Cytoplasmic Delayed Neuronal Death in the Myenteric Plexus of the Rat Small Intestine after Ischemia

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Summary. The present study demonstrates light and electron microscopic changes in neurons in the myenteric plexus of the rat ileum following four-hour ischemia. Macroscopically, an intestinal constriction occurred at the damaged portion at three weeks after ischemia; the segment oral to the constriction markedly swelled at four weeks. In light microscopy, at three weeks after ischemia, the myenteric neurons appeared spongy or foamy, containing many vacuoles in their somatic cytoplasm. At four weeks, the neuronal cytoplasm and nerve fiber bundles had disintegrated to form vacant spaces in the myenteric plexus. The neuronal nucleus of the damaged plexus did not show positive nick-end labeling. In electron microscopy, neuronal cytoplasm revealed degenerative signs already at one week after ischemia: a distended endoplasmic reticulum and swollen mitochondria with fragmentary cristae. The nerve fibers also showed destruction of the mitochondria, and degenerative changes in the postsynaptic sites appeared earlier than the presynaptic terminals. The results suggest that intestinal ischemia causes delayed neuronal death, which differs from the apoptotic process previously demonstrated in the ischemia-damaged brain.

It has been shown that a complete interruption of intestinal blood circulation for 4 h causes the irreversible disappearance of intrinsic reflexes accompanied by degenerative changes of neuronal elements in the canine myenteric plexus (Hukuhara et al., 1961a). Such neuronal changes do not occur immediately after the circulatory interruption, but during 7 weeks after ischemic insult, almost all the ganglionic cells show degenerative changes or cellular vacuolization (Hukuhara et al., 1961a, b). Furthermore, it is suggested that the destruction and loss of myenteric neurons causes constriction of the ischemic segment which, in its turn, induces dilation of the segment oral to it as seen in Hirschsprung’s disease (Hukuhara et al., 1961b; Scheuermann et al., 1986; Miura et al., 1996).

It has been reported that transient ischemic damage induces apoptotic neuronal death in the gerbil hippocampus (Nitatori et al., 1995). The present study aims to investigate histological and cytological processes of neuronal death in the myenteric plexus in the rat ileum after complete interruption of its blood supply, and shows that delayed neuronal death proceeds from cytoplasmic degeneration.

MATERIALS AND METHODS

Animals and surgical procedures
Twenty-eight adult female Wistar rats (7–8 weeks old, 200–300 g) were used. Twenty experimental animals were divided into 4 groups, each of which consisted of 5 rats. Eight control rats were also grouped into four groups.

The experimental animals were anesthetized with ethyl ether and their abdominal cavity was opened with a midline incision. Blood supply to a loop (about 5 cm) of the ileum was temporarily stopped by clamping vessels, including collateral routes, using plastic vascular clips (disposable vascular clip, TKS-1, Medical Division of the Kyowa Precision Instruments Corp., Japan) (Fig. 1a). After 4 h ischemia by the stoppage of blood circulation, the vascular clips were removed to release the clamped vessels, and the abdomen was closed. Control animals were anesthetized in the same way, and sham-operated.

Specimens for light and electron microscopy
At 1, 2, 3 and 4 weeks after operation, each group of the experimental and control animals was examined.
The animals were anesthetized with ethyl ether, and their abdominal cavity was opened again. Their abdominal organs were perfused through the thoracic aorta with Ringer’s solution, and successively with a mixture of 4.0% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) or a buffered 4.0% paraformaldehyde. Then, the ischemia-damaged segments of the intestines were excised. The control animals were also perfusion-fixed with the same mixture and similar ileal segments were excised.

**Light microscopy**
The excised ileal segments were cut into 3 mm-thick blocks which included the entire intestinal wall, and immersed in the same fixative for 6 h or longer. The tissue blocks were embedded in paraffin, and cut into sections of 10–15 µm thickness. The deparaffinized sections were stained with cationic iron colloid at a pH value of 4.0, treated with 1% K$_4$Fe(CN)$_6$ in 1% HCl aqueous solution for Prussian blue reaction, and counter-stained with nuclear fast red (MURAKAMI et al., 1986). The stained sections were cover-slipped, and observed with a light microscope (Olympus, BX-50).

