains subjected to transient or permanent focal ischemia.

Results—Four hours after ischemia, diffuse cathepsin-B spillage into cytoplasm, suggesting lysosomal leakage, was observed within neurons immunoreactive for the active form of caspase-3 (p20). Ischemic neurons with a leaky plasma membrane (positive for propidium iodide) were colabeled with caspase-cleaved actin fragment and exhibited TUNEL-positive nuclei having apoptotic morphology. At 72 hours, up to 27% of cells with caspase activity displayed morphological features suggestive of secondary necrosis.

Conclusions—These data, demonstrating an early and concurrent increase in caspase-3 and cathepsin-B activities followed by appearance of caspase-cleavage products, DNA fragmentation, and membrane disintegration, suggest that subroutines of necrotic and apoptotic cell death are concomitantly activated in ischemic neurons and that the dominant cell death phenotype is determined by the relative speed of each process. **(*Stroke*. 2004;35:2189-2194.)**

Key Words: apoptosis ■ caspases ■ cathepsins ■ cerebral ischemia, focal ■ necrosis

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Brain infarction was traditionally considered to be a classic example of liquefactive necrosis. On the basis of evidence showing that inhibition of protein synthesis reduced brain injury, Linnik et al[1] suggested that ischemic cell death was not simply a passive process but involved synthesis of new proteins as in programmed cell death. Subsequently, several morphological and biochemical features observed in apoptosis have been documented in ischemic brains.[2–4] Further supporting the role of apoptotic mechanisms in ischemic cell death, overexpressing the antiapoptotic proteins or knocking out the genes encoding the proapoptotic proteins have been demonstrated to confer resistance to ischemia.[5,6] Similarly, pharmacological inhibition of caspase activity provided substantial neuroprotection.[7,8]

However, electron microscopic studies indicate that ischemic neuronal death is characterized by a mixture of apoptotic and necrotic ultrastructural changes.[9] Proteases that are not primarily involved in apoptosis (eg, cathepsins, calpains) have also been found to be activated in ischemic neurons, and their inhibition increases brain resistance to ischemic injury.[10,11] Biochemical studies have shown concurrent appearance of spectrin breakdown products cleaved by caspase-3 as well as calpain in ischemic brain extracts.[11,12] However, despite extensive data from in vitro studies and indirect evidence from in vivo work, the interactions between different modes of cell death in the ischemic brain have not been characterized at the cellular level in vivo. In this study we investigated whether markers generally associated with apoptotic or necrotic mechanisms are colocalized in the same cell or appear in discrete cell groups after focal cerebral ischemia in intact mouse brain. We chose the 30-minute transient and permanent middle cerebral artery (MCA) occlusion models because apoptotic features are more prevalent after brief ischemia and necrosis becomes the predominant form of cell death as ischemia duration increases. Moreover, we used propidium iodide (PI), a commonly accepted marker of necrosis in cultured cells, in in vivo conditions, which has been very instrumental in colocalizing markers of caspase activation and DNA fragmentation with loss of membrane integrity.

Materials and Methods

Experimental Procedures

Animal housing, care, and application of experimental procedures were done in accordance with institutional guidelines. Swiss mice

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From the Department of Neurology (I.Ü.-Ç., Y.G.-Ö., T.D.), Faculty of Medicine, and the Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara, Turkey; the Department of Neurology (M.K.), Faculty of Medicine, Baskent University, Ankara, Turkey; and the Department of Histology and Embryology (A.C.), Faculty of Medicine, Ankara University, Ankara, Turkey.

