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Letter to the Editor

Intravenously administered propidium iodide labels necrotic cells in the intact mouse brain after injury

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Dear Editor,

Apoptosis and necrosis have been identified as two morphologically distinct types of cell death.[1–3] However, under some conditions, the same cell may display both apoptotic and necrotic features.[3,4] Although several markers of both pathways are available to study the complex nature of cell death *in vitro*, the markers that can be used *in vivo* (especially in the brain) are limited.[5] Since loss of membrane integrity is a pathognomonic feature of necrotic cell death, adapting techniques using membrane-impermeant dyes such as propidium iodide (PI) to *in vivo* conditions may help investigating mechanisms of cell death *in vivo*.

PI is a nucleic acid stain usually used as a counterstain in multicolor fluorescent techniques.[6] Once the dye is bound to nucleic acids, its fluorescence is enhanced by 20–30 fold.[7] In tissue sections, it is used as a nuclear marker and sometimes employed to identify nuclei showing apoptotic changes.[5,6] On the other hand, since PI is membrane impermeant, it is also used to detect necrotic cells in culture. When the cell membrane is disrupted, it leaks into the cell and binds to DNA and RNA and, therefore, only necrotic cells fluoresce red. In the present study, we demonstrate that intravenous (i.v.) as well as intracerebroventricular (i.c.v.) administration of PI can be employed as a marker of necrotic cell death in the intact brain after ischemia.

PI (P-3566, Molecular Probes, 20 mg/kg) was introduced via the tail vein in 10–15 min to mice subjected to 30 min of middle cerebral artery (MCA) occlusion (by the filament method) and 6 (N=4) or 72 h (N=4) of reperfusion. PI (0.5 μg in 0.5 μl distilled water) was given i.c.v. to another group of mice subjected to 6 (N=3) or 72 h (N=3) of permanent MCA occlusion or to 30 min transient MCA occlusion and 6 (N=3) or 72 h (N=3) of reperfusion. Mice were perfused transcardially with 4% formaldehyde 20 min after i.c.v. and 5.5 h after i.v. injection. Brains were kept in formaldehyde for 2 days and then cryoprotected in 20 and 30% sucrose in PBS for 12 h at +4°C. 10 μm thick, Coronal sections, were cut and coverslipped with Hoechst-33258 (H-3569, Molecular Probes).

In sham-operated mice brains, PI fluorescence was only seen in the ventricular lining at the i.c.v. injection side without cellular labeling in white and gray matter. Mice subjected to transient or permanent focal ischemia showed numerous fluorescent-labeled cells in ischemic areas (Figure 1). No PI-positive cells were detected in the contralateral hemisphere and in ipsilateral areas supplied by anterior or posterior cerebral arteries. Contrary to *in vitro* or *ex vivo* studies in which RNAase treatment prevents cytoplasmic PI labeling, we observed cytoplasmic as well as nuclear staining (Figure 1). When given i.v., a similar pattern of PI fluorescence was observed except that the signal intensity was lower than in i.c.v. injections. We varied the time between i.v. injection and killing between 1 and 5.5 h in preliminary experiments to find out the optimum time of PI administration. Although we observed labeled cells when PI was given 3 h before killing, 5.5 h of circulation with PI provided a longer penetration time into the ischemic area and a satisfactory fluorescence in injured cells.

At 6 h after ischemia, there were occasional cells labeled with PI (Figure 1). The number of PI-positive cells and the fluorescence intensity dramatically increased 72 h after transient or permanent ischemia. Terminal transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells, which were scarce 6 h after ischemia, were also dramatically increased at 72 h (Figure 1). Double labeling of tissue sections with TUNEL (Apoptosis Detection System, Fluorescein kit, Promega) revealed that PI labeling and TUNEL positivity were detected in discrete cell populations in areas of mild injury such as peri-infarct regions. In areas displaying more severe ischemic changes, some cells displayed a mixed phenotype (i.e. a TUNEL-positive nucleus in a PI-labeled cell) in addition to discretely TUNEL- or PI-labeled cells. PI was retained inside cells after processing the frozen brain sections for hematoxylin staining (N=4), which also confirmed that most PI-labeled cells displayed necrotic features such as pyknotic nuclei, irregular cell counters or loss of nuclear stainability with hematoxylin (Figure 1).

In the central nervous system (CNS), PI has mainly been used as a retrograde tracer in neuroanatomical studies after its focal injection into several brain regions.[8] In only one study, Hussain *et al.*[9] administered multiple i.v. injections of 0.1 mg of PI over a period of up to 8 h (total, 0.7–1.0 mg) to mice and searched for PI cytofluorescence in the CNS. They found that PI did not extravasate into the cerebral gray or white matter except at a few regions with leaky BBB such as the median eminence. We administered approximately the same dose (0.6 mg) of PI to mice but as a single i.v. injection. Nevertheless, we did not observe any adverse effects within the 5.5 h postinfusion period. In agreement with their study, we detected no PI fluorescence in the brain of sham-operated animals or in the nonischemic areas. On the other hand, numerous PI-positive cells were observed in the ischemic area, possibly because ischemia-induced disruption of the BBB allowed the tracer to reach the brain parenchyma, where it leaked into the cells with disrupted membranes.

