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Elevation of serum fortilin levels is specific for apoptosis and signifies cell death in vivo

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ABSTRACT

Background: Billions of cells undergo apoptosis each day in the average normal adult. The ability to readily assess the degree of apoptosis in human diseases is hampered by the lack of sensitive and specific serum biomarkers of apoptosis. Fortilin is a novel prosurvival molecule that protects cells against various noxious stimuli. While fortilin is secreted into the extracellular space under certain conditions, the relationship between the serum concentration of fortilin and the presence and extent of apoptosis in vivo remains unknown.
Methods & results: Using a newly developed fortilin ELISA system, we show here that fortilin exists in the normal human and mouse circulation. We further demonstrate that fortilin serum levels are significantly elevated in patients with solid cancer, in response to anti-cancer chemo- or radiation therapy. The elevation of fortilin serum levels is more robust and sensitive than that of such previously-reported serum biomarkers of apoptosis as fragmented cytokeratin-18, cytochrome c, and nucleosomal DNA. In addition, targeted apoptotic liver damage induced by Jo2 anti-Fas (CD95) antibody consistently and significantly increased serum fortilin levels in C57BL/6J mice. Finally, when challenged by anti-human-Fas IgM antibody, Jurkat leukemic T cells apoptosed and released fortilin into the medium before plasma membrane integrity was compromised.
Conclusions: Taken together, these data suggest that serum fortilin levels reflect the degree and extent of apoptosis occurring in vivo.
General significance: Fortilin is a viable serum biomarker of in vivo apoptosis and can be utilized to noninvasively assess the status of in vivo apoptosis in humans.

1. Introduction

Approximately 50 to 70 billion cells undergo apoptosis each day in the average normal adult [1]. Serum biomarkers of apoptosis – molecules that can be readily and objectively measured as indicators of normally and pathologically occurring apoptosis at tissue and organ levels – would allow clinicians to easily monitor the status of apoptosis associated with the diseases and conditions they treat—such as apoptosis-induced skeletal muscle atrophy resulting from cancer (cachexia), aging (sarcopenia) [2], starvation, denervation, disuse, and inflammation [3]. Cancer cells undergo apoptosis at a higher rate than do normal cells and massively apoptose in response to radiation therapy and chemotherapy [4]. Serum biomarkers of apoptosis could thus allow clinicians to screen patients for certain cancers or to monitor the response of patients with cancer to anti-cancer chemo- or radiation therapy [5].

Thus far, three serum biomarkers of apoptosis have been reported in the literature including the fragmented cytokeratin-18 (fCK18, detectable by the M30 antibody), nucleosomally-cleaved genomic DNA (n-DNA), and cytochrome c (Cyt C) [5]—each with notable limitations to their utility. The utility of fCK18 is limited to apoptosis occurring in cells of epithelial origin [6]. The utility of circulating n-DNA is diminished because it can be rapidly degraded by serum DNases [7]. Cyt C is reportedly released from both apoptotic [8] and necrotic cells [9], depending on the extent of cellular damage, thus limiting its specificity. Further, these candidate serum apoptosis biomarkers have not been extensively characterized or validated at clinical, whole animal, and cellular levels.

Fortilin, also known as translationally controlled tumor protein (TCTP) and IgE-dependent histamine releasing factor (HRF), is a 172-amino acid nuclear-cytosolic shuttle protein that was originally cloned

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in 1989 by Gross and others as a molecule abundantly expressed in tumor cells [10]. A multifunctional protein implicated in various cellular functions [11–16], fortilin possesses potent anti-apoptotic activity [12, 17–22]. It binds the sequence-specific DNA binding domain of p53 and prevents p53 from transcriptionally activating the proapoptotic gene Bax [23]. Fortilin also binds to and stabilizes MCL1 [22], a macrophage survival factor [24,25]. In addition to being intracellularly located, fortilin can be trafficked into exosomes – small secretory vesicles – and eventually be released into the extracellular space in an ER/Golgi-independent fashion [15]. However, it remains unknown if fortilin actually circulates in the blood of normal humans and animals. Further, it is not known if serum fortilin levels change in response to various pathophysiological conditions, including apoptosis occurring in body tissues.

Since it is a potent anti-apoptotic molecule that can be secreted into the extracellular space, we hypothesized that fortilin could be a viable serum apoptosis biomarker. Here, we report for the first time that fortilin is present in the blood of healthy humans and mice. We also show that anti-cancer chemo- or radiation therapy causes serum fortilin levels to increase, more robustly, sensitively and specifically than fCK-18, Cyt C, or n-DNA in humans. Strikingly, the release of fortilin from the cell precedes any signs of compromised plasma membrane integrity. We therefore propose that serum fortilin is a sensitive and robust biomarker of apoptosis occurring in vivo.

