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Cellular Accumulation of Extravasated Serum Protein and DNA Fragmentation Following Vasogenic Edema

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ABSTRACT

Accumulation of serum protein has been demonstrated in injured brain cells following vasogenic brain edema. The present study was conducted to test whether this phenomenon is also observed in apoptotic cells as well as in necrotic cells. Apoptotic cell death has been implicated in a variety of brain injuries, including ischemia and trauma. Cold injury and focal cerebral ischemia-reperfusion were used to induce both vasogenic edema and apoptotic cell death. Evans blue extravasation was used to determine the cellular accumulation of serum albumin. Apoptotic cell death was evaluated by both morphological alterations and by terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end labeling (TUNEL) staining. Evans blue accumulation in cells was observed not only in the surrounding zone of the lesion after cold injury and in the entire ischemic area after focal ischemia, but was also detected in the regions remote from the primary injury site. Some of these cells demonstrated nuclei fragmentation. TUNEL staining confirmed that apoptosis was induced in the region where apoptotic cells were morphologically detected. These observations suggest that accumulation of the extravasated serum component is accompanied by apoptotic cell death following vasogenic brain edema.

Key words: blood-brain barrier; cerebral ischemia; cold injury; DNA fragmentation; serum protein; TUNEL staining; vasogenic brain edema

INTRODUCTION

BRAIN EDEMA is a pathophysiological condition coexisting with a variety of brain injuries, including ischemia, hemorrhage, trauma, infection, and brain tumor. Vasogenic brain edema is one of the subtypes of brain edema and results from disruption of the blood-brain barrier (BBB), causing extravasation of macromolecules such as serum proteins (Fishman, 1975; Klatzo et al., 1958). There is no direct evidence that disruption of the BBB in itself can cause cell injury. However, it has been demonstrated that mild traumatic brain injury, causing prominent vasogenic edema with minimal hemorrhage, induces expression of heat shock protein as evidence of cell stress in the site corresponding to the regional pattern of the BBB disruption (Tanno et al., 1993). As a consequence of the BBB disruption, serum proteins are extravasated not only to extracellular space, but also are localized to the cytoplasmic compartment of brain cells even in the regions remote from the focus of vasogenic brain edema following cold injury, administration of hyperosmotic agents, and transient hypertension (Bright-

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man et al., 1970; Loberg and Torvik, 1991; Povlishock et al., 1979; Sokrab et al., 1988; Tanno et al., 1992; Tengvar, 1986; Tengvar and Olsson, 1982). The serum accumulates in the injured cells at the site or surrounding area of the primary lesion. However, uptake of the extravasated serum proteins has also been noted in the intact cells distant from the primary lesion, and this is caused by the retrograde transport of axons passing through the lesion. These observations indicate that the brain may be affected by a specific substance originating from the spreading edema fluid, even in the region distant from the focal BBB disruption.

Apoptosis is an active mode of cell death, exhibiting morphologically and biochemically characteristic features, including cell shrinkage, chromatin condensation, apoptotic bodies, and double-strand DNA breaks into multiples of 180 bp, which shows characteristics of the DNA laddering pattern in DNA electrophoresis (Arends et al., 1990; Gavrieli et al., 1992; Kerr et al., 1972; Wyllie et al., 1984). Recent evidence has demonstrated that apoptotic cells maintain integrity of mitochondrial function and plasma membrane, which are lost in necrotic cells (Darzynkiewicz et al., 1992; Lizard et al., 1995). Apoptotic cell death has been known to be induced in the central nervous system (CNS), not only in the embryonic development process, but also in various CNS injuries, including ischemia, trauma, and excitotoxicity (Li et al., 1995; Linnik et al., 1995; MacManus et al., 1993; Nitatori et al., 1995; Pollard et al., 1994; Rink et al., 1995).

We hypothesized that if cellular accumulation of extravasated serum proteins results from impairment of plasma membrane integrity, the serum proteins would not be localized to the cytoplasmic compartment of injured brain cells. We are interested in the possible relationship of apoptosis induction and cellular accumulation of serum proteins following vasogenic brain edema. The purpose of the present study was to determine whether cellular accumulation of extravasated serum protein is accompanied by apoptotic cell death, as well as necrotic cells.

MATERIALS AND METHODS

Induction of Vasogenic Brain Edema

Both cold injury and focal cerebral ischemia-reperfusion were used to induce both vasogenic brain edema and apoptotic cell death. CD-1 mice (3-month-old males, 35–45 g; five each) were subjected to both injuries.

Cold injury was induced as previously described with minor modifications (Chan et al., 1991; Murakami et al., 1997b). Animals were anesthetized with chloral hydrate (350 mg/kg) and xylazine (4 mg/kg) and were placed in a stereotaxic frame. A cold metal probe, 4 mm in diameter, cooled with dry ice, was applied to the right parietal skull with a force of 300 *g* for 1 min.

Focal cerebral ischemia-reperfusion was induced by intraluminal middle cerebral artery occlusion (MCAO) (Yang et al., 1994). Animals were anesthetized with 2.0% isoflurane in 30% O_2 and 70% N_2O. The skin was incised on the midline of the ventral neck. The left external carotid artery was exposed and its branches were electrocoagulated. An 11-mm 5-0 monofilament nylon suture (Dermaron, Davis + Geck, Manati, Puerto Rico) was introduced from the left external carotid artery stump into the left internal carotid artery. The common carotid artery was also occluded using a surgical clip. After 1-h MCAO, the clip and suture were removed to restore blood flow.