In line with Garcia *et al.*'s[10] seminal studies, the number of PI-labeled cells and the intensity of the signal increased

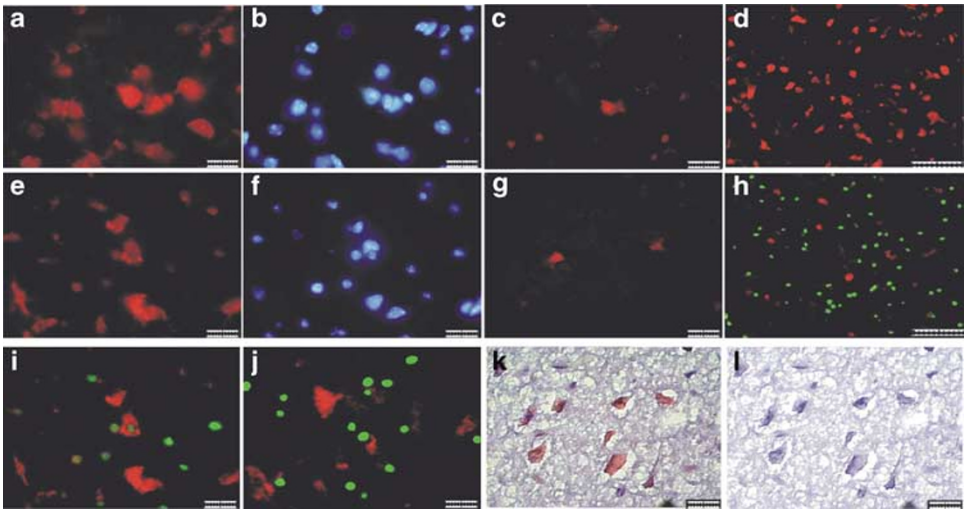

Figure 1 PI labels necrotic cells that lost their membrane integrity in the ischemic brain following its i.c.v. (upper row) or i.v. (middle and lower rows) administration. (**a, e h-k**) PI-labeled ischemic cells 72 h after 30 min of MCA occlusion, (**b** and **f**) Hoecst-33258 stainings of the same areas in (**a**) or (**e**) illustrate that PI stains the nucleus and cytoplasm in most of the cells. PI fluorescence was less intense 6 h after permanent **(c)** or transient MCA occlusion **(g)**. (**d**) A strong PI signal in ischemic cells was detected 72 h after permanent MCA occlusion. PI labeling and TUNEL-positivity were detected in discrete cell populations in areas of mild injury **(h, j)**. In areas displaying more severe ischemic changes, some PI-labeled cells had a TUNEL-positive nucleus **(i)**. (**k**) PI fluorescence was retained within cells after processing for hematoxylin counterstaining. Superimposition of fluorescent and bright-field photomicrographs disclosed that PI-labeled cells displayed necrotic features such as pyknotic nuclei, irregular cell counters or loss of nuclear stainability. (**l**) Bright-field photomicrograph with hematoxylin counterstaining of the same area in (**k**) Scale bar denotes 100 μm in (**d**) and (**h**) and, 20 μm in all other photomicrographs.

significantly from 6 to 72 h in the MCA area evolving to infarct. However, PI may also have labeled apoptotic cells that lose their membrane integrity at late stages.[11] Most of the PI-labeled cells were not TUNEL positive, indicating that PI preferentially labeled necrotic cells with a leaky membrane. However, some TUNEL-positive cells in severely ischemic areas were also labeled with PI to varying degrees. These cells may have gone to secondary necrosis after the development of apoptotic DNA fragmentation or may have entered the final stage of apoptosis with loss of membrane integrity. It should be noted that ischemic cell death is a mixed form and the contribution of apoptosis and necrosis to cell death may vary depending on the severity of ischemia.[12] In a study similar to ours, Wilde *et al.*[13] injected PI combined with kainic acid i.c.v. to detect excitotoxicity-induced cellular damage and showed that PI labeled necrotic neurons in the rat hippocampus.

In conclusion, PI can safely be used as a marker of disrupted plasma membrane integrity of necrotic cells under *in vivo* conditions after i.v. as well as i.c.v. administration. The i.v. administration is technically simple and gives acceptably intense fluorescent signals to detect the necrotic cells in brain. However, it requires the presence of a leaky BBB and prolonged postinjection survival times to reach sufficiently high levels in the injured cells.

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