**DNA nick-end labeling**
In some paraformaldehyde-fixed sections, the nick end labeling or TUNEL method (GAVRIELI et al., 1992) was applied to examine DNA fragmentation. Briefly, the sections were pretreated with 20 µg/ml proteinase K (Boeringer-Mannheim) in 0.01 M tris buffer, pH 7.4, for 15 min at room temperature. After treatment with 2% H$_2$O$_2$ aqueous solution, they were incubated with 300 U/ml terminal deoxynucleotidyl transferase (TdT) (Gibco BRL) and 40 mM biotinylated 16-2’-dUTP (Boeringer-Mannheim) in TdT buffer (Gibco BRL) for 1 h at 37°C. The sections were then blocked with 1% bovine serum albumin, and treated with peroxidase-conjugated streptavidin (Vector). Labeled sites were visualized with diaminobenzidine reaction.

**Electron microscopy**
The ileal tissues, which had been perfused with a
buffered paraformaldehyde and glutaraldehyde mixture, were sliced into small blocks (1 x 2 x 2 mm), and immersed for 6 h in the same fixative at 4°C. The tissue blocks were postfixed with 1% OsO₄ for 2 h after the aldehyde fixation, and embedded in epoxy resin. The ultrathin sections were cut and stained with uranyl acetate and lead citrate. All sections were observed under a transmission electron microscope (H-700, Hitachi) (Central Research Laboratory of our school).

RESULTS

General observations
All the operated animals survived until the reopening of their abdominal cavity for excision of the ileal tissue samples. Eight control or sham-operated rats showed no particular macroscopical pathologic change in their small intestine, except slight peritoneal adhesion. In the experimental or ischemia-treated rats, no significant macroscopic changes were seen in the vascular-clipped segments of the small intestine until two weeks after surgery. At three weeks after ischemic damage, the ileal segments showed a constriction at the center of the clipped region, whose oral segments were slightly dilated. At four weeks, the intestinal dilatation became prominent (Fig. 1b).

The present microscopic observation concentrated on the myenteric plexus of the experimented region because of the ease of comparison in changes of the
plexus. As our preliminary experiment indicated that the ischemia-operated rats died of ileus at five weeks after the operation, the present observation was performed for four weeks.

**Light microscopy**

In cationic iron colloid stained sections of the control ileal myenteric plexus (Fig. 2), the nucleolus of nerve cells showed a distinct Prussian blue reaction. Their cytoplasm was also stained slightly with the cationic iron colloid. The nerve fiber bundle regions were also stained with the iron colloid, showing a strong Prussian blue reaction. The glial cells or Schwann's cells were stained only with nuclear fast red. In ischemia-treated rats, no significant changes were noted in the mucosal, submucosal or muscular layers other than the myenteric plexus.

At one (Fig. 3a) or two weeks (Fig. 3b) after the ischemic damage, the cytoplasm and nucleolus of the neurons showed a decrease in stainability against cationic iron colloid. At two weeks, the neuronal cytoplasm swelled into a rounded shape. Nuclear structure including the nucleoli of neurons was well maintained. No other significant changes were recognized in any of the other elements of the myenteric plexus.

At three weeks after ischemia, some nerve cell somata showed vacuolation in their cytoplasm, so that these cells appeared spongy (Fig. 3c). Nucleoli of the neurons were still clearly visible. The nerve fiber bundle regions were slightly stained with cationic iron colloid. The glial cells had proliferated in number but showed no significant changes in their stainability or appearance.

At four weeks after the ischemic insult, the neurons and nerve fiber bundles in the myenteric plexus showed further degenerative features (Fig. 3d). The most damaged region was the midpoint of the ischmic segment, where the nerve cells and fiber bundles were mostly destroyed to disappear leaving large vacant spaces in the plexus; smooth muscle fibers showed atrophic degeneration.