In both the cold injury and focal ischemia-reperfusion models, rectal temperature was monitored and maintained at 37.0 ± 0.5°C with a homeothermic blanket (Harvard Apparatus, South Natick, MA) and a heating pad; brain temperature was not monitored.

Immediately after the cold injury or restoration of blood flow, 0.1 ml of 4.0% Evans blue (Sigma, St. Louis, MO) in saline was administered i.v. to assess the extravasation of serum albumin.

Evans blue solution was injected into experimental animals as a control group. We also studied the effect of osmotic BBB opening as an additional control group in which Evans blue solution was injected after intraarterial administration of mannitol. In these groups, a microcatheter (PE-10) was cannulated into the left common carotid artery, and 0.1 ml of 25% mannitol was administered; then the Evans blue solution was injected i.v.

Fluoromicroscopic Evaluation of Morphology and Evans Blue Accumulation in Damaged Cells

Morphological features of damaged cells and cellular accumulation of the extravasated Evans blue were determined by fluorescent microscopy.

Experimental mice were anesthetized with ketamine (200 mg/kg) and xylazine (10 mg/kg) and sacrificed by transcardial perfusion fixation at 24 h after the onset of cold injury or focal ischemia-reperfusion. Perfusion fixation was performed with 200 ml of 10 U/ml heparin in saline and 10% formaldehyde in phosphate-buffered saline (PBS). Brains were removed and stored in 3.7% formaldehyde overnight. Then, the brains were cut using a vibratome, and the slices were put onto slide glass. Sections were dried at room temperature in a dark box, and were then stained with hematoxylin and eosin (H&E) to determine the detailed histological findings and observed using a light microscope.

Adjacent sections from the same brain were subjected to fluoromicroscopic evaluation of Evans blue leakage

and stained with 2.5×10^{-1} μg/ml Hoechst 33258 (Molecular Probes, Eugene, OR) in PBS for nuclear staining. After rinsing in ddH_2O, the sections were mounted with glycerol. These sections were observed using a microscope (Axioplan, Zeiss, Germany) under fluorescent light (HBO W/2, Zeiss). Nuclear staining with Hoechst 33258 and the localization of the extravasated Evans blue were observed at excitation 355 nm, emission > 415 nm, and at excitation 510–550 nm, emission > 580 nm, respectively. Photomicrographs of these fluoromicroscopic findings were taken by double exposure to nuclear staining and Evans blue, and the cells with fragmented nuclei or cellular uptake of Evans blue were quantitatively evaluated on the photographs. These cells were counted in the area surrounding the cold injury and the medial striatum in the MCAO.

In Situ *Labeling of DNA Fragmentation*

To confirm that the cells that morphologically exhibit fragmented nuclei were, in fact, undergoing the apoptotic process, free 3′-OH end labeling was studied by termi-