Correspondence to Dr Turgay Dalkara, Department of Neurology, Hacettepe University Hospitals, Ankara 06100, Turkey. E-mail tdalkara@hacettepe.edu.tr

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(30 to 35 g; Hacettepe University Experimental Animal Facility, Ankara, Turkey) were anesthetized with chloral hydrate (300 mg/kg IP), and proximal occlusion of the right MCA was performed with a nylon filament as previously described.[13] Briefly, after the right common and external carotid arteries were ligated, a silicone-coated filament (8/0) was inserted proximal to the carotid bifurcation and advanced in the internal carotid up to the origin of the MCA. Mice were subjected to 30 minutes of ischemia and 6 (n=6) or 72 hours (n=17) of reperfusion or 6 (n=9) or 72 hours (n=7) of permanent MCA occlusion. Several coronal brain sections from each animal were examined with different cell death markers on frozen (10-μm-thick) or paraffin-embedded (5-μm-thick) sections. Four additional mice were subjected to 1 hour of ischemia and 4 hours of reperfusion to study caspase-3-p20 and cathepsin-B colocalization. Thicker (20-μm) cryosections were obtained from these animals to better illustrate colocalization of caspase-3-p20 immunoreactivity with extralysosomal cathepsin-B immunoreactivity in a greater number of cells.

Detection of Leaky Membranes by PI

Half of a microliter of PI (P-3566, Molecular Probes; 1 mg/mL in distilled water) was injected into the right lateral ventricle. Twenty minutes after injection, mice were perfused transcardially with 4% formaldehyde. Brains were removed and kept in formaldehyde solution for 2 days at 4°C. Later the brains were transferred into 20% and then 30% sucrose for 12 hours each at 4°C. Ten-micrometer-thick cryosections were further processed for TUNEL, fractin, caspase-3-p20, and cathepsin-B (see below). The specificity of immunoreactivity was tested by omitting each primary antibody from the incubation medium. Hoechst-33258 was added to the mounting medium to counterstain the nuclei. Neurons and astrocytes were labeled with mouse monoclonal antibodies against microtubule-associated protein-2 (MAP-2; Sigma) and glial fibrillary acidic protein (GFAP; Sigma). Sections were incubated first with primary antibodies (1:100) at 37°C for 90 minutes and then with secondary antibodies (1:200; FITC goat anti-mouse IgG; Jackson ImmunoResearch) for 90 minutes.

Detection of DNA Fragmentation by TUNEL Method

DNA fragmentation was detected in situ by 3′-end labeling with the Apoptosis Detection System, Fluorescein kit (Promega). Briefly, the frozen brain sections obtained from mice treated with PI were washed in fresh PBS, permeabilized with proteinase K, and pre-equilibrated in an equilibration buffer. DNA strand breaks were labeled with fluorescein-12-dUTP. For negative controls, TdT enzyme was not included in the incubation buffer.

For paraffin-embedded sections, a kit containing TdT and digoxigenin-11-dUTP was used (ApopTag; Oncor). Diaminobenzidine (DAB) was used as chromogen, and the background was stained with methyl green. Positive and negative controls were included in every trial. Apoptotic neurons were identified by the presence of various types of chromatin condensation or apoptotic bodies, whereas cells showing diffuse cytoplasmic labeling or nuclear staining without apoptotic chromatin changes were considered necrotic (Figure 1). Positively-labeled cells were manually counted in 6 selected regions (hindlimb and forelimb areas, parietal cortex, insular cortex, preoptic area, lateral and medial striatum) at ×400 magnification.[14]

Detection of Caspase-Mediated Actin Cleavage

PI-labeled frozen sections were incubated with polyclonal rabbit fractin antibody (1:100; provided by Drs Fusheng Yang and Greg Cole of University of California at Los Angeles) for 90 minutes and then with secondary antibodies (Cy2 goat anti-rabbit IgG; 1:200; Jackson ImmunoResearch) for 90 minutes at 37°C.

Formalin-fixed, paraffin-embedded brain sections were deparaffinized overnight, dehydrated in graded xylene and alcohol solutions, and incubated with hydrogen peroxide in methanol for 10 minutes. Antigen retrieval was achieved by microwaving for 5 minutes twice in 1 mmol/L EDTA buffer (pH=8). The sections were rinsed with PBS and were incubated in blocking solution (Zymed) for 10 minutes, followed by incubation with antibody (1:5000 in 3% bovine serum albumin) for 60 minutes at room temperature. Sections were then labeled with biotinylated secondary antibody and streptavidin-peroxidase (Zymed) for 10 minutes. DAB was used as chromogen and hematoxylin as counterstain. Positively-labeled cells were counted in the 6 aforementioned regions.

