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Regional Variability in DNA Fragmentation After Global Ischemia Evidenced by Combined Histological and Gel Electrophoresis Observations in the Rat Brain

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Abstract: We have studied whether the delayed cell death induced by transient forebrain ischemia is associated with an internucleosomal cleavage of DNA into oligonucleosome-sized fragments. The integrity of genomic DNA in various brain regions after a 20-min four-vessel ischemia was examined using gel electrophoresis. We found typical ladders of oligonucleosomal DNA fragments in the striatum and in the Ammon's horn. In the latter we also often found a random DNA degradation as a smear pattern. These findings were reinforced by a specific in situ labeling of DNA breaks in tissue sections. A dark staining of nuclei was observed in the cell bodies of neurons—in particular in the head of the caudate and in the vulnerable CA1 hippocampal area. With biochemical and histological approaches, there was no evidence of DNA degradation in regions that are resistant to the injury. We conclude that the association of multiple mechanisms of cell damage may occur after a global ischemia. The regional variability in DNA fragmentation stresses the importance of using histological approaches in parallel with gel electrophoresis. **Key Words:** Apoptosis—Endonuclease—In situ DNA nick-end labeling—Hippocampus—Striatum.
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The mechanism underlying the delayed neuronal death following ischemic injury is not yet understood (for a review, see Schmidt-Kastner and Freund, 1991). A massive transient glutamate release (Benveniste et al., 1989) and an intracellular calcium accumulation have been demonstrated following a global ischemia in the experimental rodent brain (Deshpande et al., 1987, Dux et al., 1987; Araki et al., 1990). This increase in the free cytoplasmic calcium concentration presumably activates calcium-dependent enzymes, which may lead to the degeneration of neurons. Recently, activation of a programmed cell death has been suggested to be involved in the delayed neuronal death following a global (Goto et al., 1990; Shigeno et al., 1990; Papas et al., 1992) or a focal (Linnik and Zobrist, 1992) ischemia, based on protection offered by protein synthesis inhibitors. Furthermore, an increase in protein synthesis following anoxia has also been reported in rat hippocampal slices (Charriaut-Marlangue et al., 1992). Since Kerr et al. (1972), programmed cell death or apoptosis has been extensively studied in developmental biology. It is described as an active process requiring metabolic energy as well as RNA and protein syntheses that are characterized by a cleavage of DNA into oligonucleosome-sized fragments. This is demonstrated by a typical ladder pattern after DNA gel electrophoresis.

We now report that this typical DNA degradation occurs after a transient global ischemia in the rat brain. In addition, we have adapted a method that enables us to demonstrate histologically in situ DNA breaks in neurons. With this method we have been enabled to detect apoptotic signs 24 and 48 h after the insult in subpopulations of striatal and hippocampal neurons.

MATERIALS AND METHODS

Ischemia

Animal care procedures were conducted entirely in accordance with the guidelines set by the European Community Council Directives 86/6091 EEC. Experiments were performed on male Wistar rats (Charles River, France) weighing 280–320 g. Transient global forebrain ischemia was performed according to the four-vessel occlusion model of Pulsinelli and Brierley (1979). In brief, vertebral arteries were electrocoagulated, and 24 h later common carotid arteries were clamped for 20 min. Body temperature was maintained at 37 ± 0.5°C. Rats (n = 6) with only electrocoagulated vertebral arteries were used as controls. At 24, 48, and 80 h after recirculation (n = 6–8 each), rats were decapitated, and the brain was rapidly removed. One cerebral hemisphere was frozen for morphological and in situ DNA nick-end labeling studies. From the second hemi-

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Abbreviations used: RT, room temperature; TE buffer, 10 m*M* Tris-HCl (pH 8.0) and 1 m*M* EDTA.

sphere, cerebellum, striatum, and hippocampus (Ammon's horn and dentate gyrus) were dissected out onto an ice-cold plate and used for DNA gel electrophoresis analysis.

DNA extraction and electrophoresis

Each region was homogenized in 10 volumes (wt/vol) of TE buffer [10 m*M* Tris-HCl (pH 8) and 1 m*M* EDTA] with a hand-operated homogenizer. The homogenate was then incubated in 3 volumes of extraction buffer [10 m*M* Tris-HCl (pH 8.0), 10 m*M* EDTA (pH 8.0), 0.5% sodium dodecyl sulfate, and 20 μg/ml of RNase] for 1 h at 37°C and incubated further for 3 h at 50°C after addition of proteinase K (100 μg/ml). Genomic DNA was extracted using 1 volume of phenol (pH 8) (twice) and 1 volume of chloroform/isoamyl alcohol (24:1, vol/vol) and precipitated with 3.0 *M* sodium acetate (0.1 volume) and ethanol (2 volumes). The final DNA pellet was dissolved in TE buffer, and the DNA content was measured by spectrophotometry at 260 nm. Ten micrograms of DNA samples was electrophoresed on 1% agarose gels, stained with ethidium bromide, and photographed under UV illumination.