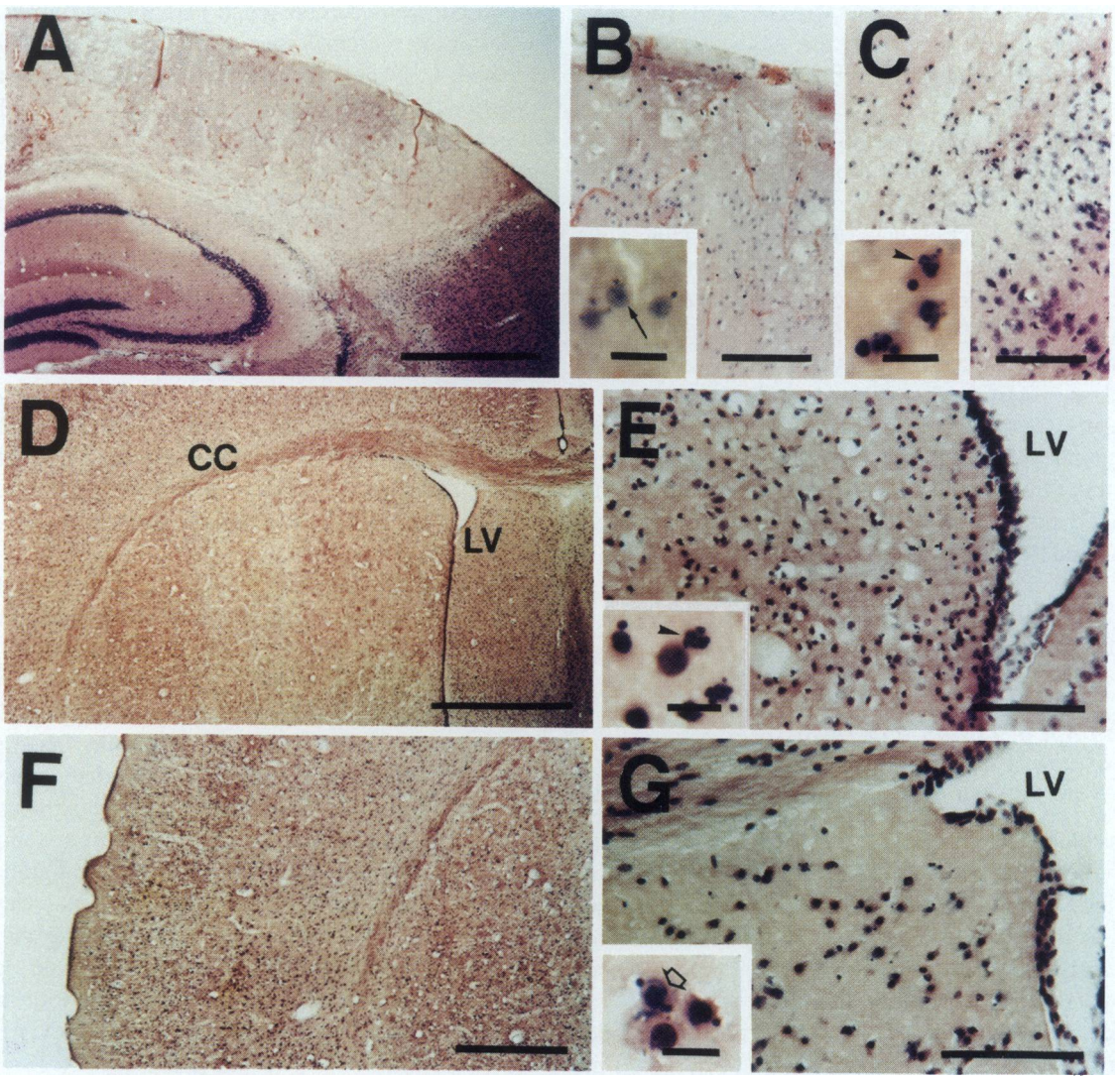


FIG. 1. Photomicrographs of H&E-stained sections showing histological changes following cold injury (A–C) and focal cerebral ischemia-reperfusion (D–G). The cold injury lesion was clearly defined in the frontoparietal cortex and penetrated the external capsule at 24 h after the cold injury (A). Brain cells exhibited oval-shaped nuclei with decreased hematoxylin stainability in the core of the lesion (B, arrow). Some cells demonstrated fragmented nuclei or small apoptotic bodies in the surrounding zone (C, arrowhead). The ischemic area involved the entire striatum and MCA territory cortex (D,F). Cells with nuclei fragmentation were frequently observed in the inner boundary zone (E, arrowhead). Some cells also showed fragmentation of the lateral septal nucleus in the contralateral hemisphere (G, open arrow). CC, corpus callosum; LV, lateral ventricle. Bar = 1,000 μm (A,D,F), 100 μm (B,C,E), and 10 μm (magnified photomicrographs in B,C,E,G).

nal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end labeling (TUNEL) staining.

Brain samples were separately prepared by the following procedures. The experimental animals were anesthetized with an overdose of methoxyflurane and were sacrificed by decapitation at 24 h after onset of cold injury or focal ischemia-reperfusion. The brains were rapidly removed and frozen in −20°C 2-methyl-butane. Brain sections, 20 μm in thickness, were taken using a cryostat and stored at −80°C.

Frozen brain sections were fixed with 3.7% formaldehyde for 45 min. Endogenous peroxidase was inactivated with 60 mM hydrogen peroxide and 100 mM sodium azide for 30 min. After the slides were washed with PBS, they were immersed in terminal deoxynucleotidyl transferase (TdT) buffer (Gibco BRL, Gaithersburg, MD) at room temperature for 15 min and incubated with TdT and biotin-16-uridine-5′-triphosphate (Boehringer Mannheim, Indianapolis, IN) at 37°C for 60 min. The reaction was stopped by washing with 6 mM sodium citrate and 60 mM sodium chloride for 30 min. Then the slides were incubated with 2% bovine serum albumin in PBS. After washing with PBS, the sections were incubated with avidin-biotin horseradish peroxidase (ABC kit, Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature, and staining was visualized with 3 mM 3,3′-diaminobenzidine tetrahydrochloride and 18 mM hydrogen peroxide in PBS. These sections were also stained with methyl green for nuclear staining.

RESULTS

Localization of Lesion and Evans Blue Extravasation Following Cold Injury and Focal Ischemia-Reperfusion

Evans blue is bound to serum albumin immediately after i.v. injection (Wolman et al., 1981). In the present study, intravascular Evans blue was washed out at the time of sacrifice. Therefore, Evans blue fluorescence observed in the brain parenchyma indicates extravasated and retained serum albumin in the brain tissue.

In the control study group without the cold injury and ischemia, no histopathological change was observed in H&E staining. Furthermore, fluorescence of extravasated Evans blue was not detected, indicating that Evans blue–labeled albumin did not leak into the brain parenchyma and that the BBB was not disrupted. In the mannitol-treated group, however, mortality was unexpectedly high (50%). Histopathology demonstrated a massive ischemic infarct in the left hemisphere, indicating that the occlusion of the left common carotid artery for cannulation is critical for inducing ischemic injury to the ipsilateral hemisphere. Therefore, in this group, the hyperosmotic BBB opening effected the histopathology. Evans blue leakage, and intracellular uptake, were not evaluated. The primary lesion observed after the cold injury was clearly defined and localized mainly in the parietal cortex, and it penetrated the external capsule and reached the hippocampus in the ipsilateral hemisphere (Fig. 1A). Furthermore, this method of cold injury induced prominent vasogenic brain edema, as has been well established and characterized by many other investigators. Some cells demonstrated piknotic and fragmented nuclei in the marginal zone of the lesion, while the nuclei had an oval shape with decreased stainability for hematoxylin in the core of the lesion (Fig. 1B,C). Evans blue fluorescent signal showing the extent of edema fluid was intense and clearly defined the lesion corresponding to the lesion observed in H&E staining. Extravasated Evans blue was also found to have migrated to the contralateral hemisphere along the corpus callosum (Fig. 2C). Since intense fluorescence of Evans blue was not detected in the cingulate gyrus of the contralateral hemisphere where the more intense cold injury might be induced than at the corpus callosum, Evans blue observed in the septal nucleus or striatum of the contralateral hemisphere is caused not by direct disruption of the BBB following cold injury, but, in fact, by the migration of extravasated serum albumin from the primary lesion in the ipsilateral cortex.