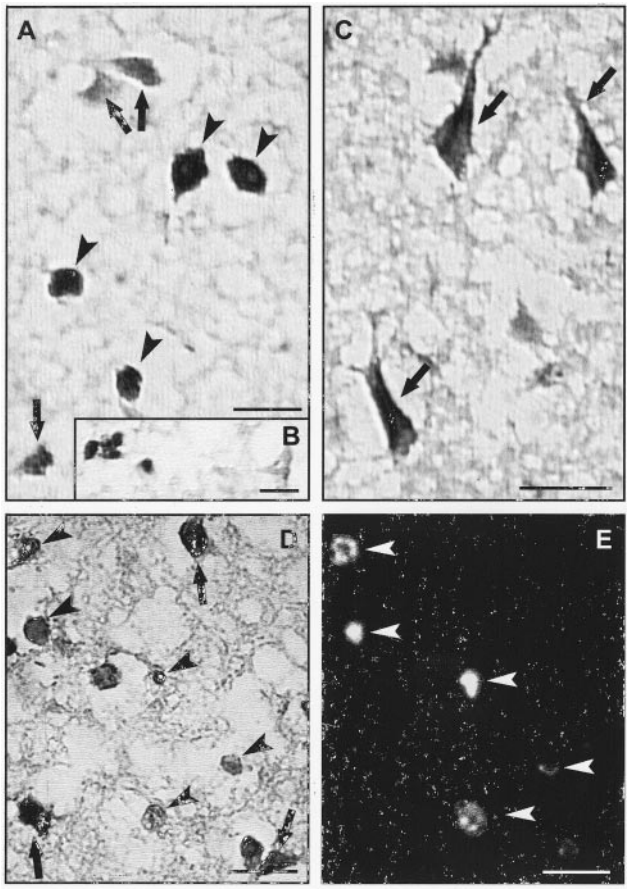

Figure 1. TUNEL staining labeled necrotic neurons in addition to apoptotic nuclei 72 hours after 30-minute ischemia. Apoptotic nuclei (arrowheads) exhibited peripheral chromatin clumping, blebbing (A, D, E), and fragmentation (B), whereas cytoplasm of necrotic cells was labeled (A, C, D; arrows). TUNEL plus methyl green counterstaining suggests that these cells were neurons (note necrotic cells in C). Hoechst staining of the same area in D better discloses apoptotic nuclear morphology (E). Bars=20 μm.

Detection of Caspase-3 and Cathepsin-B Activation

The brains were perfusion-fixed with formaldehyde. Brain slices were stained by double-immunofluorescence histochemistry with rabbit polyclonal antibodies against caspase-3-p20 (Cell Signaling) and cathepsin-B (Upstate). Frozen sections were incubated with primary antibody (1:200) for 90 minutes and then with secondary antibodies (1:200; Jackson ImmunoResearch) for 90 minutes at 37°C. Tyramide amplification technique (Molecular Probes) was used to enhance the caspase-3 signal.

Bright-field examinations were performed with a Nikon Eclipse E600 upright microscope with the use of appropriate filter sets. Specimens were further analyzed by a Zeiss LSM-510 confocal laser-scanning microscope. Single optical sections were collected at 2048×2048 pixel resolution. Digitized images were pseudocolored according to their original fluorochromes.

Data are expressed as mean and SE. Numbers in parentheses indicate the number of animals from which the data were obtained.

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Several coronally-cut brain sections from each animal were examined.

