Delayed Neuronal Death in the CA1 Pyramidal Cell Layer of the Gerbil Hippocampus following Transient Ischemia Is Apoptosis

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The CA1 pyramidal neurons in the hippocampus are selectively vulnerable to transient ischemic damage. In experimental animals, the CA1 pyramidal neurons undergo cell death several days after brief forebrain ischemia. It remains, however, unknown whether this delayed neuronal death is necrosis or apoptosis. To investigate the degenerating processes of the CA1 pyramidal neurons in gerbil hippocampus after brief ischemia, lysosomal and nuclear alterations in the cells were examined using immunocytochemistry, in situ nick-end labeling, and Southern blotting. By light and electron microscopy, immunoreactivity for cathepsins B, H, and L, representative lysosomal cysteine proteinases, increased in the CA1 pyramidal neurons 3 d after ischemic insult, which showed cell shrinkage. By morphometric analysis, the volume density of cathepsin B-positive lysosomes markedly increased 3 d after ischemic insult, while that of autophagic vacuole-like structures also increased at this stage, suggesting that cathepsin B-immunopositive lysosomes increasing in the neurons after ischemic insult are mostly autolysosomes. Nuclei of the CA1 neurons were nick-end labeled by biotinylated dUTP mediated by terminal deoxytransferase 3 and 4 d after ischemic insult, but not in the prior stages. Simultaneously, dense chromatin masses appeared in nuclei of the neurons. By Southern blotting, laddering of DNA occurred only in CA1 hippocampal tissues obtained 4 d after ischemic insult. Confocal laser scanning microscopy demonstrated that the fragmented DNA in the CA1 pyramidal layer was phagocytosed by microglial cells. The results suggest that delayed death of the CA1 pyramidal neurons after brief ischemia is not necrotic but apoptotic.

[Key words: Mongolian gerbil, hippocampus, brief cerebral ischemia, autolysosomes, cysteine proteinases, DNA fragmentation, apoptosis]

The CA1 pyramidal neurons in the hippocampus have been shown to undergo selective cell death both in experimental animals and in humans after ischemic insult (Ito et al., 1975;

Pulsinelli and Brierley et al., 1979; Kirino, 1982; Kirino and Sano, 1984; Petito et al., 1987; Bonnekoh et al., 1990). Neuronal cell death, however, does not occur soon after ischemic insult, but the neurons survive several days. Physiological as well as biochemical analyses using animal models have demonstrated that the CA1 pyramidal neurons are normal 24 hr after brief forebrain ischemia (Suzuki et al., 1983a,b; Arai et al., 1986; Monmaur et al., 1986). The CA1 pyramidal neurons of gerbil hippocampus degenerate within 2 to 4 d after transient ischemia (Petito et al., 1987) and only 5.8% of the neurons survive 3 weeks later (Bonnekoh et al., 1990).

There has been no evidence whether this delayed neuronal death is necrosis or apoptosis. According to the morphological criteria of apoptosis, dying cells show chromatin condensation and cell shrinkage, followed by heterophagocytosis (Kerr et al., 1987; Clarke, 1990). Until the present, no study has reported that chromatin condensation appears in nuclei of the CA1 pyramidal neurons after ischemic impact. Microglial cells, however, invade the CA1 pyramidal layer, when the pyramidal neurons degenerate (Kirino and Sano, 1984). Moreover, in the course of the study on the delayed death of the CA1 pyramidal neurons after transient ischemia, we noticed that shrinkage of these neurons suddenly occurs 3 or 4 d after ischemic insult. These results suggest the possibility that delayed death of the CA1 pyramidal neurons after brief ischemia is not necrotic but apoptotic.

To determine whether the delayed neuronal death occurring in the CA1 pyramidal layer of gerbil hippocampus after brief ischemia is necrotic or apoptotic, we investigated the dying processes of CA1 pyramidal neurons with respect to cell shrinkage and nuclear changes. To detect the cytoplasmic changes, we immunocytochemically examined alterations in lysosomes, which have been shown to participate in the dying processes of some embryonic cells by forming autolysosomes (Clarke, 1990). Moreover, to detect nuclear changes, in situ nick-end labeling of biotinylated dUTP mediated by terminal deoxytransferase (TUNEL reaction) and Southern blot analysis were applied to the tissue. Participation of microglial cells in the degenerative processes of the CA1 neurons was also examined by specific staining of microglia.

Materials and Methods

Animals

Ninety-seven adult male Mongolian gerbils (12 weeks of age, ca. 70–100 gm) were used: 15 animals for the control group and the rest for the experimental groups.
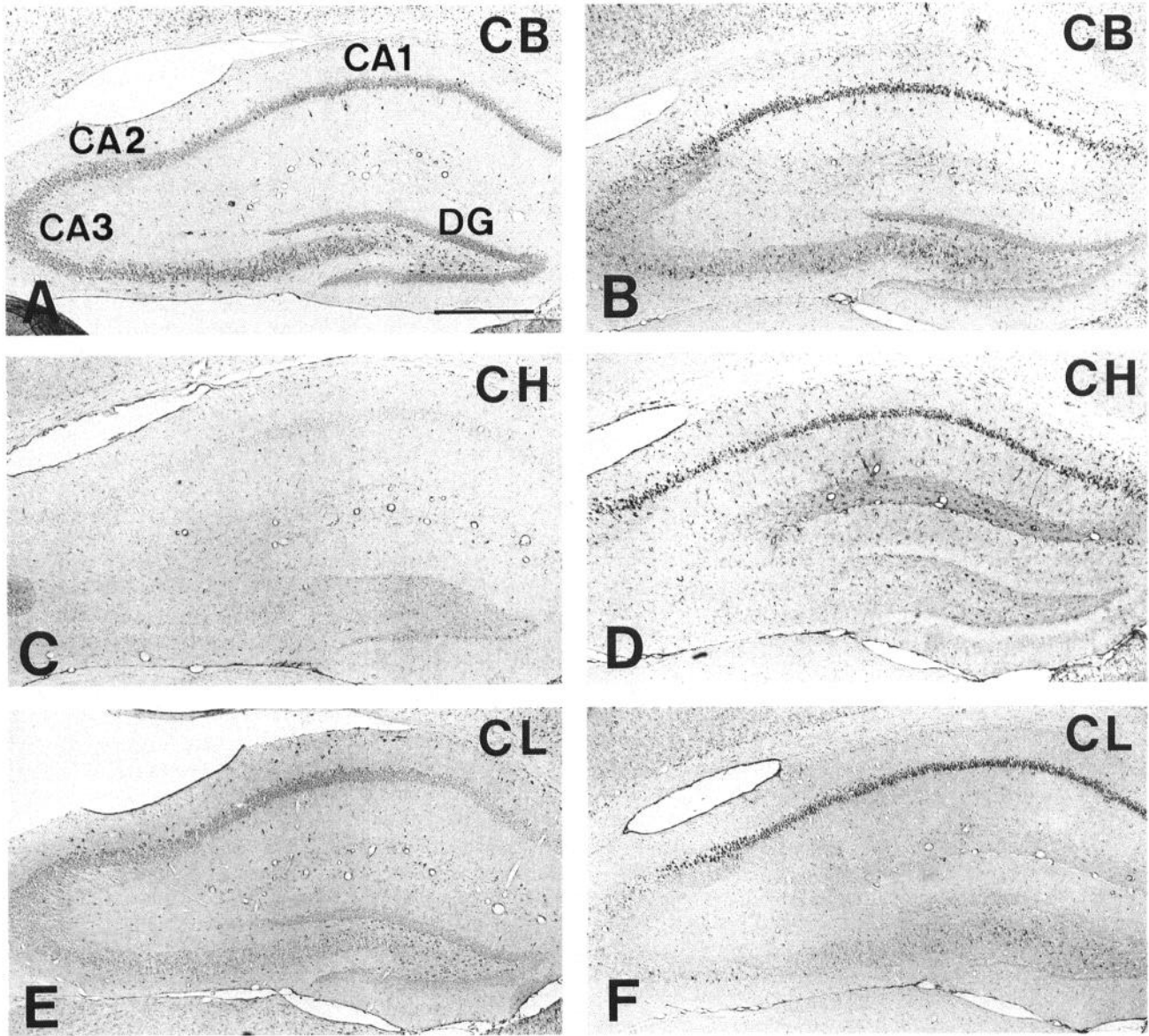
Figure 1. Changes in immunoreactivity for cathepsins B (*CB*), H (*CH*), and L (*CL*) in the hippocampus obtained from a sham-operated control gerbil brain (*A, C,* and *E*), and from gerbil brains 3 d (*B, D,* and *F*) after the bilateral occlusion of the common carotid arteries for 5 min. The immunoreactivity for cathepsins B and L appears in the pyramidal and granular layers of the control hippocampus (*CA1, CA2,* and *CA3*) and dentate gyrus (*DG*), respectively, while that for cathepsin H is detected in perivascular regions (*A, C,* and *E*). Three days after ischemic insult, the immunoreactivity for these three enzymes increases mainly in the CA1 pyramidal layer, but not in other regions (*B, D,* and *F*). Note that the immunoreactivity for cathepsins B and H also increases in perivascular regions (*B* and *D*). Scale bar, 500 μm

