Cyclophilin A release as a biomarker of necrotic cell death

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Necroptosis is a form of programmed necrotic cell death mediated by the activity of receptor-interacting protein 1 (RIP1) kinase (1). This type of cell death is caspase-independent and marked by early membrane permeabilization and does not exhibit any other hallmarks of apoptotic cell death such as phosphatidyserine externalization or DNA strand breaks (1). Thus, the markers available to characterize apoptotic cell death cannot be used to analyze necroptosis. Only one marker of necrosis has been identified, and that is the release of the chromatin protein high mobility group B1 (HMGB1) (2). However, we have found the release of HMGB1 to occur at a late stage of death. In this study we have found that cyclophilin A (CypA), a cytosolic peptidyl-prolyl cis-trans isomerase, is released early in necroptosis. We propose that the release of CypA may be used as a biomarker for necroptosis and other cell death processes when the integrity of the plasma membrane is compromised.

Since necroptotic cells exhibit early permeabilization of the plasma membrane (1), we hypothesized that the release of intracellular protein(s) may be used as a biomarker for necroptosis. Treatment of L929 cells with zVAD.fmk induces necroptosis, which can be inhibited by treatment with the RIP1 kinase inhibitor, necrostatin-1 (Nec-1) (1). We observed the appearance of a strong band around 17 kD by SDS-PAGE in the supernatant of zVAD-treated L929 cells at an early stage of cell death (Supplemental Figure 1, top panel). Mass spectrometry analysis identified this band as cyclophilin A (CypA), a soluble cytosolic protein that catalyzes the cis-trans isomerization of proline bonds. The release of CypA was further confirmed by western blot (Supplemental Figure 1, bottom panel). In contrast to the necrosis marker HMGB1, the timing of CypA release precedes or occurs around the same time as that of HMGB1 (Figure 1A). Nec-1 blocks the release of CypA into the supernatant (Figure 1B).

To determine if the release of CypA from the cells was specific to necroptosis, we tested the supernatant of apoptotic cells. We were unable to detect CypA released from Jurkat cells treated with TNFα and CHX to induce apoptosis, even though intracellular CypA appears to be degraded at 24h when cell death was completed (Figure 1C). However, FADD-deficient Jurkat cells, which undergo necroptosis in response to TNFα, did release CypA (Figure 1C).
We also tested different inducers of apoptosis for the ability to release CypA. Treatment of
doxorubicin and staurosporine caused a small amount of CypA release at later stages of cell
death, hours after caspase-3 activation (Supplemental Figures 2-3). Thus, CypA may also be
released after permeabilization of the plasma membrane in late phase of apoptotic cell death.
However, since apoptotic cells are efficiently engulfed \textit{in vivo}, the release of CypA by
apoptotic cells may not occur \textit{in vivo}.

To examine if CypA could be released by permeabilization of the cells, we treated cells with
the detergent digitonin, which led to cell membrane permeabilization. We found that
digitonin permeabilized cells also release CypA (Supplemental Figure 4). This may explain
why CypA is released during necroptosis, which exhibit early permeabilization of the
plasma membrane (1).

Elevated serum CypA levels have been found in inflammatory diseases such as rheumatoid
arthritis and CypA is secreted \textit{in vivo} by progressive solid tumors (3, 4). CypA release from
necrotic cells may play a role in the effects of necrosis on surrounding tissue. Necrosis is
typically associated with inflammation. Necroptosis occurs in models of neuronal ischemia
and traumatic brain injury (1, 5). Treatment with Nec-1 significantly reduces cell death and
inhibits the recruitment of neutrophils to the injured area (5). Extracellular CypA has been
shown to induce a rapid inflammatory response upon injection \textit{in vivo} and to recruit
neutrophils. This recruitment is dependent on the CypA receptor, CD147 (EMMPRIN) (3).
Signaling of CypA through CD147 is proposed to contribute to the migration of neutrophils
into the joints in rheumatoid arthritis and possibly in the destruction of bone and cartilage in
the disease (6).

We propose that CypA may be used as a biomarker for necroptosis. The release of CypA, in
combination with Nec-1, a specific inhibitor of necroptosis, may be used as an assay for the
activation of necroptosis, both \textit{in vitro} and \textit{in vivo}.

\textbf{Supplementary Material}

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Cyclophilin A is released from necroptotic cells as a result of cytoplasmic membrane permeabilization. **A**, L929 cells were treated with 100μM zVAD.fmk for indicated hrs. The supernatants were collected, concentrated, and analyzed by SDS-PAGE followed by western blotting using anti-CypA and anti-HMGB1. **B**, L929 cells were treated with 100μM zVAD.fmk in the presence or absence of 30μM Nec-1. The cell lysate and supernatant were analyzed by western blotting using anti-CypA. Anti-Tubulin was used as a control. Cell viability was determined by ATP assay and indicated as a bar graph below. **C**, FADD-deficient Jurkat cells (left panel) or wt Jurkat cells (right panel) were treated with 10ng/ml hTNFα to induce necroptosis, or 10ng/ml hTNFα + 0.5μg/ml CHX to induce apoptosis, respectively. The cell lysate and supernatant were analyzed by western blotting using anti-CypA. The percentages of cell death were determined by ATP assay and indicated below.