Results

Colocalization of PI and TUNEL Labeling in Ischemic Cells

Numerous PI-labeled cells in the ischemic MCA area were observed in mice brains subjected to 30-minute transient or permanent focal cerebral ischemia (Figure 2A to 2O). Contrary to in vitro studies in which RNAse was applied, nucleic acids in the cytoplasm as well as nucleus were stained; hence, not only the nucleus but the whole cell fluoresced red.[15] PI-labeled cells were present 6 hours after transient (n=3 mice) or permanent (n=3 mice) ischemia. The number of PI-positive cells and the intensity of fluorescence increased dramatically at 72 hours (n=6 mice). PI labeling was observed in MAP-2–positive neurons and GFAP-positive astrocytes (Figure 2K and 2L).

Although TUNEL is not a specific marker for apoptosis, it is instrumental in identifying nuclei showing apoptotic DNA fragmentation when combined with changes in nuclear morphology (eg, chromatin clumping, nuclear fragmentation, and pyknosis). Hence, we routinely counterstained fluorescent or DAB-labeled slides with Hoechst-33258 to better evaluate the nuclear morphology[15] (Figure 1). TUNEL-positive nuclei displaying apoptotic morphology, which were scarce at 6 hours after ischemia ($7\pm2/mm^2$; n=9 mice), increased strikingly at 72 hours ($108\pm14/mm^2$; n=17 mice). Double-labeling studies (n=6 mice) revealed that PI and TUNEL fluorescence were present in discrete cell populations in areas of mild injury such as peri-infarct regions (Figure 2M). The TUNEL-positive cells exhibited apoptotic nuclear features in contrast to the TUNEL-negative, PI-positive cells displaying large, round, or triangular nuclei (Figure 2M and 2N). However, in areas displaying more severe ischemic changes, several cells displayed a mixed phenotype (ie, a TUNEL-positive nucleus in a PI-labeled cell; Figure 2A through 2D) in addition to discretely TUNEL- or PI-labeled cells. To determine the proportion of TUNEL-positive necrotic cells, we counted labeled cells on paraffin-embedded sections, which are more suitable to evaluate cellular morphology (Figure 1). The percentages of cells exhibiting necrotic-type TUNEL staining in reference to total number of TUNEL-positive cells were 27±4% (n=14 mice) and 5±2% (n=4 mice) in transient and permanent ischemia groups, respectively, 72 hours after ischemia.

Colocalization of PI Positivity and Caspase Activity in Ischemic Cells

We assessed caspase activation by appearance of the 32-kD actin fragment (fractin) generated by caspase-mediated cleavage of actin.[16] The antibody used is specific for the 32-kDa fragment, and it does not recognize the intact actin or actin fragments produced by calpain. Six hours after ischemia (n=9 mice), fractin immunoreactivity was confined to cytoplasm (Figure 2P). The cytoplasm, pyknotic, or segmented nuclei and apoptotic bodies were all positively-stained 72 hours after ischemia (Figure 2F). At 72 hours, the number of fractin-positive cells in coronal sections passing through the anterior commissure ($252\pm25/mm^2$ and $327\pm38/mm^2$ in transient [n=13 mice] and permanent [n=4 mice] ischemia, respectively) paralleled that of TUNEL-positive cells ($127\pm12/mm^2$ [n=14 mice] and $179\pm48/mm^2$ [n=4 mice], respectively). There was a strong correlation between the number of fractin- and TUNEL-positive cells when sections from the same animal were compared ($r=0.9$, $P<0.01$). However, fractin-positive cells outnumbered TUNEL-positive cells in every animal studied. Similar to the observations with TUNEL staining, 27±5% (n=13 mice) and 7±1% (n=4 mice) of cells with fractin-positive nuclei (in transient and permeant ischemia, respectively) did not exhibit apoptotic morphology.

Additional evidence illustrating the presence of caspase-cleaved actin fragments in necrotic cells was obtained by demonstration of fractin immunofluorescence in PI-labeled cells (Figure 2G and 2H). Interestingly, fractin-positive cells were colabeled with PI not only 72 hours after transient (n=3 mice) or permanent (n=3 mice) ischemia, but also at earlier stages of cell death (6 hours after transient ischemia, n=3 mice; Figure 2S), indicating that this colocalization was not a coincidence caused by membrane rupture developing at the end stage of cell death.