DNA nick-end labeling of tissue sections

Cryostat-cut sections (15 μm) were adhered to gelatin-coated slides and stored at −80°C until use. Labeled sections were processed according to the method of Gavrieli et al. (1992). In brief, sections were treated with RNase (20 μg/ml) and incubated with proteinase K (20 μg/ml) for 15 min at room temperature (RT). Endogenous peroxidases were inactivated with 2% H_2O_2 for 5 min at RT. Terminal deoxynucleotidyl transferase (0.3 EU/μl) and biotinylated dUTP were then added to cover the sections and incubated in a humid atmosphere at 37°C for 60 min. The reaction was stopped by transferring the slides in citrate buffer (300 m*M* NaCl and 30 m*M* sodium citrate) for 15 min at RT. After a 10-min immersion in 2% bovine serum albumin, the sections were covered with streptavidin–biotin peroxidase complex (Dako) for 1 h at RT. Labeling was revealed with diaminobenzidine. Alternate sections were stained with toluidine blue for morphological analysis.

RESULTS

The present data are based on 24 experimental animals (24, 48, and 80 h of recirculation). For these animals, histological observations, performed on one hemisphere, showed a typical time course and extent of CA1 injury in agreement with the observations of Pulsinelli et al. (1982) (data not shown). In the striatum and cerebellum, no noticeable morphological modifications were seen 48 h after ischemia.

The bulk of the genomic DNA extracted from the different brain regions of control animals demonstrated that the integrity of DNA was roughly preserved, as shown by its molecular size, being ~40 kb after gel electrophoresis (Fig. 1A). In contrast, in the striata studied of 48-h postischemic animals (n = 3), there was a typical DNA ladder pattern with oligonucleosome-sized fragments of ~200 bp (Fig. 1B). In the hippocampal formation, several patterns of DNA were observed, as illustrated in Fig. 1C: (a) no DNA degradation for most 24- and 48-h postischemic animals (lane 1; n = 10); (b) DNA degradation into slight bands of oligonucleosome-sized fragments at 24 h (lane 2; n = 3) and more discrete bands at 48 h of recirculation (lane 3; n = 2); (c) a genomic DNA of lower molecular size (lane 4; n = 3); and (d) a smear pattern suggesting random DNA degradation (n = 3) without (lane 5) or with (lane 6) the presence of a ladder in 80-h postischemic animals. In contrast, no obvious DNA degradation was found in the dentate gyrus and cerebellum (Fig. 1D) of all the 80-h postischemic rat brains (n = 8).

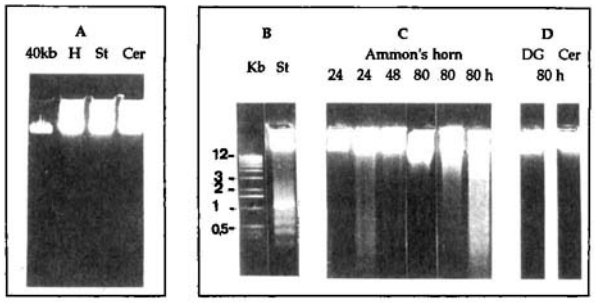

FIG. 1. Gel electrophoresis of the genomic DNA from control and ischemic rats. DNA samples were electrophoresed on 1% agarose gels and stained with ethidium bromide. **A:** Typical example of DNA obtained from hippocampus (H), striatum (St), and cerebellum (Cer) of control rats. The λ phage DNA used as a size marker is indicated at 40 kb. **B:** One-kilobase DNA ladder (GIBCO BRL; Kb) and DNA obtained from the striatum (St) of a 48-h postischemic rat. **C:** DNA from Ammon's horn of postischemic rats killed at various times following recirculation (24–80 h, indicated above the lanes). **D:** DNA from dentate gyrus (DG) and cerebellum (Cer) of 80-h postischemic rats.

Using a method allowing an in situ labeling of DNA breaks in tissue sections, we observed a staining of clustered cells in several brain regions 24 and 48 h postischemia. Omission of either the terminal transferase or its biotinylated substrate gave negative results. In the striatum there was a marked labeling, typically confined to clusters of neurons located within the rostral part of the caudate putamen (Fig. 2A and B). The staining faded for cells located more caudally in the caudate putamen or in the globus pallidus. In the hippocampal fields, the labeling was restricted to clusters of neurons exclusively located within the subicular and CA1 pyramidal cell layers at 24 h of recirculation (Fig. 2D). In contrast, neither the CA3 pyramidal cells (data not shown) nor the granule cells (Fig. 2E and F) were labeled. No degradation was found in the cerebellum at any time (data not shown).