Antisera

Rabbit antibodies to rat cathepsins B, H, and L, were purified by affinity chromatography, as previously reported (Kominami et al., 1984, 1985; Bando et al., 1986). These antibodies were immunologically different from each other and showed no cross-reactivity (Kominami et al., 1984).

Surgical procedures

The bilateral common carotid arteries of gerbils under light ether anesthesia were exposed at the neck and were occluded with aneurysm clips for 5 min. The clips were then released to resume the normal blood flow in the forebrain. Gerbils operated on without occluding the carotid arteries were used as sham operated controls.

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Figure 2. Changes in immunoreactivity for cathepsins B (*CB*), H (*CH*), and L (*CL*) in the CA1 pyramidal layer of the hippocampus obtained from a sham-operated control gerbil brain (*A, E,* and *G*), and from gerbil brains 12 hr (*B*), 24 hr (*C*), and 3 d (*D, F,* and *H*) after the bilateral occlusion of the common carotid arteries for 5 min. The immunoreactivity for cathepsin B in pyramidal neurons after ischemic insult progressively increases, compared with that in the control (*A–D*). Particularly, coarse granular immunodeposits for these three enzymes are densely localized in the neurons 3 d after ischemic insults (*D, F,* and *H*). Phagocytic microglia-like cells showing the intense immunoreactivity appear in the pyramidal cell layer (*arrows*). Scale bar, 20 μm.
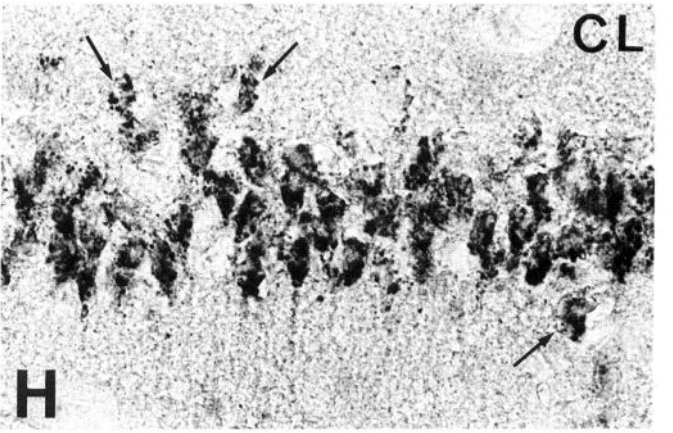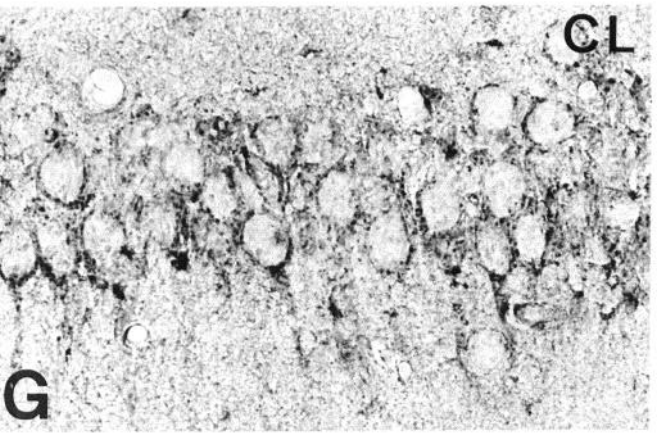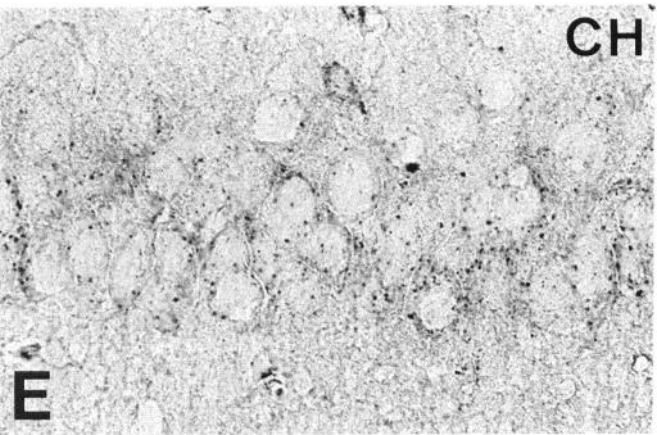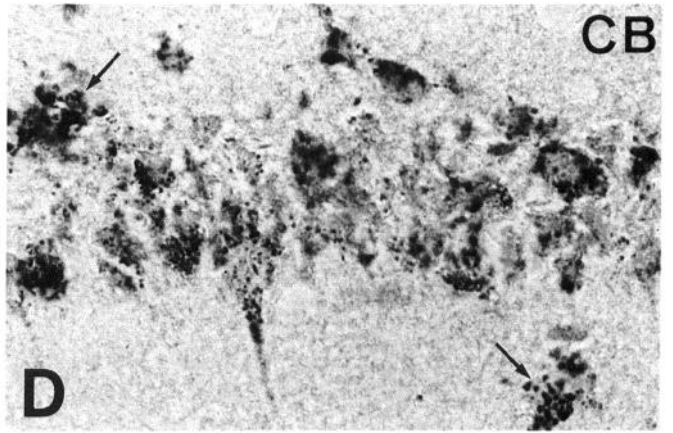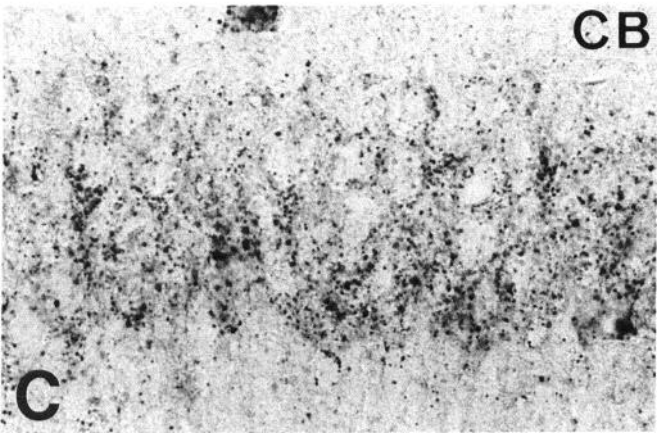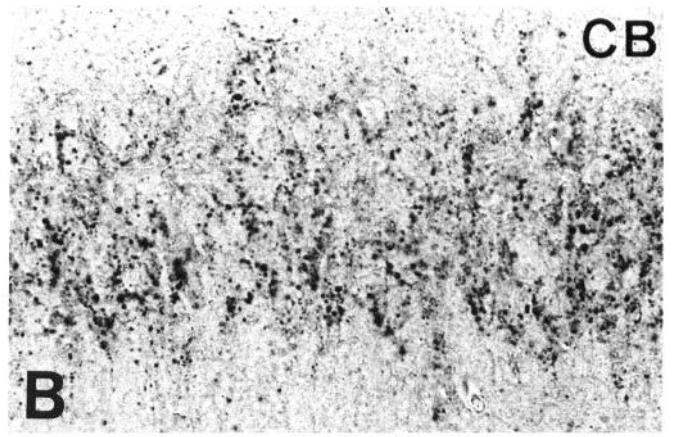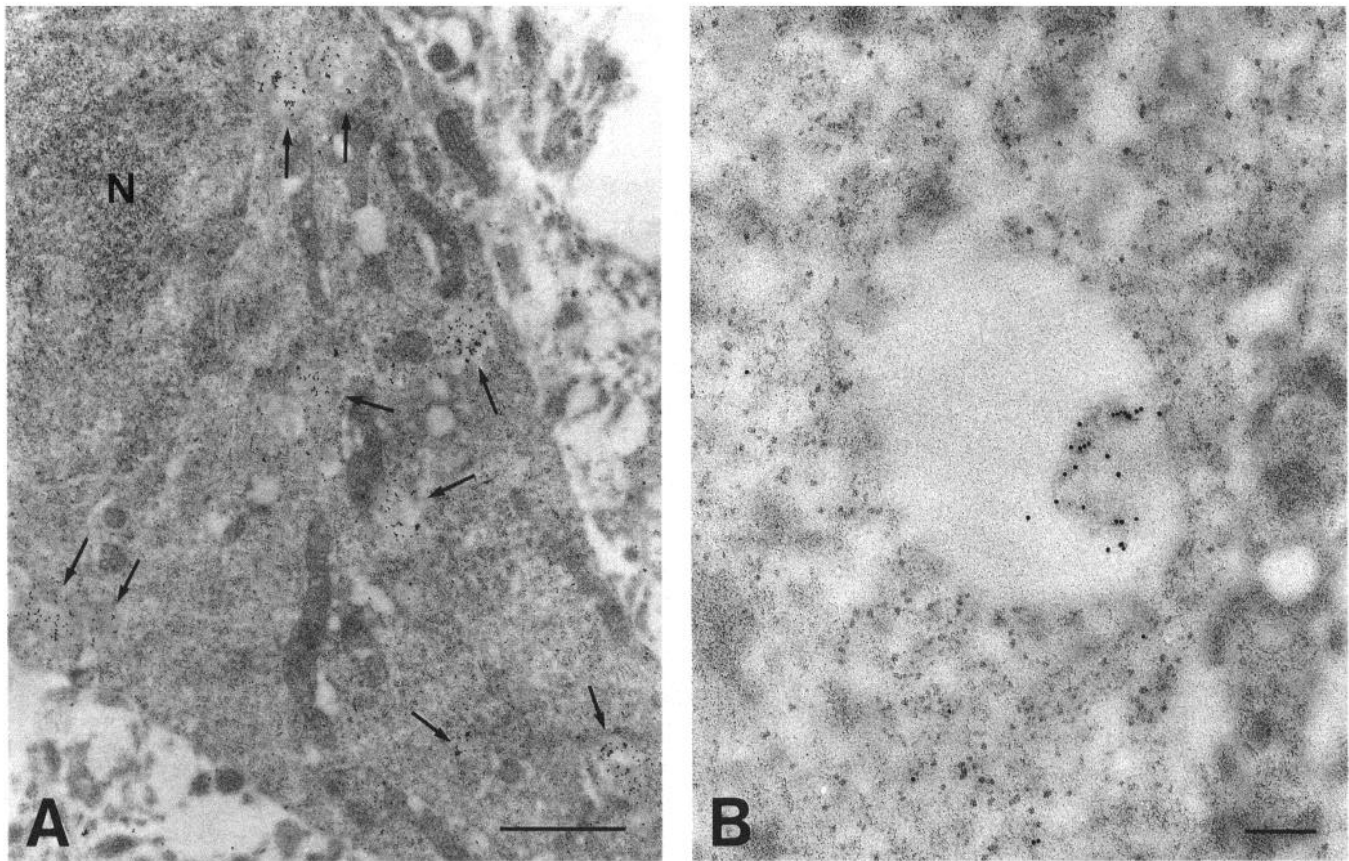
Figure 3. Immunocytochemical demonstration of cathepsin B in the CA1 pyramidal neurons 3 d after the bilateral occlusion of the common carotid arteries for 5 min. *A,* Immunogold particles indicating cathepsin B are localized in numerous lysosomes of the neuron (*arrows*). *B,* A vacuolar structure containing amorphous structures, where the immunogold particles are localized, can be seen in the perikarya of the neuron. *N,* nucleus. Scale bars: *A,* 1 μm; *B,* 0.2 μm.