2. Materials and methods

2.1. Cell culture and cell lines

The Jurkat cell lines (Clone E6-1) were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were maintained in high-glucose Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in an atmosphere containing 10% CO_2.

2.2. Cell-based assay of biomarkers release and plasma membrane disruption

Jurkat cells were seeded in 18 wells of 6-well plates (5×10^5 cells/well) in RPMI medium with 5% FBS. The next day, the cells were washed once with phosphate buffered saline (PBS) and re-suspended in 1 mL of RPMI medium containing 12.5 ng/mL of anti-human Fas IgM (clone CH11). At each time point (0, 0.5, 1, 2, 4, and 8 h, N = 3 for each time point), we harvested 500 μL of cell suspension, centrifuged it at 100 *g* for 5 min, transferred the medium to fresh microfuge tubes, and froze both the media and cell pellets separately at −80 °C until the assays for LDH, n-DNA, fortilin, Cyt C, and fCK-18, were performed. For 7-AAD staining, we added 20 μL of 7-AAD solution (BD Pharmingen) to 400 μL of cell suspension and incubated it for 10 min at room temperature, shielded from light. We counted total and 7-AAD-positive cells under the fluorescence microscope as described previously [26]. The integrity of the plasma membrane of the cells with positive 7-AAD signal is compromised. At least 200 cells were counted and the 7-ADD index was calculated as (the number of 7-AAD-positive cells) / (the number of total cells) * 100.

2.3. Mouse model of targeted liver apoptosis

All animal procedures were performed according to a protocol approved by the UTMB Institutional Animal Care and Use Committee (IACUC), in accordance with the National Institutes of Health guidelines and the "Position of the American Heart Association on Research Animal Use." We induced apoptosis in the liver of C57BL/6J male mice (12 weeks of age) by intraperitoneal administration of the Jo2 anti-Fas antibody (1.25 μg/body weight in gram, resulting in approximately 25 μg of antibody per mouse): PBS was used as a control. Once injected, the mice became ill within 3 h; half of them were dead within 6 h, as described previously [27]. At 5–9 h after anti-Fas injection, when they were clinically moribund, the mice were sacrificed, their blood collected, and the organs harvested for further analyses. Jo2 antibody binds the mouse Fas antigen and induces Fas-mediated apoptosis in the liver without affecting any other tissues as reported previously [27]. Transmission electron microscopic examination reportedly showed a lack of phagocytosis of apoptosed cells. Also, there was no gross leakage of cell contents into the extracellular space observed by the same examination [27].

2.4. Caspase-3 activity

Caspase-3 assays were performed as we described previously [28]. In brief, cells were suspended in cell lysis buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.01% Triton X-100), subjected to three freeze-thaw cycles, and centrifuged at 14,000 *g*. Aliquots of cleared cell lysates were incubated with 2.5 mM rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide)(Z-DEVD-R110) substrate (Invitrogen-Molecular Probes, Grand Island, NY). Caspase-3 activities were determined every 5 min for 90 min by measuring fluorescence (excitation/emission = 496/520 nm), using the SpectraMax Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA), and expressed as relative fluorescence units (RFU).

2.5. TUNEL staining

TUNEL staining was performed as we previously described [23], using the FragEL™ DNA Fragmentation Detection Kit (EMD Millipore, Calbiochem, Billerica, MA), according to the manufacturer's instructions. At least 600 cells were counted and TUNEL indices were calculated as the number of TUNEL-positive cells divided by the number of total cells counted and expressed as percentages.

2.6. Lactate dehydrogenase (LDH) activity assay

Serum LDH activity was measured by the LDH Activity Assay Kit (Sigma-Aldrich, St. Louis, MO; Catalog Number: MAK066) according to the manufacturer's instructions.

2.7. Serum Cyt C assay

Serum Cyt C was quantified by the Human Cytochrome C Quantikine ELISA kit (R&D Systems, Minneapolis, MN; Catalog Number: DCTC0) according to the manufacturer's instructions.

2.8. Serum alanine transaminase (ALT) assay

Serum ALT was quantified by the mammalian liver profile rotor and VETSCAN VS2 (Abaxis, Union City, CA) according to the manufacturer's instructions.

2.9. DNA fragmentation assay

The Cell Death Detection ELISA PLUS kit (Roche, Indianapolis, IN, Catalog Number: 11774425001) was used according to the manufacturer's instruction and modifications described previously [23]. We added 20 μL of serum from a patient into a well of a streptavidin-coated 96-well plate, in triplicate. We then added 80 μL of incubation buffer (PBS supplemented with 1% BSA, 0.5% Tween 20 and 1 mM EDTA), containing peroxidase-conjugated mouse anti-DNA antibody (MCA-33) and mouse biotinylated anti-histone antibody (H11-4) to the wells and incubated the plate for 2 h at room temperature on a shaker. The plate was then washed three (3) times with incubation buffer before we added 100 μL of 2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ATBS) solution to each well,