**DNA nick-end labeling**

No diaminobenzidine reaction indicating a positive nick-end labeling was seen in the myenteric plexus and also myenteric neurons at one, two, three and four weeks after ischemia (data not shown).

**Electron microscopy**

In the electron microscopy of control specimens, the neurons in the myenteric plexus showed a cytoplasm rich in cellular organellae such as rough-surfaced endoplasmic reticula, mitochondria and polyribosomes, and possessed large and round nuclei with a homogeneous karyoplasm (Fig. 4a). Around the cisterns of the rough-surfaced endoplasmic reticulum, there were numerous polyribosomes in rosette-like aggregates of four to six monoribosomes (Fig. 4a inset). Glial cells had large irregularly shaped nuclei (Fig. 4b). Neuronal processes, which contained mitochondria, smooth-surfaced endoplasmic reticula, microtubules and neurofilaments, were grouped in bundles between glial processes. Their presynaptic terminals showed numerous synaptic vesicles (Fig. 4b).

At one week after the ischemia-treatment, the neuronal perikaryonal and axoplasmic mitochondria swelled into a round shape, and mitochondrial cristae lost their original arrangement (Fig. 5a, b). The cisterns of the rough-surfaced endoplasmic reticulum distended (Fig. 5a). However, the nucleus, including the nucleolus, revealed no significant changes.

At two weeks after ischemic damage, the destruction of the mitochondrial cristae progressed; in the cytoplasm of myenteric neurons, fragmentation and vacuolation of some mitochondria were noticed (Fig. 6a). Cisterns of the rough-surfaced endoplasmic reticulum showed further distended features (Fig. 6a inset). In the nerve fibers or axons, mitochondria were swollen and vacuolated in appearance. The neuronal processes were degenerated with distention (Fig. 6b).

At three weeks after neuronal ischemic insult, almost all mitochondria in the neuronal cytoplasm (Fig. 7a) and in the axoplasm (Fig. 7b) showed swelling and vacuolation, so that they completely lost their normal structures. The endoplasmic reticulum in the neuronal cytoplasm dilated into vacuoles. The periphery of the neuronal somata contained empty spaces as the result of vacuolation. The polyribosomes showed no significant changes (Fig. 7a inset). In the nerve fibers, the neuronal processes became markedly swollen or distended. Particularly, the postsynaptic sites became empty and swelled with large vacuoles. The synaptic vesicles disappeared or decreased in number (Fig. 7b).

At four weeks after the ischemia-operation, the areas of the nerve fiber bundles showed vacant spaces, where only destroyed fragments and collagen fibrilar bundles were visible (Fig. 8).