Samplings for immunohisto/cytochemistry and electron microscopy

The sham-operated controls and ischemia-treated animals at 12 hr, and 1, 2, 3, 4, and 7 d after the resumption of the cerebral blood flow were deeply anesthetized with pentobarbital (25 mg/kg, i.p.) and fixed by cardiac perfusion with 4% paraformaldehyde buffered with 0.1 M cacodylate-HCl buffer containing 4% sucrose, pH 7.2, for light microscopic histochemistry and immunohistochemistry ($n = 5$ for each stage), with 4% paraformaldehyde-0.1% glutaraldehyde buffered with the same buffer for electron microscopic immunocytochemistry ($n = 3$ for each stage), or 2% paraformaldehyde-2% glutaraldehyde buffered with the same buffer for ordinal electron microscopy ($n = 3$ for each stage). The brains were then removed from each animal and cut coronally into about 3 mm-thick sections including the hippocampal area. For light microscopic histochemistry and immunohistochemistry, the samples were further immersed in the same fixative overnight at 4°C and embedded in OCT compound (Miles, Kankakee, IL) after cryoprotection with 15% and 30% sucrose solutions for cryosectioning. For electron microscopic immunocytochemistry, samples were dehydrated with graded alcohols and embedded in LR White at −20°C. For ordinal electron microscopy, samples were postfixed with 2% osmium tetroxide buffered with 0.1 M cacodylate-HCl, pH 7.2, dehydrated with graded alcohols, and embedded in Epon 812.

Immunohisto/cytochemistry

Light microscopy. Cryosections cut at 10 μm with a cryostat (Reichert-Jung, Frigocut 2700, Germany) were immunostained using a method fully described elsewhere (Uchiyama et al., 1990). Briefly, they were treated with 0.3% H_2O_2 in methanol for 30 min and incubated with 2% normal goat serum for 20 min at room temperature (RT). Then they were incubated with the following first antibodies for 3 d at 4°C: anti-cathepsin B (5 μg/ml), anti-cathepsin H (7 μg/ml), and anti-cathepsin L (3 μg/ml). Further incubations were performed with biotinylated goat anti-rabbit IgG (Histofine SAB-PO kit, Nichirei; Tokyo, Japan) and peroxidase-conjugated streptavidin (Nichirei) at RT for 20 min. After each step, sections were rinsed thoroughly in 0.1 M phosphate-buffered 0.5 M saline (pH 7.2), containing 0.1% Tween 20 (Sigma; St. Louis, MO). Staining for peroxidase was performed using 0.0125% diaminobenzidine (DAB) and 0.002% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) for 10 min. Immunostained sections were in some cases counterstained with hematoxylin.

Electron microscopy. Thin sections were cut with an ultramicrotome (Ultracut N, Reichert-Nissei, Tokyo, Japan) and mounted on nickel grids. After incubation with 5% normal goat serum (NGS) for 20 min at RT, the thin sections were incubated with anti-cathepsin B (11 μg/ml) at 4°C overnight. Then they were treated with gold-labeled goat anti-rabbit IgG for 1 hr; the gold particles were coated and their sizes (8 or 15 nm in diameter) adjusted according to the method of Slot and Geuze (1985). Between each step, the grids were rinsed in 0.02 M Tris-HCl buffered 0.5 M NaCl, pH 8.2, containing 0.1% bovine serum albumin (BSA; Sigma). After the immunoreactions, sections were stained with a saturated aqueous solution of uranyl acetate and lead citrate and observed with a Hitachi H-7100 electron microscope.