On the other hand, the ischemic infarction was localized throughout the entire MCA territory, including the striatum and cortex (Fig. 1D,F). Injured cells with fragmented nuclei were frequently observed in the medial striatum adjacent to the lateral ventricle (Fig. 1E). Furthermore, some of these cells were also dispersed in the septal nucleus adjacent to the lateral ventricle in the contralateral hemisphere (Fig. 1G). Evans blue extravasation was relatively mild even in the ischemic core compared with that in the cold injury (Fig. 3C), but migration of edema fluid containing extravasated Evans blue was also observed along the corpus callosum, reaching the contralateral hemisphere. Cells with Evans blue accumulation were observed to be dispersed throughout the ischemic area and some of these cells were found even in the contralateral hemisphere (Fig. 3D).

Fluoromicroscopic Evaluation of Evans Blue Accumulation and Apoptosis Morphology

Cellular accumulation of the Evans blue and morphological features of these cells were evaluated by fluoromicroscopy. Since cold injury induced such massive

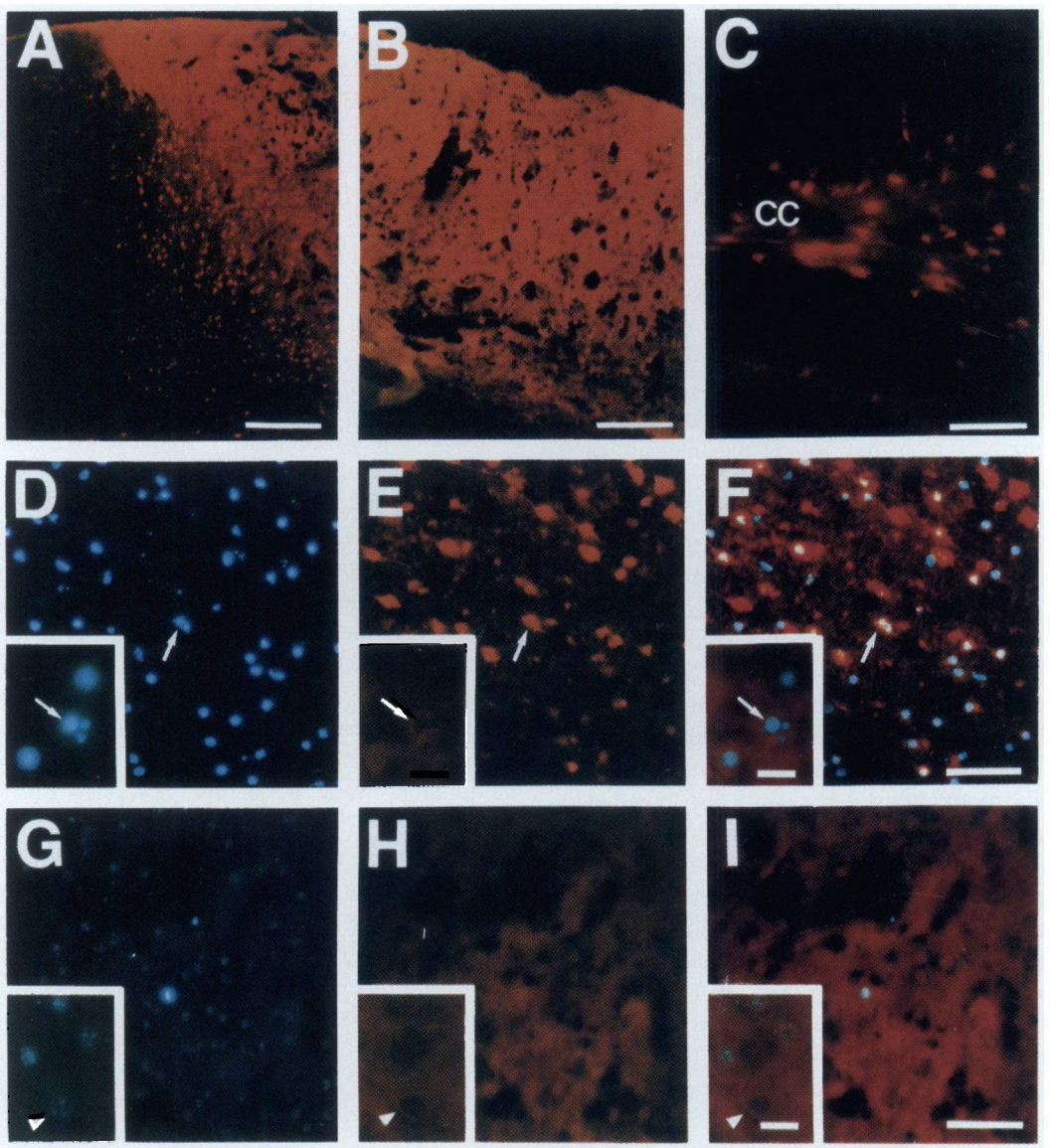


FIG. 2. Fluorescent photomicrographs showing extravasation and cellular accumulation of Evans blue following cold injury. The cold lesions demonstrated extremely intense red fluorescence showing massive extravasation of serum albumin due to BBB disruption (A,B). The edema fluid was extended to the surrounding zone and migrated to remote regions such as the parietal cortex in the contralateral hemisphere, along with the corpus callosum (C). Cellular accumulation of Evans blue was frequently observed in the surrounding area, exhibiting the shape of cells such as axons and dendrites. Fragmented nuclei were seen in some of these cells. However, in these cells, the fluorescent signal was detected in the space surrounding the small fragmented nuclei, but the nuclei definitely lacked Evans blue accumulation (D–F, arrows). Nuclei had a round shape with weak Hoechst labeling as observed in H&E staining (G–I, arrowheads). CC, corpus callosum. Bar = 200 μm (A,B), 100 μm (C), 50 μm (D–I), and 10 μm (magnified photomicrographs in D–I).