Coactivation of Cathepsin-B and Caspase-3 Soon After Ischemia

Although cathepsin-B is involved in activation of some forms of apoptotic cell death, diffuse cytoplasmic release of cathepsin-B is considered a marker of loss of lysosomal membrane integrity and hence of necrotic cell death.[10,17,18] In nonischemic cells, cathepsin-B–immunoreactive fine granules were dispersed in the cytoplasm in agreement with predominantly lysosomal localization of cathepsin-B, and there was no caspase-3-p20 immunolabeling. In the MCA territory, we observed a prominent caspase-3-p20 immunoreactivity in neurons 4 hours after reperfusion, in accordance with previous reports[12,19] (Figure 2T). In these neurons, the size and immunoreactivity of cathepsin-B granules were increased (suggesting an increase in lysosomal cathepsin-B); additionally, there was diffuse cytoplasmic cathepsin-B immunofluorescence (indicating lysosomal leakage, n=4 mice; Figure 2U and 2V). Caspase-3-p20 and diffuse cathepsin-B immunoreactivities in the same cell overlapped to varying degrees.

Discussion

The mode of ischemic neuronal death has been a matter of controversy. The initial enthusiasm about the role of apoptotic mechanisms has recently been replaced by a more cautious approach emphasizing the complicated nature of ischemic neuronal death.[20–23] Available evidence based primarily on the detection of TUNEL-positive nuclei and active forms of caspases has led to the conclusion that necrosis predominates in the severely stressed core area, whereas apoptotic neurons are concentrated in the border zone of the ischemic territory, where the insult is milder.[3,24] However, this contention is only partly valid because many TUNEL-positive neurons with unambiguously apoptotic nuclear morphology and caspase activity can be detected in the core areas amid apparently

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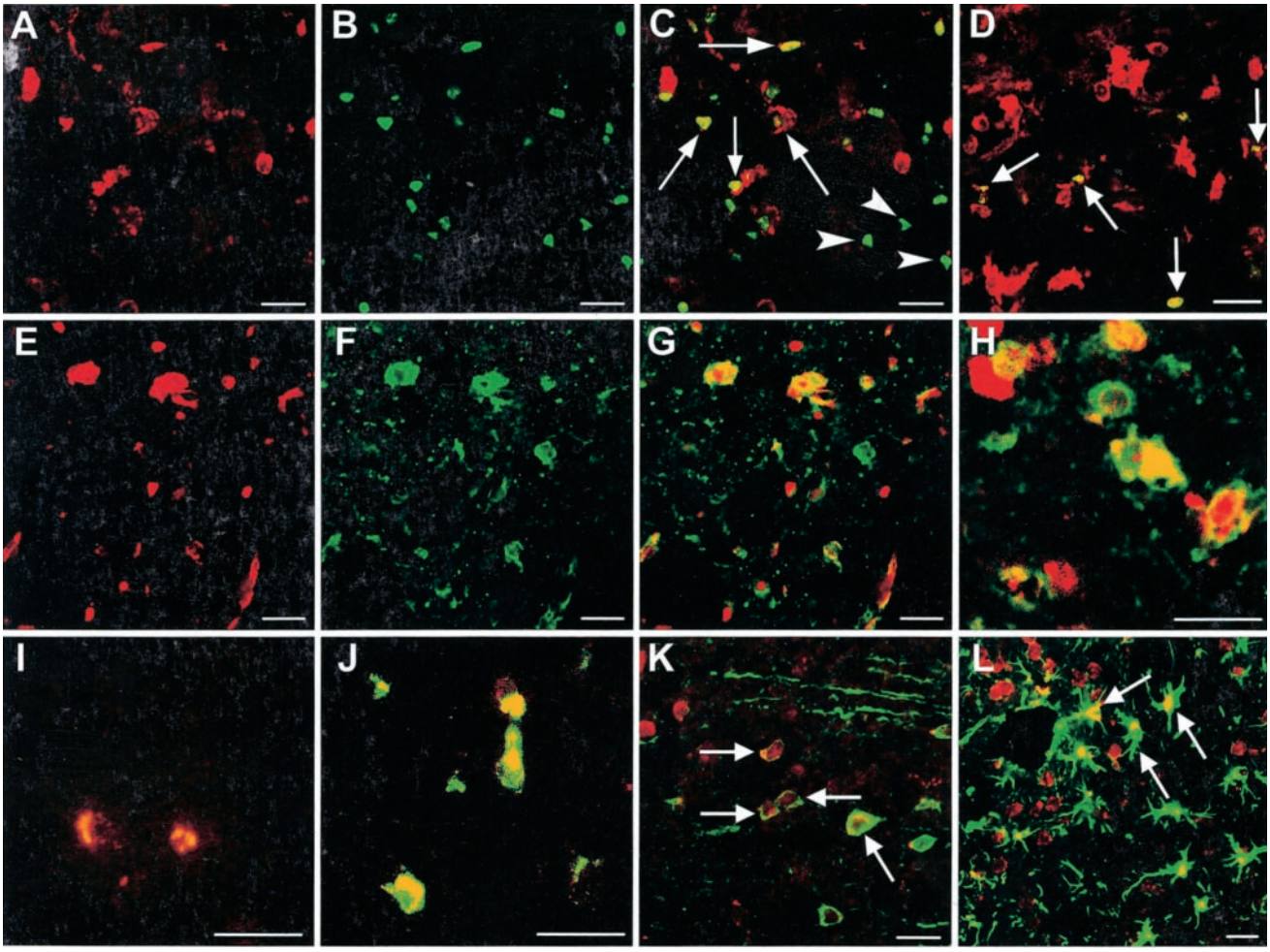