Control experiments. Control cryosections were incubated with anti-cathepsins B, H, and L adsorbed by rat liver cathepsins B, H, and L, respectively, or with a nonimmune rabbit serum diluted to 1:1000, followed by incubation with biotinylated goat anti-rabbit IgG and streptavidin-peroxidase. Some sections were directly incubated with the second antibody without any preceding incubation with the first antibodies. For immunoelectron microscopy, thin sections were incubated with the adsorbed antibody or with the nonimmune rabbit serum diluted to 1:200, followed by the gold-labeled second antibody. Some sections were directly incubated with the gold-labeled second antibody without pretreatment with the first antibody.
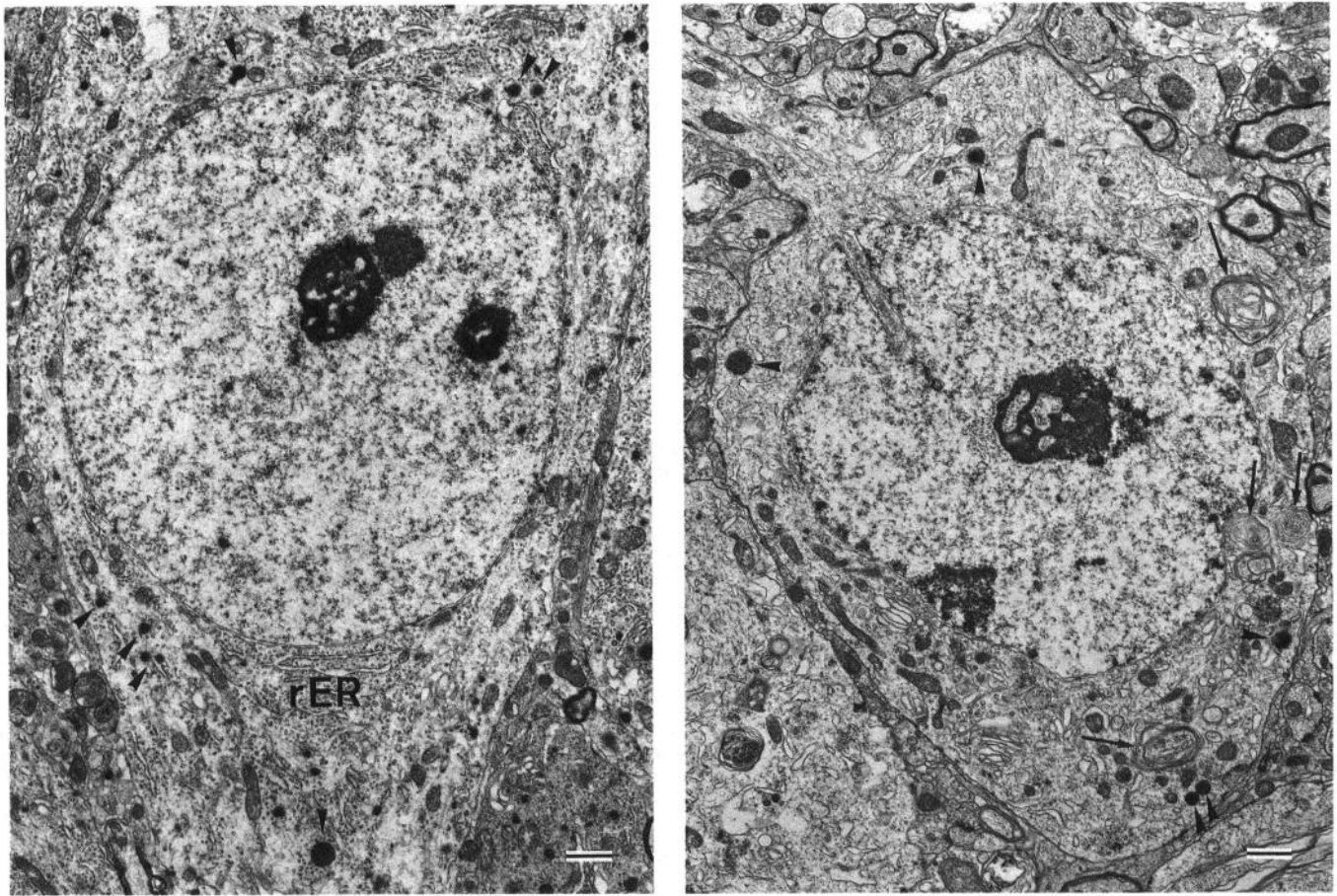
Figure 4. Electron micrographs of CA1 pyramidal neurons obtained from a sham-operated control gerbil brain (*A*) and from that 3 d (*B*) after the bilateral occlusion of the common carotid arteries for 5 min. The control neuron has a large round nucleus with the homogeneous karyoplasm and contains well-developed rough endoplasmic reticulum (*rER*) and several dense bodies (*arrowheads*) *A,* Three days after ischemic insult, the neuron has an indented nucleus with the homogeneous karyoplasm and contains dense bodies (*arrowheads*) and membrane-bounded vacuoles containing membranous structures and parts of the cytoplasm (*arrows*). *B,* Note that parallelly arranged rER disappears from the cytoplasm 3 d after ischemic insult, while mitochondrial profiles appear intact in both the control and ischemia-treated neurons. Scale bars, 1 μm.

All controls confirmed the specificities of immunoreactivity in light and electron microscopy.

Ordinal electron microscopy. Thin sections of the CA1 pyramidal layer embedded in Epon 812 were cut with an ultramicrotome. After stained with uranyl acetate and lead citrate, they were observed with an electron microscope.

Morphometry. To examine the volume densities of cathepsin B-immunopositive lysosomes, and dense bodies and autophagic vacuoles in the CA1 pyramidal neurons of the control and ischemia-treated gerbil brains, the point-counting method was applied to the immunoelectron and ordinal electron micrographs (Watanabe and Uchiyama, 1988). Thirty CA1 pyramidal fields of immunostained and ordinal sections obtained from the control group and ischemia-treated groups at 12 hr, 24 hr, and 3 d after ischemic insult were randomly chosen and photographed at the magnifications of × 5,000 for immunoelectron microscopy and × 3,500 for ordinal electron microscopy. The volume densities were analyzed at 10 times the original magnifications using a double-lattice system with a 1.5 cm-spacing (235 test points). Statistical analysis was performed using Student's t test.

DNA nick-end labeling. To detect DNA fragmentation in nuclei of the cells, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) reaction was applied to the cryosections according to the modified method of Gavrieli et al. (1992). Briefly, the cryosections were pretreated with or without 20 μg/ml proteinase K (Sigma) at RT for 15 min. After treatment with 0.3% H_2O_2 in methanol for 30 min, they were incubated with 100 U/ml TdT and 10 nmol/ml biotinylated 16-2'-dUTP (Boehringer-Mannheim-Yamanouchi, Osaka, Japan) in TdT buffer (100 mM sodium cacodylate, pH 7.0, 1 mM cobalt chloride, 50 μg/ml gelatin) in a humid atmosphere at

37°C for 60 min. Further incubation with peroxidase-conjugated streptavidin or Texas red-conjugated avidin (Nichirei) was carried out for 60 min at RT. Staining for peroxidase was performed as described above.

Griffonia lectin histochemistry. To detect microglial cells, the cryosections were also stained with 20 μg/ml *Griffonia simplicifolia* lectin-HRP or -FITC conjugate (Sigma) in 0.01 M phosphate-buffered 0.15 M saline (pH 7.2), containing 0.1% Triton X-100 at RT for 2 hr (Streit, 1990). Staining for peroxidase was performed as described above.

To examine the correlation of microglia with dying cells, double staining of *Griffonia* lectin and nick-end labeling of dUTP (Texas red labeling) was also carried out and viewed with a confocal laser scanning microscope (LSM-GB 200, Olympus, Tokyo, Japan).

Southern blotting. Hippocampal tissues including the CA1 zone were dissected from gerbil brains of the sham-operated controls and of those 2, 3, and 4 d after ischemic insult under a binocular microscope (*n* = 5 for each stage). Genomic DNA from each hippocampal tissue was prepared by a modification of the method of Sambrook et al. (1989). Each sample was subjected to electrophoresis on a 1.8% agarose gel and transferred to a nylon membrane (Sambrook et al., 1989). To detect the transferred DNA, the membrane was hybridized with ^{32}P-labeled EcoRI-digested gerbil brain DNA. The membrane was then subjected to autoradiography.