vasogenic brain edema in the core of the lesion, fluorescence of extravasated Evans blue was so intensely diffused that it was not possible to observe whether the fluorescence was limited only to the cytoplasm (Fig. 2B,H). Nuclei in this area were shrunken and a round shape, and stainability to Hoechst 33258 was remarkably decreased. The nuclei with weak Hoechst staining exhibited a lack of Evans blue fluorescent signal, showing a round defect of red fluorescence (Fig. 2G–I). On the other hand, cellular accumulation of Evans blue was clearly demonstrated in the surrounding area of the lesion (Fig. 2A). Evans blue was observed to have accumulated in the cytoplasmic space in the surrounding area and these cells clearly exhibited the shapes of the soma, dendrites and

axon. Some of these cells demonstrated fragmentation of nuclei, indicating a morphological alteration of apoptotic-like cells. Evans blue fluorescence was observed to have migrated to the region remote from the primary injury, along the corpus callosum, and reached the septal nucleus and the parietal cortex in the contralateral hemisphere (Fig. 2C).

Ischemia also induced vasogenic brain edema indicated by the extent of Evans blue extravasation. However, the fluorescent signal of Evans blue was not as intense as that shown in the cold-injured brain; cellular accumulation was clearly detected (Fig. 3C). These cells were more frequently observed in the striatum than in the ischemic cortex, which may reflect the intensity of ischemia. Evans blue, which accumulated in the cells, filled the cytoplasmic space of these cells, exhibiting cell shapes such as those of dendrites and axons (Fig. 3C,D,J,K,L). Some of these cells showed typical fragmentation of the nuclei and apoptotic bodies (Fig. 3A,G). These cells also showed Evans blue signal with intense staining of Hoeschst 33258 in fragmented nuclei. However, necrotic cells showed low stainability to Hoechst 33258 and demonstrated irregularly shaped nuclei with intense signals of accumulated Evans blue. Cells with apoptotic-like morphology were mainly distributed in the medial striatum adjacent to the lateral ventricle. Furthermore, Evans blue migrated to the contralateral hemisphere along the corpus callosum as observed in the cold-injured brain. Evans blue accumulation was also observed in some cells in the lateral septal nucleus and the cortex adjacent to the cingulum (Fig. 3B,D,F,I,L,O). In the lateral septal nucleus, these cells were morphologically damaged, some cells showed nuclei fragmentation (Fig. 3B).

Quantitative Evaluation of Fluoromicroscopic Apoptosis Morphology and Evans Blue Accumulation

Cells with apoptotic-like morphology and Evans blue accumulation were counted on the photomicrographs and expressed as per mm^2. Figure 4 demonstrates the number of cells showing characteristic apoptotic-like morphology, cells with Evans blue accumulation, and the ratio of apoptotic cells in Evans blue-positive cells. Between the cold injury and MCAO, there was no statistically significant difference. Apoptotic-like cells were $346.67 \pm 309.30/mm^2$ and $356.70 \pm 75.64/mm^2$ in cold injury and MCAO, respectively. There were more cells with Evans blue accumulation than apoptotic-like cells, and were, $1{,}111.11 \pm 209.36/mm^2$ in the cold injury and $795.57 \pm 194.51/mm^2$ in the MCAO. The ratio was also the same level, and was $69.01 \pm 34.14\%$ and $73.09 \pm 13.70\%$ in the cold injury and MCAO, respectively.