Figure 2. Loss of plasma and lysosomal membrane integrity, caspase activity, and DNA fragmentation are colocalized in the same cell after cerebral ischemia. Seventy-two hours after 30-minute transient (A, B, C) or permanent (D) MCA occlusion, several cells in core cortical areas lost their membrane integrity, as indicated by penetration of membrane-impermeant fluorescent dye PI (A, C, D; red). Nuclei of some of these cells exhibited apoptotic-like features and were TUNEL-positive (B, C; green). Merging PI and TUNEL images discloses colocalization (C, D; yellow; arrows) of necrotic and apoptotic features in the same cell. Another group of cells with TUNEL-positive nuclei preserved their membrane integrity (C; arrowheads). PI-positive cells (red) were also immunoreactive for another marker of apoptosis, the caspase-cleaved actin fragment (F; green) 72 hours after transient (E, F, G) or permanent (H) ischemia (core areas). Merged images (G, H) illustrate colocalization (yellow) of fractin and PI fluorescence. PI labeling (red) was also colocalized (yellow) with the active, cleaved form of caspase-3 (I; green; peri-infarct area) and with diffuse cytoplasmic cathepsin-B immunoreactivity (J; green; core) 72 hours after permanent ischemia. PI-labeled cells were MAP-2–positive neurons (K; green) or GFAP-positive astrocytes (L; green; peri-infarct, 72-hour permanent ischemia). In I to L, only overlays are illustrated. Bars=20 μm.

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necrotic neurons and inflammatory cells.[25] Similarly, electron microscopic studies and analysis of DNA fragmentation in the ischemic brain raise the question of whether necrotic and apoptotic mechanisms are independently involved in ischemic neuronal death.[9,26] Although these observations together with in vitro findings from neuronal cell cultures led several authors to propose that ischemic neuronal death is a "messy" form of cell death, possibly displaying a mixture of many death subroutines in varying combinations at different stages of cell demise,[20–23,27] the validity of this view was not tested under in vivo conditions. In this study we have demonstrated that markers of caspase activation (fractin or caspase-3-p20 immunoreactivity), apoptotic DNA fragmentation (TUNEL positivity along with apoptotic nuclear morphology), early loss of plasma (PI labeling), and lysosomal membrane integrity (cathepsin-B spillage) are indeed colocalized in ischemic neurons in the intact brain in vivo.