Results

Cytoplasmic changes

Immunohisto/cytochemistry. In the sham-operated gerbil brains, immunoreactivity for cathepsins B, H, and L was detected in
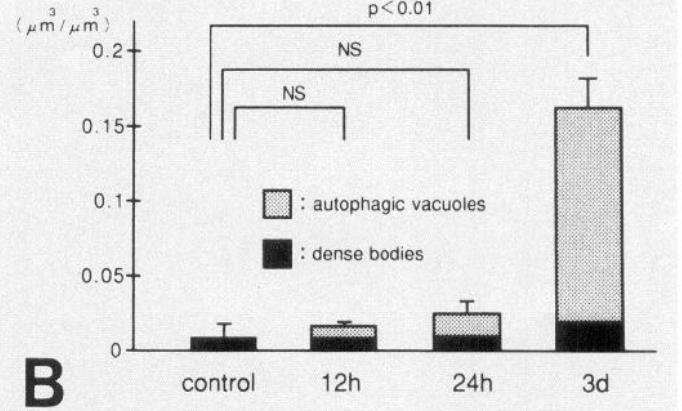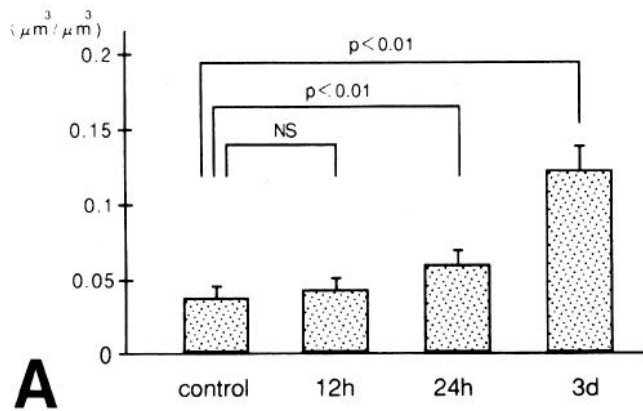
Figure 5. Changes in the volume densities ($\mu m^3/\mu m^3$) of cathepsin B-immunopositive lysosomes (*A*), and of dense bodies (*B*) and autophagic vacuole-like structures (*B*) in the CA1 pyramidal neurons of the hippocampus obtained from a sham-operated control gerbil brain, and from gerbil brains 12 hr, 24 hr, and 3 d after the bilateral occlusion of the common carotid arteries for 5 min.

the CA1, CA2, and CA3 pyramidal layers of the hippocampus, and in the granular layer of the dentate gyrus, although that for cathepsin H was faint or weak (Fig. 1*A,C,E*). The immunoreactivity for these cysteine proteinases was also found in the perivascular regions, especially that for cathepsins B and H. Three days after ischemic insult, the immunoreactivity for these enzymes was intensely elevated in the CA1 pyramidal layer, but not in other regions (Fig. 1*B,D,F*). The immunoreactivity for cathepsin H was also increased in the perivascular regions.

Granular immunodeposits for cathepsins B, H, and L were detected in the CA1 pyramidal neurons of the sham-operated control gerbil brains, but those for cathepsin H were less in number (Fig. 2*A,E,G*). The immunodeposits for these cysteine proteinases gradually increased and became coarse in the pyramidal neurons after ischemic insult (Fig. 2*B–D,F,H*). In particular, 3 d after ischemic insult, the CA1 pyramidal neurons, which became shrunken, were densely immunostained by anticathepsin B, H, or L (Fig. 2*D,F,H*). At this stage, microglial cells showing the immunoreactivity for these cysteine proteinases also appeared close to the CA1 pyramidal neurons (Figs. 2*D,F,H*; 9*B*).

By electron microscopy, immunogold particles indicating cathepsin B were localized in lysosomes of the neurons 3 d after ischemic insult (Fig. 3*A,B*). Large vacuoles containing dense amorphous materials, where immunogold particles showing cathepsin B were deposited, often appeared in the neurons at this stage (Fig. 3*B*).

Electron microscopy. As the immunoreactivity for cysteine proteinases increased in the CA1 pyramidal neurons 3 d after ischemic insult, when the profiles of the neurons were shrunken, we examined the ultrastructure of the neurons at this stage. The control CA1 pyramidal neurons possessed well-developed rough endoplasmic reticulum (rER), numerous polysomes, and several dense bodies in the perikarya (Fig. 4*A*). Three days after isch-

emic insult, profiles of rER and polysomes decreased from the perikarya of the neurons, whereas numerous dense bodies and membrane-bounded vacuoles containing membranous structures and parts of the cytoplasm, which seemed to be autophagic vacuoles, appeared in them (Fig. 4*B*). Profiles of mitochondria and the Golgi complexes appeared intact 3 d after ischemic insult.

To examine the correlation of cathepsin B-immunopositive lysosomes with dense bodies or autophagic vacuole-like structures appearing in the CA1 neurons after ischemic insult, we analyzed the volume densities of these structures using immunostained and ordinal electron microscopic sections. As shown in Figure 5*A*, the density of cathepsin B-immunopositive lysosomes significantly increased in the neurons 3 d after ischemic insult and was about 3.4 times higher than that of the control. The density of dense bodies did not significantly change in the neurons after ischemic insult, but that of autophagic vacuole-like structures clearly increased 3 d after ischemic insult (Fig. 5*B*). Distinct increases in the volume densities of both cathepsin B-immunopositive lysosomes and autophagic vacuole-like structures 3 d after ischemic insult suggest that most of the cathepsin B-immunopositive lysosomes are autolysosomes corresponding to membrane-bounded (autophagic) vacuoles.

Nuclear changes

TUNEL reaction. To detect nuclear changes in the CA1 pyramidal neurons after ischemic insult, we examined the hippocampal tissue by the TUNEL reaction. Positive staining of the TUNEL reaction was weakly detected in nuclei of the CA1 pyramidal neurons 3 d after ischemic insult, but not in those obtained in the earlier stages nor in the sham-operated control brains (Fig. 6). Four days after ischemic insult, the positive staining in the neurons was not only localized in their cell bodies, but also in their dendrites forming the molecular layer (Fig.

