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Evidence of caspase-3 activation in hyposmotic stress-induced necrosis.

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Abstract

Primary culture of dentate gyrus was submitted to a hyposmotic stress that induces a rapid cell death that is necrosis morphologically. Surprisingly, we observed a rapid and dramatic upregulation of the active form of caspase-3 (caspase-3a) in both neurons and glial cells. Caspase-3a immunoreactivity appears as early as 1 min after hyposmotic treatment, when some neurons are still alive, suggesting that caspase-3a may contribute to further necrotic cell death.

Keywords: Dentate gyrus culture, electron microscopy, immunocytochemistry.
Necrosis and apoptosis are two modes of death originally defined by unequivocal morphological criteria (11). Their underlying molecular mechanism has also been believed to be radically different. Classically, apoptosis is induced by a genetic program initiated by expression of immediate early genes and of death effector proteins, that by a variety of mechanisms activate a cascade of proteolytic enzymes called caspases. In contrast, necrosis is thought to be a passive phenomenon induced by ion fluxes and cell explosion (7). However, recent studies suggest that necrosis can also be a programmed cell death that shares biochemical features with classical apoptosis (1-2, 8-10). In this study, we tried to determine whether massive necrotic cell death induced by a strong hyposmotic stress requires the activation of such a program.

Dentate gyrus cell cultures were prepared as described elsewhere (8) from postnatal day 3 (P3) Wistar rat pups. At day in vitro 7, cultures were submitted to hyposmotic shock by exchanging the culture medium for sterile distilled water (Gibco). Cell survival was assessed with propidium iodide assay and ultrastructural studies were performed as described elsewhere (8). Immunocytochemical studies using rabbit polyclonal antibody to the p20 active fragment of caspase-3 (caspase-3a) (R&D system; AF835) were
performed as described previously (8). Caspase-3 immunoreactivity was confirmed with the cleaved caspase-3 (Asp175) antibody (Cell Signaling, #9661). The staining was performed similarly with some modifications. Cultures were incubated with the cleaved caspase-3 (Asp175) antibody (1:50) overnight at 4°C. Staining specificity was confirmed using cleaved caspase-3 (Asp175) blocking peptide (Cell Signaling, #1050) as described by the manufacturer.

Hyposmotic treatment of dentate gyrus cultures induced a rapid death of a majority of neurons and a rapid decrease of cell density, reflecting a complete disappearance of cell bodies. Ultrastructural studies confirmed that both glial cells and neurons displayed a necrotic morphology, characterized by the swelling of the cytoplasm and organelles with a relatively intact nucleus and an unbroken nuclear membrane (Fig. 1). Apoptotic cell death is usually characterized by preservation of the cytoplasm and cell membrane, while the nuclear membrane dissolves and chromatin is condensed into large masses (3-4, 6). By contrast, in necrotic death, the nuclear membrane remains intact and the nucleus is relatively preserved at early stages, while cytoplasmic organelles swell and the cell membrane is disrupted, as seen in our preparations and in earlier studies (8, 11). However some cells were still excluding propidium
iodide after 1 min of hyposmotic stress (Fig. 2 D) and surprisingly, nearly all of them were caspase-3\textsubscript{a} immunoreactive (Fig. 2C). This fast activation of caspase-3\textsubscript{a} was confirmed with the 9661 antibodies and could still be observed after 15 min of hyposmotic treatment. Caspase-3 immunoreactivity revealed by AF835 or 9661 antibodies was blocked when they were pre-incubated with cleaved caspase-3 (Asp175) blocking peptide (data not shown).

Caspase-3 activation in hyposmotic necrotic cells confirms earlier reports of caspase-3\textsubscript{a} involvement in necrosis. In vivo studies suggest that ischemic cell death sometimes displays both a necrotic morphology and an apoptotic-like biochemistry (1-2, 9-10). Our laboratory has shown that necrotic hypoxic neurons are immunoreactive to caspase-3\textsubscript{a} and are partially protected by caspase inhibitors including a caspase-3 inhibitor (8). A previous study has shown that mild hyposmotic stress induces a cell death blockable by caspase inhibitors (5). After 1 min of hyposmotic treatment, the fact that some dentate gyrus cells were still excluding propidium while almost all of them were caspase-3\textsubscript{a} immunoreactive, suggested that caspase-3\textsubscript{a} may contribute to their death (Fig. 2). However, caspase-3\textsubscript{a} may not be functional, since we did not observe any increase in fractin immunoreactivity, revealing actin cleavage by caspase-3\textsubscript{a} (12), even after 30 min of hyposmotic treatment (data not shown).
Dentate gyrus cells may indeed rapidly die before caspase-3\(_a\) has time to do much damage. Additional experiments will be necessary to clarify this point.

The upstream mechanism leading to hyposmotic stress-induced caspase-3 activation remains to be identified. In cyanide-induced necrotic cell death, we have previously shown that energy failure induces an early mitochondrial swelling and loss of the mitochondrial membrane potential, followed by the rapid release of cytochrome c from the mitochondria to the cytoplasm and caspase-9-dependent activation of caspase-3 (8). After hyposmotic treatment, we did not observe any cytochrome c release from the mitochondria or caspase-9 activation (data not shown), suggesting that another pathway is responsible for caspase-3 activation.

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**REFERENCES**


Legends

Fig. 1 Hyposmotic stress induces necrosis in primary culture of dentate gyrus cells. Ultrastructure of control (A and C) and hyposmotic (3 min of treatment; B and D) neurons (A and B) and astroglial cells (C and D) was studied by electron microscopy. Treated cells have a characteristic necrotic morphology, characterized by a severe swelling of cytoplasm and organelles and relatively intact nucleus. Bars = 1 μM.

Fig. 2 Hyposmotic stress triggers caspase-3 activation in primary culture of dentate gyrus cells. Control cells exclude propidium iodide (B) and do not express caspase-3α immunoreactivity (A). After 1 min of treatment, nearly all cells are caspase-3α immunoreactive (C) while some of them are still excluding propidium iodide (D; see arrows).
Figure 1