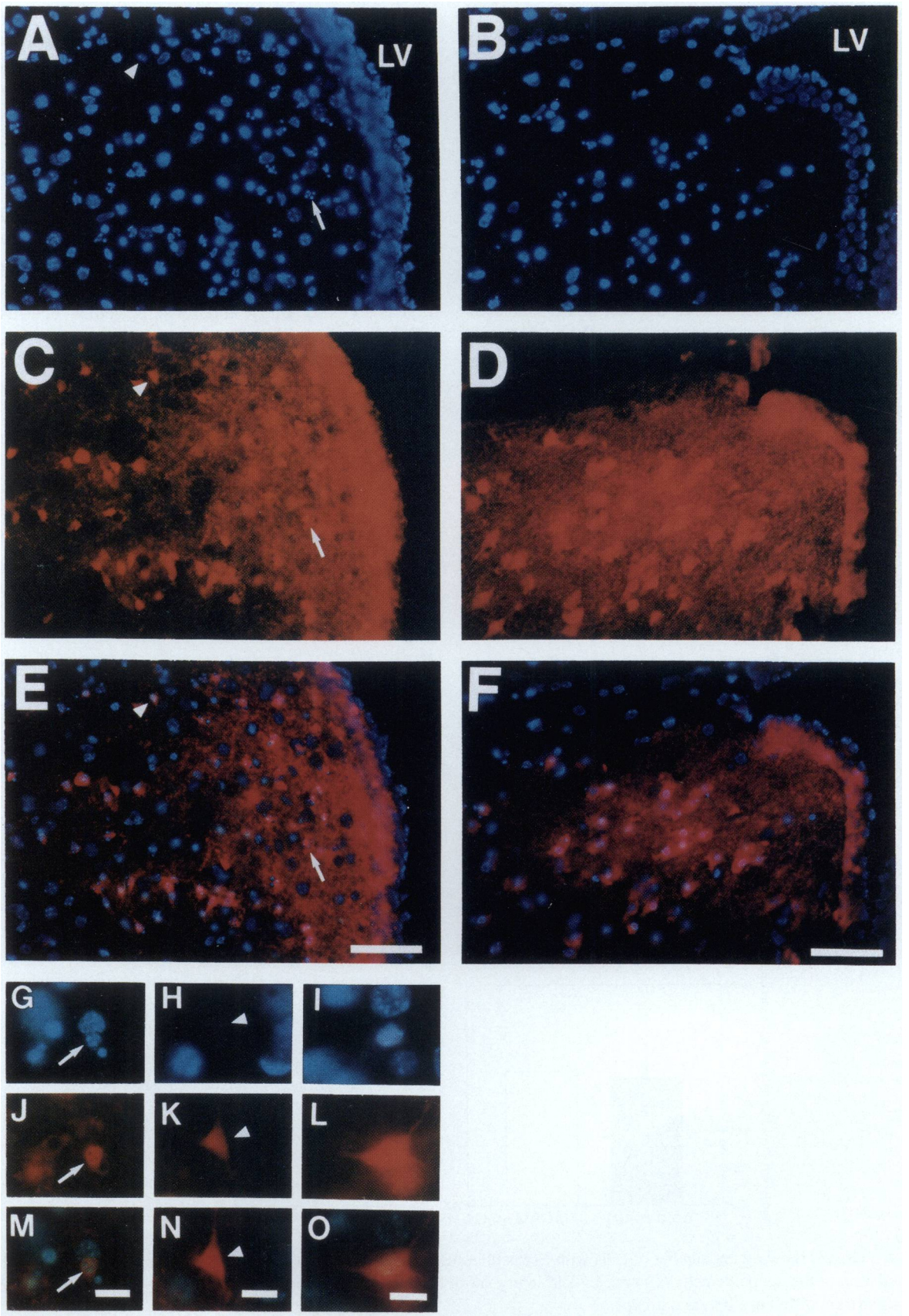
In Situ *Detection of DNA Fragmentation by TUNEL Staining*

Some injured cells with apoptotic-like morphology were specifically labeled by TUNEL staining, both in cold injury and focal ischemia-reperfusion. In the cold-injured brain, TUNEL-labeled cells were mainly localized to the surrounding zone, but were not detected in the core of the lesion (Fig. 5A,B). These cells showed condensation of the nuclei when stained with methyl green, occasionally exhibiting fragmentation. TUNEL-labeled cells were also observed in the contralateral hemisphere, including the lateral septal nucleus and the deep cortex adjacent to the cingulum, where cells with Evans blue accumulation were frequently observed by fluoromicroscopy (Fig. 5C). Cells with DNA fragmentation were also detected following focal ischemia-reperfusion. TUNEL-labeled cells were dispersed throughout the entire ischemic area, but were mainly distributed in the medial striatum adjacent to the lateral ventricle (Fig. 5D,E). These findings were consistent with those from the morphological analysis.

DISCUSSION

Vasogenic brain edema is caused by increased permeability of the BBB, resulting in the extravasation of serum components into the brain parenchyma. Macromolecules extravasated from the disrupted BBB, including albumin, fibrinogen, and fibronectin, have been described as accumulating in the cytoplasmic space of cells in the primary injury site corresponding to the BBB disruption and even in the area distant from the lesion following vasogenic brain edema (Brightman et al., 1970; Loberg and

FIG. 3. Fluorescent photomicrographs showing Evans blue extravasation and morphology of cells with Evans blue accumulation. Although Evans blue was not as intense as observed in the cold-injured brain, Evans blue accumulation in cells was observed in the entire ischemic area. Cells with nuclei fragmentation were frequently seen in the medial striatum coexisting with cells with necrosis morphology (A,G,H, arrows and arrowheads). Some of these cells represent cellular accumulation of extravasated Evans blue (C,E,J,K,M,N). Evans blue was observed to migrate to the contralateral hemisphere and accumulate in cells in the lateral septal nucleus, exhibiting morphological alterations including apoptosis and necrosis (B,D,F,I,L,O). LV, lateral ventricle. Bar = 50 μm (A–F) and 10 μm (G–O).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Torvik, 1991; Povlishock et al., 1979; Sokrab et al., 1988; Tanno et al., 1992; Tengvar, 1986; Tengvar and Olsson, 1982). It has been demonstrated that cellular accumulation of serum proteins appears as two patterns: a diffuse pattern suggesting dysfunction of the plasma membrane in severely injured cells, and a granular pattern suggesting increased pinocytosis in mildly damaged cells. Moreover, retrograde transport of the axon, which passes through the primary lesion, is also likely to contribute to the serum protein accumulation in the cytoplasmic space.