Several TUNEL-positive neurons with apoptotic nuclear features in mildly ischemic areas were PI-negative, suggesting that they still generated some energy and maintained membrane integrity. However, in severely ischemic areas, some of the PI-labeled cells also had a TUNEL-positive nucleus with apoptotic morphology. They did not look like the cells that lost their membrane integrity at the final stages of apoptosis[28] because their nuclei did not display advanced apoptotic changes. Interestingly, mixed phenotype was more common in transient ischemia compared with permanent ischemia, possibly because more cells had a chance to activate the apoptotic mechanisms before the collapse of energy metabolism and loss of membrane integrity in this

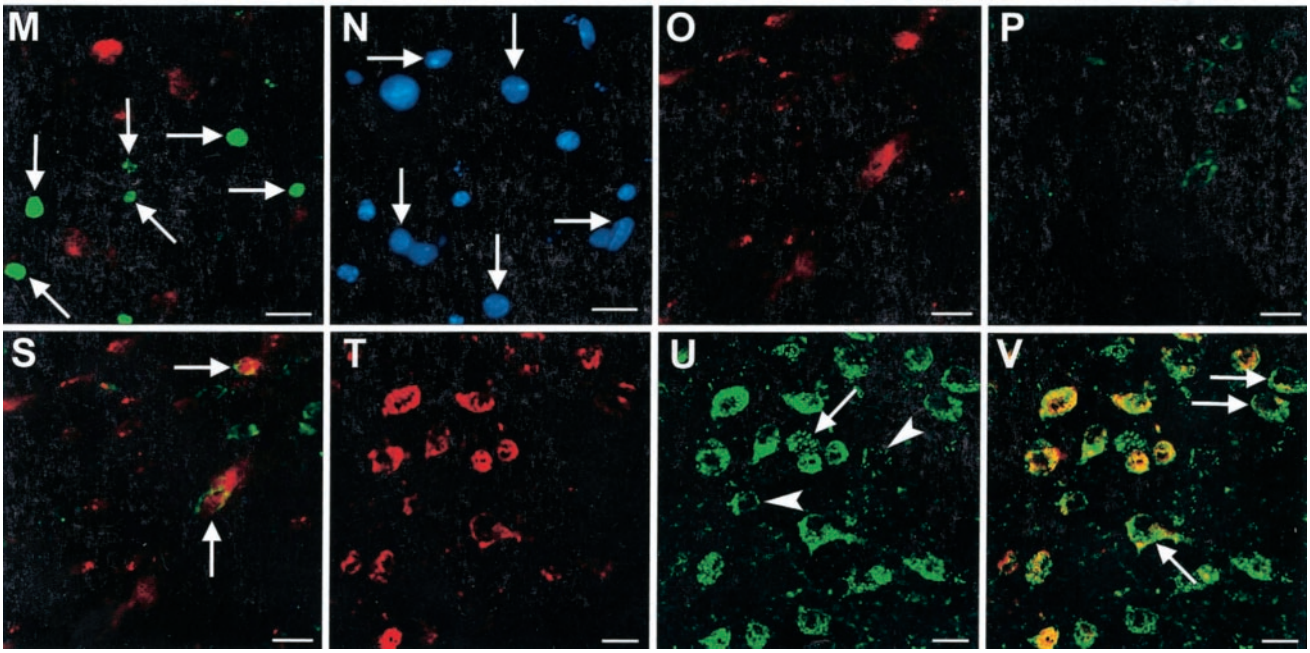

Figure 2 continued. In peri-infarct areas that were exposed to milder ischemic injury, TUNEL positivity (green) and PI labeling (red) were observed in separate cells 72 hours after 30-minute ischemia (M). TUNEL-positive nuclei were pyknotic or fragmented (M; arrows), whereas PI-positive cells had larger, triangular nuclei (N; arrows). N is the Hoechst-33258 staining (blue) of the same section in M. Colocalization of PI labeling (O; red) with caspase-cleaved actin immunoreactivity (P; green) was noticeable as early as 6 hours after 30-minute ischemia (S; arrows; core). Diffuse cytoplasmic cathepsin-B immunoreactivity (U; green) indicating lysosomal leakage was evident in ischemic cortical cells 4 hours after reperfusion following 1-hour ischemia. Note also cathepsin-B–immunoreactive fine (intact lysosomes; arrowheads) and enlarged granules (arrow). Merged images illustrate colocalization (V; yellow) of diffuse cathepsin-B and caspase-3-p20 (T; red) immunoreactivities. The overlap was partial in some cells (V; arrows). Bars=20 μm.

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milder form of ischemia. On the basis of these findings, one may conclude that rapid development of energy collapse in severely injured cells at the onset of ischemia leads to early membrane disruption as in necrosis, whereas apoptotic mechanisms continue to dismantle the irreparably damaged cells with residual ATP levels, and some of these cells may later develop necrotic features on total failure of energy metabolism.[4,29,30] The fact that fractin-positive cells outnumbered TUNEL-positive cells suggests that some neurons could not sustain caspase-3 activity beyond actin cleavage to induce DNA fragmentation and supports this possibility. In agreement with this idea, 27% of fractin-positive cells in the transient ischemia model did not display apoptotic nuclear morphology but displayed necrotic features, and fractin immunofluorescence was found to be colocalized with PI-labeled cells. Detection of caspase-mediated cleavage products is considered evidence for activation of the caspase cascade.[25,31] Therefore, our findings clearly indicate that caspase activity was present in cells that lost their plasma membrane integrity.

