Published in final edited form as:

Crit Care Med. 2015 November ; 43(11): 2506–2508. doi:10.1097/CCM.0000000000001289.

Imaging Apoptosis in Sepsis – a technology we would die for!

Richard S. Hotchkiss, M.D. and

Washington University School of Medicine, St. Louis, MO, 660 South Euclid, St Louis, MO 63110

Elliott Crouser, M.D.

Ohio State University Medical College, Columbus, OH

Richard S. Hotchkiss: hotchkir@anest.wustl.edu

Keywords

sepsis; apoptosis; cell death; imaging; lymphocyte

Cells die in a multitude of different ways; among these is a well-established sequence of cellular events called apoptosis (1). Apoptotic cell death is activated in mice and in men in sepsis (2,3) and is important because of its effects on immune effector cells and gastrointestinal epithelial cells. Lymphocyte apoptosis occurs in organs and lymph nodes throughout the body, results in profound depletion of immune effector cells, and is one contributing factor to the development of the immunosuppressive phase of the disorder (4). New immuno-adjuvant therapies of sepsis are either underway or in the planning stages and several of these therapies, i.e., interleukin-7 and anti-programmed cell death ligand-1 (anti-PD-L1), have potent anti-apoptotic effects to prevent lymphocyte cell death (5). Therefore, development of a non-invasive, imaging modality to detect and serially follow apoptotic cell death could be helpful in evaluating efficacy of immuno-adjuvant therapies both in pre-clinical animal models and potentially in septic patients. Work by Zou et al. provides one potential means to accomplish this difficult goal (6).

Zou and colleagues used near-infrared (NIR) imaging of a probe, i.e., fluorochrome-labeled annexin V, that literally “lights up” cells undergoing apoptosis in an *in vivo* mouse cecal ligation and puncture (CLP) sepsis model. Early in the process of cellular apoptosis, phosphatidylserine translocates from the inner leaflet of the plasma membrane to the outer leaflet. Annexin V has a strong affinity for phosphatidylserine and fluorescently-labeled annexin V is widely used to detect apoptosis by flow cytometry and fluorescence microscopy (1). Zuo et al. also evaluated whether toll-like receptor (TLR3) deficient mice had amelioration of sepsis-induced lymphocyte apoptosis. TLR3 recognizes pathogen-associated molecular patterns (PAMPs) and activates host immune defenses to control microbial invasion.

Studies by Zuo and associates demonstrated increased annexin-V imaging in thymus, spleen, and liver of septic versus sham mice. In addition, mice with more severe sepsis had greater uptake of annexin-V in these three organs compared to mice with milder sepsis. The increased sepsis-induced apoptosis detected by NIR imaging of annexin-V was verified by flow cytometry of isolated thymocytes and splenocytes for active caspase 3 and by TUNEL. Other results showed decreased apoptosis in spleen but not thymus in TLR3 null mice. Flow

cytometry for active caspase-3 and immunohistochemical staining for nuclear strand breaks confirmed increased apoptosis in spleen and thymus.

The authors are to be commended for their efforts to develop a novel non-invasive method to track apoptotic cell death *in vivo* and for their use of additional, traditional methods to confirm their new imaging approach. However, there are a number of issues which will limit the utility of this method of following annexin-V imaging to detect apoptotic death in sepsis. First, imaging of apoptotic cells by annexin-V lacks sensitivity because apoptotic cells are rapidly engulfed by professional phagocytic cells and therefore, are only present in the tissues for a brief time period (7). Indeed, this was one reason why apoptosis was not appreciated as a method of cell death until relatively recently. Thus, it takes a tremendous amount of apoptotic cells (which temporarily overwhelm the body's phagocytic capacity) in order to be detected. In the initial phase of sepsis, there is a huge "burst" of immune cell apoptosis occurring in thymus, spleen, lymph nodes, and in gastrointestinal-associated lymphoid tissues. However, after this initial phase, the degree of apoptosis remains elevated over baseline but, is much less than the initial phase. Therefore, there would most likely be a poor signal-to-noise ratio when using imaging techniques to detect apoptosis in sepsis if it were conducted at 48-72 hours after sepsis onset.