Recent investigations have demonstrated several features characterizing two different modes of cell death, apoptosis and necrosis. One of the features characterizing apoptosis is the fragmentation of nuclear DNA, and is widely used to distinguish apoptosis from necrosis or living cells (Arends et al., 1990; Kerr et al., 1972; Wyllie et al., 1984). This feature was used for *in situ* detection of DNA fragmentation by labeling with free 3′OH terminal (Gavrieli et al., 1992). DNA electrophoresis revealed characteristic bands indicating multiples of 180 bp, so-called DNA laddering. Another feature is the integrity of the plasma membrane. The plasma membrane integrity of cells undergoing apoptosis is preserved and most functions of the membrane remain unchanged. In necrotic cells, however, one of the earliest changes is the loss of structural integrity and function of the plasma membrane. Therefore, apoptotic cells exclude dyes used for viability assays, such as trypan blue or propidium iodide, as do living cells. This characteristic feature of the plasma membrane in apoptotic cells has been used, particularly in *in vitro* studies, to differentiate between apoptotic and necrotic cells.

In the present study, we determined the distribution of cells with apoptosis morphology and those with accumulation of extravasated Evans blue. To induce both vasogenic brain edema and neuronal cell death, we used models of cold injury and focal ischemia-reperfusion, which have been demonstrated to induce apoptotic cell death as well as vasogenic edema (Li et al., 1995; Murakami et al., 1997a,b). Cellular accumulation of Evans blue was observed in both apoptotic and necrotic cells. The cold injury used herein caused massive vasogenic edema, as evidenced by the extent of Evans blue fluorescence, and induced apoptotic cell death mainly in the area surrounding the primary lesion. Some cells showed accumulation of the Evans blue in the surrounding area and even in the regions remote from the primary lesion, such as the lateral septal nucleus and cortex in the con-

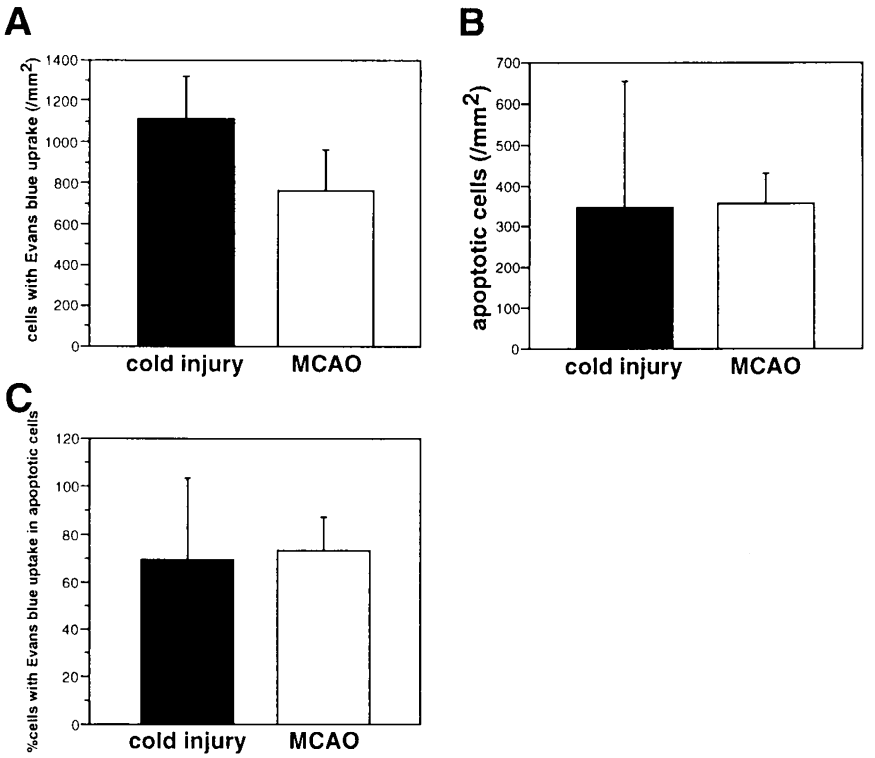

FIG. 4. Graphs showing the number of cells with Evans blue accumulation (A), apoptotic cells (B), and the ratio of Evans blue accumulation in the apoptotic cells (C) both in cold injury and focal ischemia. Values show mean ± SD. There was no statistically significant difference between groups.