DISCUSSION

This study demonstrates that a specific DNA cleavage at internucleosomal sites occurs during the process of global transient ischemia. In ischemic rat brains, the DNA obtained from the striatum exhibited a typical DNA fragmentation into oligonucleosomes. This ladder pattern is most probably due to the activation of Ca^{2+}-dependent endonucleases. The use of a specific in situ DNA nick-end labeling on conventional histological sections permitted us to localize this DNA cleavage mostly in cells located in the rostral part of the caudate putamen (the head), an area more sensitive to ischemic injury than globus pallidus (Pulsinelli et al., 1982). Furthermore, our results suggest that, in this region, a single neuronal death process of the apoptotic type could be induced by global ischemia, because a DNA cleavage was observed in undamaged neurons (as shown by our histological criteria) and involved most of the neurons in this region.

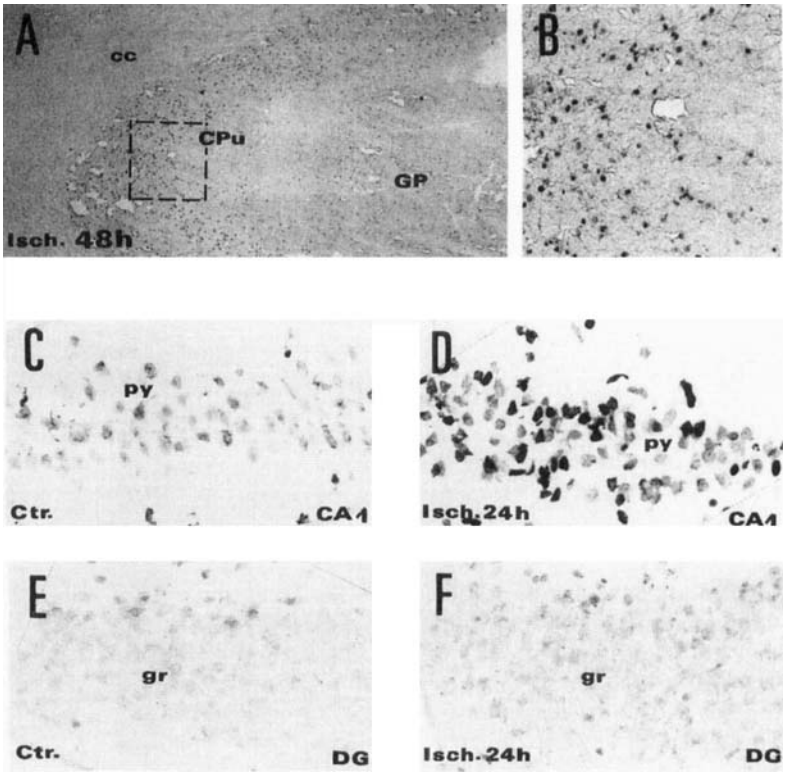

FIG. 2. DNA nick-end labeling of tissue sections from control and ischemic rats. Sagittal sections (15 μm) were prepared from the second cerebral hemisphere of control (Ctr.) and ischemic (Isch.) rats used in Fig. 1. In the ischemic rat brain, note the presence of positively labeled clustered cells within the rostral part of the caudate putamen (CPu; **A**; enlargement in **B**), at 48 h of recirculation, and throughout the CA1 pyramidal cell (py) layer in the hippocampus (**D**), at 24 h of recirculation. **C**: Control CA1, at 24 h of recirculation. No staining was seen within the granule cell (gr) layer of the dentate gyrus (DG) of the same animals (**E** and **F**). CA1, CA1 field of the Ammon's horn; cc, corpus callosum; GP, globus pallidus; A, X12.5; B, X45.5; C–F, X98.

The study of Tominaga et al. (1993) also reported an endonuclease activation in the caudate putamen 48 h after a focal ischemia.

In contrast to the striatum, our observations suggest a hitherto unsuspected degree of variability in the pattern of ischemic cell death of the pyramidal neurons of the highly vulnerable CA1 hippocampal region: The pattern of DNA degradation was clearly more complex, with a pattern of DNA degradation to oligonucleosome-sized fragments of 200 bp, a smear pattern resulting from random DNA degradation, or both patterns simultaneously. It is likely that this variability reflects a continuum spectrum of DNA degradation in the CA1 neurons. Our working hypothesis is that with a given degree of ischemic severity, the rise in intracellular Ca^{2+} content that follows the ischemic episode will activate endonucleases, leading to a ladder pattern in subpopulations of highly vulnerable CA1 neurons. This is followed, with a longer delay postischemia, with a more generalized random degradation that occurs at a time when DNA is degraded also in adjacent populations of pyramidal neurons and that represents the late phase of CA1 neuronal death. Furthermore, we have noticed that the earlier is the occurrence of primary and late events, the more severe is the ischemic insult (as evidenced by morphological alterations). Further studies will be necessary to determine the links between the two patterns of DNA degradation. The combined use of the biochemical procedure, which involves homogenization of the entire cell population, and the in situ procedure, which enables a specific labeling of DNA breaks at the cellular level, should facilitate better definition of the regional differences toward vulnerability to ischemia.

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