A second potential drawback in using annexin-V imaging to detect apoptotic death in sepsis is that this method provides no information regarding the type of cells that are dying. This inability to discriminate the types of cells which are dying may be particularly relevant in the findings by Zou et al. who showed that there is increased apoptosis in liver of mice with sepsis using annexin-V labeling. These findings are at odds with previous studies from several investigators who used a variety of immunohistochemistry methods to demonstrate that there is very little hepatocyte death in the mouse CLP model of sepsis (8). Although our group has shown increased hepatocyte necrosis and apoptosis in autopsy samples of patients dying of sepsis (3), our work in the murine CLP model of sepsis failed to show hepatocyte apoptosis (2). How to reconcile these differences in the various studies? It is likely that much of the apoptosis that is being detected by NIR annexin-V imaging is due to apoptotic polymorphonuclear leukocytes (9), lymphocytes, and immature monocyte/macrophages that have migrated into the liver, and may also reflect circulating cell fragments from apoptotic cells that are cleared by the liver (10) during sepsis. Secondly, it is possible, given the liver's role to clear various drugs and toxins from the circulation that some increased labeling in the liver may be secondary to the liver's elimination of the annexin V label. The authors' findings of decreased bladder concentrations of the annexin-V label is consistent with decreased renal elimination of the label (conceivably due to intravascular volume depletion and/or impaired renal function in septic versus sham mice) which might have resulted in compensatory hepatocyte elimination of the indicator.

A final intriguing finding of Zuo et al is their report that compared to wild type mice with sepsis, TLR3 null mice had decreased sepsis-induced apoptosis in spleen but not thymus. Thymocytes are particularly sensitive to apoptotic stimuli because immature thymocytes contain much less of the anti-apoptotic Bcl-2 molecule compared to more mature immune cells in spleen. As much as 50-70% of thymocytes may be lost in 24-48 hours after severe sepsis in mouse models. The protection in *splenocytes* against sepsis-induced apoptosis

afforded by TLR3 knockout was relatively modest as indicated by quantitative flow cytometry and annexin V/propidium iodide staining. Nonetheless, the TLR3 experiments serve to demonstrate the potential utility of NIR imaging of annexin V for experimental purposes in the mouse.

The ability to sequentially following the magnitude of lymphocyte apoptosis as a guide to severity of sepsis and as a means to evaluate the efficacy of immuno-adjuvant therapies could be invaluable. The use of NIR spectroscopy is also limited by the penetrance of light into the tissues, which is said to be 4-5 cm for the annexin V imaging protocol. This level of penetration is adequate for mice; however, it would provide very limited information in humans. Although whole body imaging of apoptotic cell death in sepsis is unlikely to be clinically useful at the present time, it may well be feasible to follow the degree of apoptosis in *circulating* lymphocytes in septic patients by using flow cytometry. As some investigators are fond of saying: “flow is the way to go”. There is evidence that the degree of apoptosis in circulating lymphocytes from patients with sepsis correlates with sepsis severity (11). Importantly, multiple laboratories have shown that IL-7 and anti-PD-1 block apoptosis of lymphocytes from septic patients *ex vivo* (12–15). Evaluation of blood samples from septic patients who are treated with IL-7 or anti-PD-1/L1 using flow cytometry could be useful in showing which patients are responders and in gauging drug dosing. Flow cytometry offers many other advantages as well by enabling investigators to correlate prevention of cell death with improvement in cell functional status, for example, restoration of cytokine production. As demonstrated by Zuo *et al* NIR imaging of annexin V is likely to be of some limited value in terms of assessing the overall magnitude of cell apoptosis during sepsis in small animal models early during sepsis. However, unlike flow cytometry, which can detect several specific cell markers simultaneously, NIR annexin V imaging lacks sensitivity and specificity for monitoring the rate of apoptosis of lymphocytes *in vivo*.

Precision care of the septic patient is going to require understanding, assessment, and modification of the apoptosis pathway. The work by Zuo and colleagues is one tiny step along this path to precision medicine precisely because it highlights the specificity and sensitivity problems. Current generation *in vivo* probes can't make the fine distinctions among which cells etc. *Ex vivo* flow cytometry samples only a tiny fraction of the susceptible and likely important cells. Progress in sepsis care will depend strongly on improvements in both technologies, and of course agents that can selectively modify cells susceptible to this death pathway in sepsis.

Acknowledgments

Copyright form disclosures: Dr. Hotchkiss received support for article research from the National Institutes of Health (NIH). Dr. Crouser received funding from Mallinckrodt Pharmaceutical. His institution received funding from Beckman Coulter, Inc. and the NIH/NHLBI.

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