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Figure 6. TUNEL staining of the CA1 area of the hippocampus obtained from gerbil brains 2 d (*A* and *B*), 3 d (*C* and *D*), 4 d (*E* and *F*), and 7 d (*G* and *H*) after the bilateral occlusion of the common carotid arteries for 5 min. No staining is seen in the hippocampus (*A*) and the CA1 pyramidal neurons (*B*) 2 d after ischemic insult, whereas the positive staining specifically appears in the CA1 pyramidal layer 3 d (*C*), 4 d (*E*), and 7 d (*G*) after ischemic insult. Particularly, the positive staining is localized in the nuclei of the CA1 pyramidal neurons 3 d after ischemic insult (*D*), while it appears not only in their cell bodies but also in their dendritic processes forming the molecular layer on day 4. Positively stained neurons decrease in number 7 d after ischemic insult (*H*). Scale bars: *A, C, E,* and *F,* 500 μm; *B, D, F,* and *H,* 20 μm.
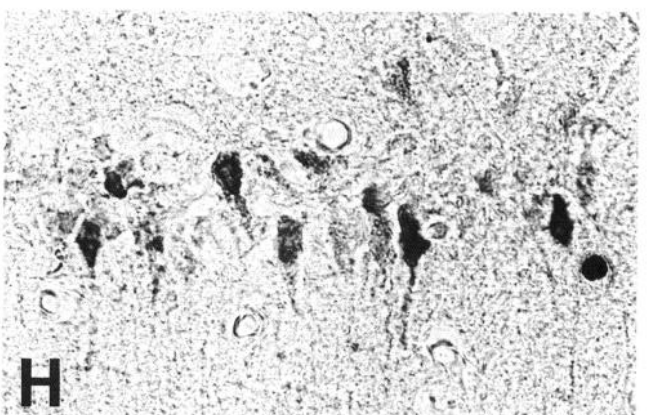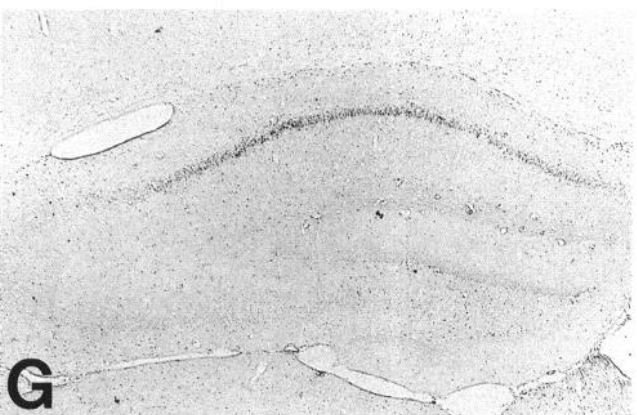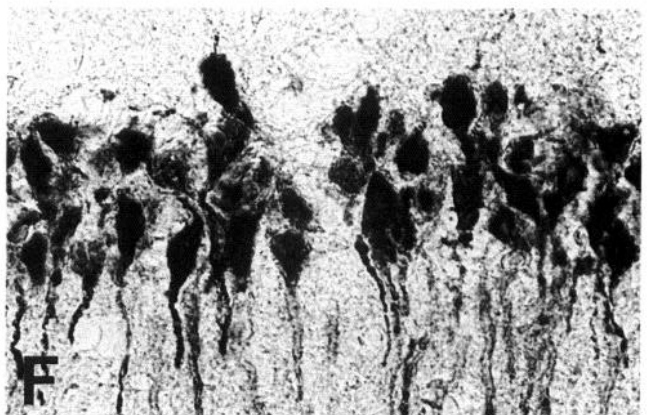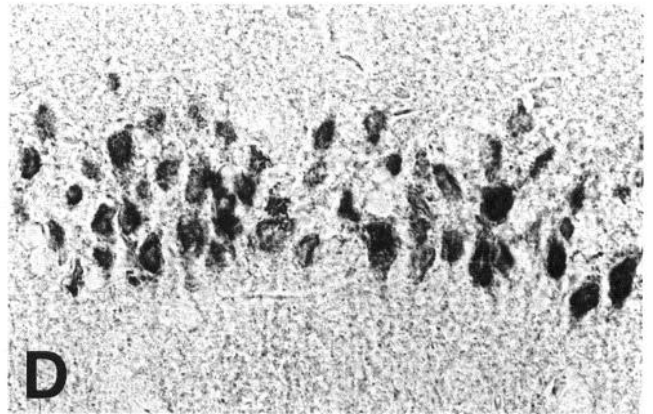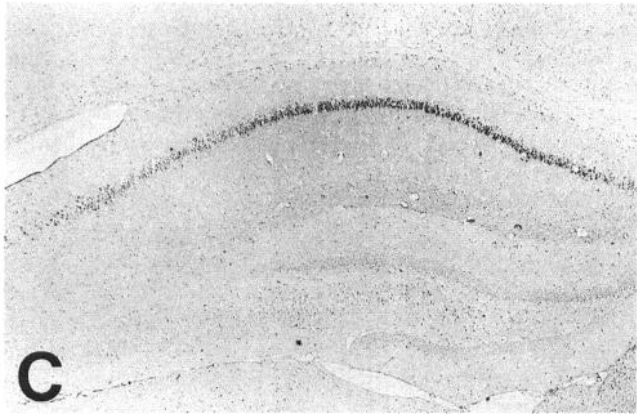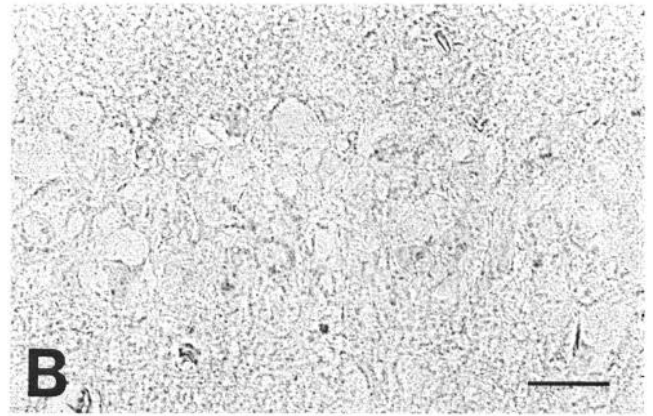
Figure 7. Southern blot analysis of hippocampal tissues containing the CA1 pyramidal layer obtained from a sham-operated control gerbil brain (*lane 1*), and gerbil brains 2 d (*lane 2*), 3 d (*lane 3*), and 4 d (*lane 4*) after the bilateral occlusion of the common carotid arteries for 5 min. DNA fragmentation into oligonucleosomal fragments appears only in the tissue 4 d after ischemic insult.

6*E,F*). The CA1 pyramidal neurons, having the positively stained nuclei, were detected even 7 d after ischemic impact, although the number of the neurons distinctly decreased in the CA1 pyramidal layer (Fig. 6*G,H*).

Electron microscopy. Electron microscopically, the CA1 pyramidal neurons of the control brains had large round nuclei with a homogeneous electron density (Fig. 4*A*). Three days after ischemic insult, distinct karyoplasmic alterations in the nuclei of the CA1 neurons were not detected, but their nuclear profiles were often indented (Fig. 4*B*). Four days later, the CA1 neurons contained irregularly-shaped nuclei with dense chromatin masses (Fig. 7*A*), while apoptotic bodies containing distinct chromatin condensation were often found near microglial cells located in the CA1 pyramidal layer (Fig. 7*B*). Moreover, elongated nuclei of the neurons extended into the dendritic processes with a relatively high electron density, which seemed to correspond with the positive staining of the TUNEL reaction in the molecular layer (Fig. 7*C*). These nuclear changes occurred concomitantly with perikaryal condensation; numerous autophagic vacuoles and vacuolar structures (clear vacuoles) appeared in the shrunken perikarya of the neurons (Fig. 7*A*).

Southern blot analysis. Since the nuclear changes in the CA1 neurons suddenly occurred 3 or 4 d after ischemic insult, we examined genomic DNA in hippocampal tissue including the CA1 region obtained from the control and ischemia-treated gerbil brains by Southern blotting. DNA fragmentation into oligonucleosomal fragments appeared only in the tissue 4 d after ischemic impact, but not in tissue at prior stages or in the control hippocampal tissue (Fig. 8).

Microglial reaction

We then examined the correlation of the dying neurons with microglial cells using *Griffonia* lectin histochemistry. *Griffonia* lectin-positive cells were not detected in the pyramidal layers of the hippocampus in the control brains, but they suddenly appeared in the CA1 pyramidal layer 3 d after ischemic insult (Fig. 9*A,B*). Using a confocal laser scanning microscope, double staining demonstrated that the positively stained nuclei by the TUNEL reaction were phagocytosed by *Griffonia* lectin-positive microglial cells (Fig. 9*C–E*).

Discussion

In the present study, the following perikaryal as well as nuclear alterations were demonstrated in the CA1 pyramidal neurons of gerbil hippocampus after brief ischemia: (1) perikaryal condensation with the formation of autophagic vacuoles (autolysosomes), (2) positive nick-end labeling of the nuclei, (3) dense chromatin masses in the nuclei and apoptotic bodies, (4) laddering of DNA, and (5) heterophagocytosis by microglial cells.

Cytoplasmic alterations of the CA1 pyramidal neurons after ischemic insult

By immunocytochemistry, cathepsins B and L are localized in the lysosomes of nerve cells and astrocytes in the rat CNS, while cathepsin H is only found in perivascular microglia-like cells (Bernstein et al., 1989, 1990; Taniguchi et al., 1993). The presence of these lysosomal cysteine proteinases in the CNS tissue has also been confirmed by enzyme immunoassay; the content of cathepsin B is high in the tissue, compared with that of cathepsins L and H (Kominami et al., 1985; Bando et al., 1986). In the present study, immunoreactivity for cathepsins B and L was clearly localized in nerve cells of the hippocampus of the sham-operated control gerbils, whereas that for cathepsin H was faintly detected in the CA1 pyramidal neurons and densely present in perivascular microglia-like cells of the control brains. Therefore, it seems likely that the amount of the cathepsin H protein, even if expressed in nerve cells of the CNS tissue, is immunohistochemically very low.