**DISCUSSION**

The present study has confirmed that, after four-hour
Fig. 4. Transmission electron micrographs of the control myenteric plexus. a. The myenteric neuron (N) shows cytoplasm rich in organelae, including rough endoplasmic reticulum (small arrow), mitochondria (m) and polyribosomes (small arrowhead). Inset. Part of the cytoplasm, showing rough endoplasmic reticulum (small arrow), mitochondria (m) and polyribosomes (small arrowhead). b. The glial cell (G) possesses an irregularly shaped nucleus and fewer organelae than the neuron. In the nerve fiber bundles, the presynaptic terminal (arrowheads), postsynaptic side (arrows) and mitochondria (m) keep their structural integrity. The presynaptic endings contain numerous synaptic vesicles. a: ×9,200, Inset: ×18,000, b: ×11,500
Fig. 5. One week after ischemia. a. Mitochondrial cristae (m) in the cytoplasm lose their arrangement. The cisterns of the rough-surfaced endoplasmic reticulum (small arrow) are distended. Ribosomes (small arrowhead) show no changes. N neuron. Inset in a shows changes in mitochondrial cristae, rough-surfaced endoplasmic reticulum and ribosomes (small arrowhead) in higher magnification. b. Mitochondrial cristae (m) in the axoplasm have lost their arrangement as in the cytoplasm. The postsynaptic sites (arrowheads) and the presynaptic terminals show no structural changes (arrows). G glial cell. Inset in b shows a higher magnification of the mitochondria (m) and postsynaptic sites (arrowheads). a: ×15,000, Inset in a: ×24,000, b: ×7,700, Inset in b: ×15,000
Fig. 6. Two weeks after ischemia. a. Mitochondrial cristae are fragmented (m). Cisterns of the rough endoplasmic reticulum (small arrow) show distended features. N neuron. Inset shows a higher magnification of the mitochondria (m), cisterns of the rough endoplasmic reticulum (small arrow) and ribosomes (small arrowhead). b. The presynaptic terminals (arrowheads) and postsynaptic side (arrow) are dilated. a: ×9,400, Inset: ×17,000, b: ×14,000
Fig. 7. At 3 weeks. a. Mitochondria (m) are completely degenerated. The cisterns of the rough endoplasmic reticulum (small arrow) are dilated into vacuoles. Some ribosomes (small arrowhead) have separated into monoribosomes and decreased in number. The periphery of the neuron shows empty spaces with large vacuoles (asterisks). N neuron. Inset shows a higher magnification of mitochondria (m), cisterns of the rough endoplasmic reticulum (small arrow) and ribosomes (small arrowhead). b. The presynaptic terminals (arrowheads) and postsynaptic sites (arrow) show large vacuoles. The synaptic vesicles are decreased in number. G glial cell. a: ×5,500, Inset: ×27,000, b: ×11,500
ischemia, neurons of the myenteric plexus in the rat small intestine degenerated within four weeks (MATSUMOTO, 1997; YANO et al., 1997). HUKUHARA and his associates have also shown in the canine intestine that the intrinsic reflex completely disappeared at 49 days after four-hour ischemia, and that the ganglionic cells in the myenteric plexus degenerate with cytoplasmic vacuolization (HUKUHARA, 1973; HUKUHARA et al., 1961a). In these previous studies, the process of morphological changes in the intestinal myenteric neurons after ischemic damage was observed only by light microscopy. The present study using electron microscopy was able to demonstrate that the first distinct degenerative changes occurred at one week after ischemic insult and that essentially all neurons were completely destroyed at four weeks after reperfusion.

In the central nervous system in the gerbil, degenerative changes have been reported as delayed neuronal death after five-minute ischemia (ITO et al., 1975). Such damaged brain neurons have shown an enlargement of cisterns of the endoplasmic reticulum (KIRINO and SANO, 1984), disaggregation of ribosomes (KLEIHUES and HESSANN, 1971; KIRINO and SANO, 1984), and a positive reaction of nick end labeling (NITATORI et al., 1995). As confirmed in the present study, an ischemic duration of four hours is necessary to induce neuronal damages in the rat myenteric plexus. The present study has demonstrated that the earliest ultrastructural changes due to ischemic damage occur in the rough-surfaced endoplasmic reticulum and mitochondria of the neuronal cytoplasm. The light microscopic finding of decreased stainability in the neuronal cytoplasm against iron colloid at pH 4.0 may be due to such changes in the cytoplasmic organelae.

It is noteworthy that the nuclei of the ischemia-damaged myenteric neurons were neither positively stained by the nick end labeling, nor revealed structural changes until the last three weeks. In contrast, cytoplasmic organelae, including the rough-surfaced endoplasmic reticulum and mitochondria, showed pathological changes. These differ from hitherto reported apoptotic changes in the delayed neuronal death in the brain and spinal cord after ischemic damage (ITO et al., 1975; CHU et al., 1978; TAKAKI,
1982; Kirino and Sano, 1984; Nittatori et al., 1995). This suggests that myenteric neuronal death after ischemia may be classified as necrotic damage characterized by "peripheral organellae failure".

The present study shows that axonal changes in the myenteric plexus appeared at an early stage. First, mitochondrial cristae disintegrated into numerous vacuoles. Later, they became empty and swollen with large vacuoles, after which they disappeared completely. These pathological changes, which occur until the axons of the myenteric neurons disappear completely, are similar to those in the transitional zone of Auerbach's plexus in Hirschsprung's disease (Hukuhara et al., 1961b; Miura et al., 1996).

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