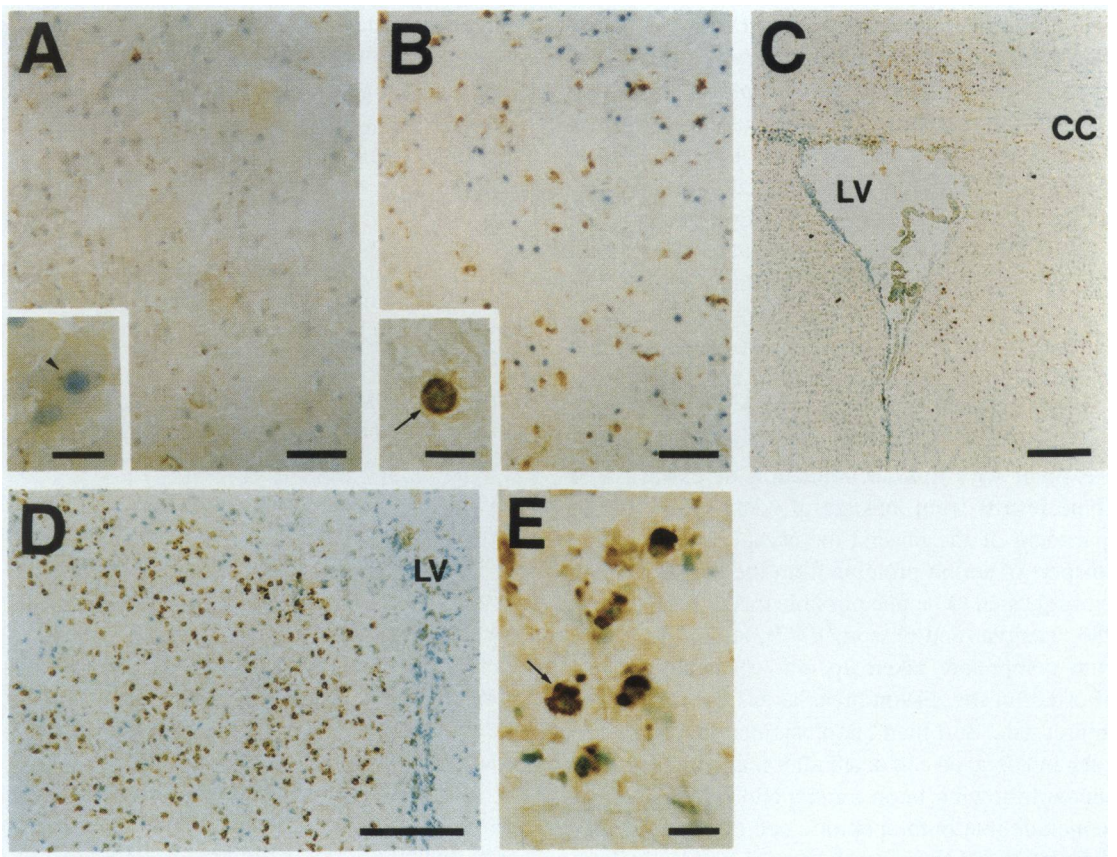

FIG. 5. Photomicrographs showing *in situ* labeling of DNA fragmented cells at 24 h after cold injury (A–C) and focal ischemia-reperfusion (D,E). TUNEL-labeled cells were rarely detected in the core of the lesion (A). These cells exhibited an oval shape with decreased staining intensity when stained with methyl green (magnified photomicrograph, A). Many cells were labeled in the surrounding zone, showing condensed nuclei (magnified photomicrograph, B), and these labeled cells were also observed in the remote regions such as the lateral septal nucleus and deep cortex adjacent to the cingulum in the contralateral hemisphere (C). TUNEL-labeled cells were also observed following focal ischemia-reperfusion and were frequently detected in the inner boundary zone (D), where TUNEL-labeled cells showed characteristic apoptosis morphology, nuclei condensation and fragmentation, and apoptotic bodies. LV, lateral ventricle; CC, corpus callosum. Bar = 50 μm (A,B), 200 μm (C), 100 μm (D), and 10 μm (E).

tralateral hemisphere. Among these cells exhibiting Evans blue accumulation, some demonstrated fragmented nuclei which were clearly labeled by Hoechst 33258 as morphological evidence for apoptotic cell death. Interestingly, however, the nuclear compartment was not labeled with accumulated Evans blue in the apoptotic cells, nor in the apoptotic bodies, although it is not understood what this finding means. Focal ischemia-reperfusion also induced vasogenic brain edema, although it was mild compared with the edema induced by cold injury. However, extravasated Evans blue also migrated along the corpus callosum to the contralateral hemisphere. In these brains, Evans blue accumulated in the cells in the lateral septal nucleus and deep cortex adjacent to the cingulum of the contralateral hemisphere, as well as in the ischemic area in the ipsilateral hemisphere. Nuclei-fragmented cells were mainly observed in the medial striatum as previously reported (Li et al., 1995; Murakami et al., 1997a), and both cellular accumulation of Evans blue and nuclei fragmentation also overlapped in some cells. Although these apoptotic cells accumulated Evans blue, the compartments of fragmented nuclei lacked labeling of the Evans blue fluorescence as was seen in the cold injury. We did not determine the cell type of Evans blue-positive cells and apoptotic cells in this study. Therefore, we note the possibility that infiltrating inflammatory cells with multilobed nuclei may phagocytose Evans blue-positive material. This interpretation will have to be investigated in future experiments, but was impossible in the present study because the fluorescent marker was used in intravenous injection into *in vivo* animals easily decays.

The present study clarifies neither the detailed mech-

anism of cellular accumulation of extravasated serum proteins nor the direct relationship between cellular accumulation of serum protein and apoptosis induction. However, since apoptotic cells were found among the Evans blue–positive cells at a ratio that ranged from 69% to 73% (Fig. 4), our study has demonstrated that the cellular accumulation of extravasated serum protein following vasogenic brain edema is associated with brain cell injury, including apoptosis. Further studies are needed to determine the temporal pattern and the cause-effect relationship of these two events after traumatic and ischemic brain injury. Since apoptotic cells have been demonstrated to preserve the integrity of the plasma membrane, which is lacking in necrotic cells, it is difficult to explain why this accumulation of extravasated Evans blue results from leakage of edema fluid due to the impairment of the plasma membrane integrity. Axonal transport of serum proteins from the primary lesion has been suggested to be one possible mechanism (Tengvar, 1986; Tengvar and Olsson, 1982), and, if this is so, the serum component taken up into cells may have a harmful effect in the distant areas as well as in the primary injured site, and then apoptosis may contribute to both acute and delayed cell death after massive vasogenic brain edema following brain trauma and ischemia.

We conclude that both apoptotic cell death and cellular accumulation of extravasated serum protein following vasogenic brain edema are likely to result from brain injury, and that both of these events are related in traumatic and ischemic brain injury.

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