The present study demonstrated that the immunoreactivity for cathepsin H appears in the CA1 pyramidal neurons from the early stage after the ischemic insult, corresponding to the increase in the immunoreactivity for cathepsins B and L. It has been shown that pyramidal neurons in the gerbil hippocampus decrease uptake of radio-labeled L-tyrosine immediately after ischemic insult, but the uptake by the neurons mostly recovers within 24 hr after ischemic impact, except for that by the CA1 pyramidal neurons (Thilmann et al., 1986). Lysosomal cysteine proteinases degrade old, unneeded proteins within lysosomes of mammalian cells into amino acids, which are reused for protein synthesis in the cells (Kirschke et al., 1980; Katunuma and Kominami, 1983). It is interesting that the decrease in the uptake of amino acids in pyramidal neurons of gerbil hippocampus after brief ischemia occurs concomitantly with the increase in the immunoreactivity for lysosomal cysteine proteinases including cathepsin H.

Autophagy consisting of the sequestration of intracellular components and their degradation by lysosomal enzymes, usually occurs in normal cells to maintain cellular turnover, while it is known to greatly increase in cells under pathological conditions that cause cell dysfunction, such as hypoxia, ischemia, endotoxin shock, and metabolic inhibitors (Glaumann et al.,
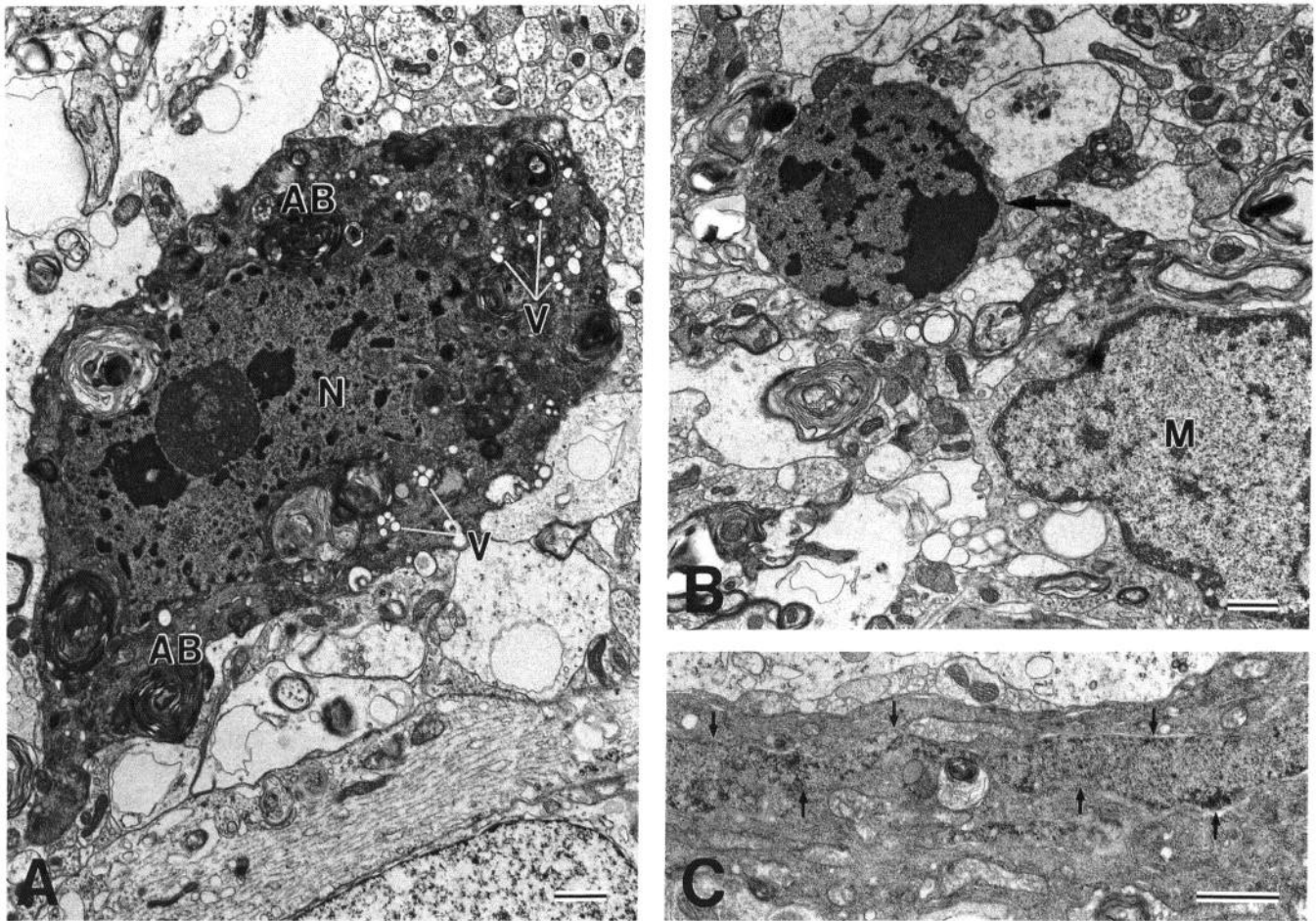
Figure 8. Electron micrographs of the CA1 pyramidal neurons 4 d after the bilateral occlusion of the common carotid arteries for 5 min. *A,* A pyramidal neuron contains an irregularly shaped nucleus (*N*) with condensed chromatin masses and shows karyoplasmic condensation with numerous vacuolar (*V*) and autophagic body-like (*AB*) structures. *B,* An apoptotic body (*arrow*) is seen near a microglial cell (*M*). *C,* Parts of an elongated nucleus (*arrows*) is seen in a dendritic process with a relatively high electron density. Scale bars, 1 μm.

1981). By electron microscopy, we found that membrane-bounded vacuoles containing membranous structures and parts of the cytoplasm appeared in the CA1 pyramidal neurons 3 d after ischemic insult, corresponding to increases in immuno-reactivity for cathepsin B in lysosomes. The present morpho-metric study indicated that cathepsin B-immunopositive lysosomes are mostly autolysosomes or autophagic vacuoles corresponding to membrane-bounded vacuoles. These events suggest that the degradation of proteins in lysosomes of the CA1 pyramidal neurons is highly activated after the ischemic insult and that this process proceeds by forming autolysosomes.

In the process of autophagic alterations frequently occurring in dying cells, the role of the autophagy is believed to protect the cells from death (Clarke, 1990). That is, lysosomes in the CA1 pyramidal neurons early in the stage after ischemic insult may serve as a source of amino acids by actively degrading proteins. Concerning this point, the increases in the immuno-reactivity for cysteine proteinases in the CA1 pyramidal neurons may play an important role in protecting them from further alterations. In contrast to the lysosomal reaction in the pyramidal neurons early in the stage after ischemic insult, cathepsin B-immunopositive autolysosomes or autophagic vacuoles rapidly increased 3 d later. In particular, the dying CA1 pyramidal neurons contained numerous autophagic vacuoles and vacuolar

structures (clear vacuoles) 4 d after ischemic insult when peri-karyal condensation occurred, although it remains unknown whether these autophagic vacuoles in the cells contain lysosomal enzymes. According to Clarke (1990), dying cells with auto-phagic vacuoles have been shown to undergo intense endocy-tosis, which can be thought of as inward blebbing and serves equally well to reduce the area of the plasma membranes. There-fore, it seems likely that the formation of autophagic vacuoles late in the stage after ischemic insult is closely associated with perikaryal condensation.