Interestingly, fractin-positive cells were colabeled with PI not only 72 hours but also 6 hours after ischemia. This latter finding suggests that PI specifically identified cells with necrotic-like membrane disruption rather than nonselectively labeling ruptured cells at final stages of various forms of cell death. Importantly, these observations also indicate that necrotic processes may be initiated soon after ischemia in ischemic cells that can sustain caspase activity. To evaluate the latter possibility, we studied the cathepsin-B degranulation conventionally associated with necrosis in cells displaying caspase-3 activity. We observed diffuse cytoplasmic cathepsin-B immunolabeling in ischemic cells, suggesting massive release of cathepsin-B after loss of lysosomal membrane integrity rather than (or in addition to) its leakage activated by apoptotic mechanisms.[10,18] Partial overlap in the same cell of caspase-3-p20 and cathepsin-B immunoreactivities supports this idea (a similar partial overlap was also observed with truncated Bid and cytochrome-*c* release in our ongoing studies). Extralysosomal cathepsin-B immunolabeling is in accord with a biochemical study reporting significant increases in released lysosomal enzymes in monkey brain 4 hours after MCA occlusion.[32] Like loss of plasma membrane integrity, lysosomal membrane disintegration also coexisted with caspase-3 activation in the same cell at early hours of ischemia. Cathepsin-B spilled into cytoplasm may contribute to development of necrotic features by digesting structural and functional proteins, whereas it reinforces apoptotic mechanisms such as Bid cleavage and caspase activation[18] that are also activated by several other factors in ischemic cells.[2–4]

In conclusion, in accordance with the mixed ultrastructural changes observed by electron microscopy,[9] ischemic neurons exhibit combined biochemical features of apoptotic and necrotic pathways in the same cell. Several cell death pathway subroutines involving caspases, cathepsins, and proteases such as calpains may concurrently be operative in ischemic cells and may facilitate each other's action.[10,11,20,27] Ischemic cell death phenotype is then determined by the relative speed of each process and the opposing action of

survival mechanisms. Consequently, ischemic cell death phenotype may display significant variation depending on the species, age, cell type, and coexisting conditions in the tissue.[20,27,33]

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