Nuclear alterations of the CA1 pyramidal neurons after ischemic insult

Gavrieli et al. (1992) have introduced the TUNEL reaction, by which nuclei of the dying cells associated with programmed cell death are positively stained. It is interesting that the nuclei of intestinal epithelial cells located in the tip of the villi are pos-itively stained by the TUNEL reaction (Gavrieli et al., 1992), although no apoptotic cells are morphologically seen at the villi tips (Kerr et al., 1987). In the present study, we found the pos-itive staining of the TUNEL reaction even in nuclei of the isch-emia-damaged CA1 pyramidal neurons. Moreover, this positive reaction occurred in the nuclei prior to the morphological al-terations with chromatin condensation. These results suggest
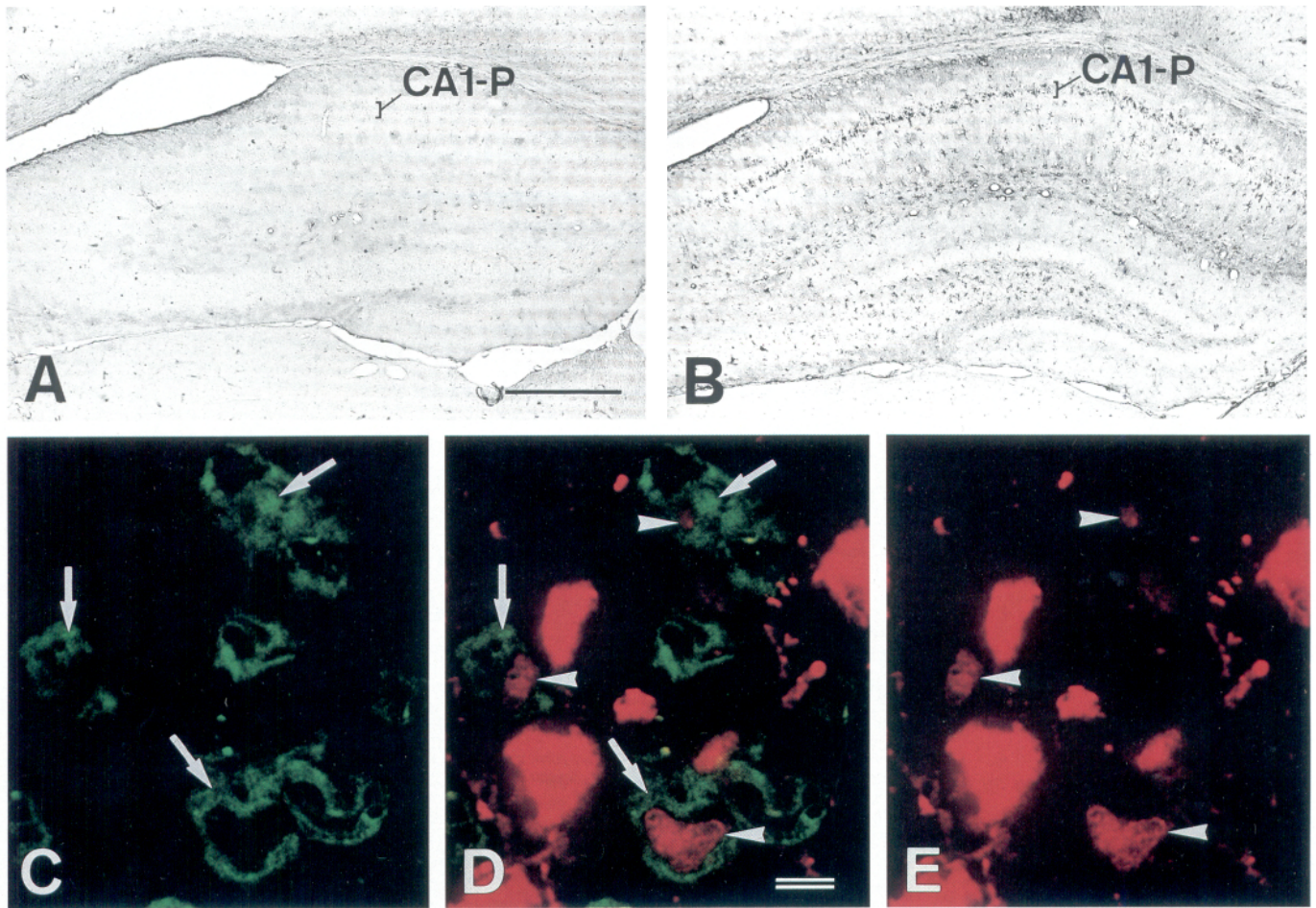
Figure 9. Demonstration of *Griffonia* lectin-positive microglial cells in the CA1 area of gerbil hippocampus. *A* and *B,* No positive staining of *Griffonia* lectin is seen in the CA1 pyramidal layer (*CA1-P*) of the control gerbil brain (*A*), whereas its positive staining is detected in the layer 3 d after ischemic insult (*B*). *C–E,* By a confocal laser scanning microscope, *Griffonia* lectin-positive microglial cells (*green*) (*arrows*) contain nuclei stained by the TUNEL reaction (*red*) (*arrowheads*) in the CA1 pyramidal layer 4 d after ischemic insult (*D*). *Griffonia* lectin-positive cells are shown in *C,* while nuclei stained by the TUNEL reaction are displayed in *E.* Corresponding cells and nuclei are also indicated by *arrows and arrowheads in C and E.* Scale bars: *A* and *B,* 500 μm; *C–E,* 5 μm.

that the TUNEL reaction more sensitively detects the nuclear changes with DNA fragmentation than morphological approaches do.

Four days after ischemic insult, the positive staining of the TUNEL reaction in nuclei of the CA1 pyramidal neurons occurred concomitantly with electron microscopic alterations: dense chromatin masses in their nuclei and apoptotic bodies with nuclei showing dense chromatin condensation in the pyramidal layer. Moreover, parts of nuclei spreading into dendrites of the CA1 neurons were detected by both the TUNEL reaction and electron microscopic analysis. By Southern blot analysis, we also found DNA fragmentation into oligonucleosomal fragments. These findings are consistent with morphological and biochemical criteria of apoptosis (Kerr et al., 1987; Clarke, 1990), indicating that the delayed neuronal death in the CA1 pyramidal layer after brief ischemia is apoptosis.

As far as we observed, no nuclear change occurred in the CA1 pyramidal neurons until 48 hr after ischemic insult, suggesting that the delayed neuronal death requires 3 to 4 d after ischemic impact, if nuclear changes play a key role in cell death. Until the present, various morphological, physiological, and biochemical changes have been noted in the CA1 pyramidal neurons

after brief ischemia (Ito et al., 1975; Pulsinelli and Brierley, 1979; Kirino, 1982; Suzuki et al., 1983a,b; Kirino and Sano, 1984; Arai et al., 1986; Monmaur et al., 1986; Petito et al., 1987; Bonnekoh et al., 1990); however, there has been no accurate definition as to what reversible or irreversible alterations are in the neurons after brief ischemia. The *in vitro* study of neonatal sympathetic neurons has shown that the decrease in protein synthesis of the cultured neurons after nerve growth factor deprivation is insufficient to cause subsequent death, whereas DNA fragmentation of the neurons occurs close to the neuronal death (Deckwerth and Johnson, 1993). This implies that DNA fragmentation in the CA1 pyramidal neurons after brief ischemia is linked to irreversible alterations of the neurons.

Microglial reaction

Microglial cells have been shown to invade into the CA1 pyramidal layer after ischemic insult (Kirino, 1982). We also found *Griffonia* lectin-positive microglial cells in the CA1 pyramidal layer 3 d after ischemic insult, which phagocytosed nerve cell debris containing nuclei showing DNA fragmentation on day 4. Heterophagy has been shown to be prominent, when cells die by apoptosis (Kerr et al., 1987; Clarke, 1990). The results may

indicate that the delayed neuronal death occurring in the CA1 pyramidal layer after brief ischemia is apoptosis.

From the present results, we concluded that the delayed neuronal death occurring in the CA1 pyramidal layer after brief ischemia is apoptosis. Considering prevention of neuronal death, it is important that initial disruption of Ca^{2+} homeostasis in the CA1 pyramidal neurons by transient ischemia may cause neurotoxicity via mechanisms triggered by Ca^{2+} overload (Choi, 1988). In fact, in vivo and in vitro studies have indicated that Ca^{2+} channel blockers, glutamate receptor antagonists, or Ca^{2+} chelators prevent neuronal death from excitotoxic and ischemic neuronal injury (Rothman, 1984; Rothman and Olney, 1987; Sheardown et al., 1990; Buchan et al., 1991; Nellgård and Wieloch, 1992). In addition to these events, the present results displaying the apoptotic death of the CA1 neurons after brief ischemia may suggest further therapeutic interventions to prevent neuronal death.

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