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Use of a Fluorescently Labeled Poly-Caspase Inhibitor for *In Vivo* Detection of Apoptosis Related to Vascular-Targeting Agent Arsenic Trioxide for Cancer Therapy

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Abstract

Arsenic trioxide (ATO, Trisenox) is a potent anti-vascular agent and significantly enhances hyperthermia and radiation response. To understand the mechanism of the anti-tumor effect *in vivo* we imaged the binding of a fluorescently-labeled poly-caspase inhibitor (FLIVO) in real time before and 3 h or 24 h after injection of 8 mg/kg ATO. FSaII tumors were grown in dorsal skin-fold window chambers or on the rear limb and we observed substantial polycaspase binding associated with vascular damage induced by ATO treatment at 3 and 24 h after ATO injection. Flow cytometric analysis of cells dissociated from the imaged tumor confirmed cellular uptake and binding of the FLIVO probe. Apoptosis appears to be a major mode of cell death induced by ATO in the tumor and the use of fluorescently tagged caspase inhibitors to assess cell death in live animals appears feasible to monitor and/or confirm anti-tumor effects of therapy.

Keywords

Anti-vascular; Apoptosis; *In vivo*; Real time

Introduction

The detection of apoptotic cells in tumor and tumor-associated vascular tissue has great value in the study of anti-tumor agents. The ability to image and/or quantify apoptosis events in the tumor over time after a given treatment may allow us to better schedule and dose new agents, monitor sensitivity of individual patients to therapy, and learn how to best sequence multi-modality therapy. There are possible applications to many other areas of medicine as well. Unfortunately, there are few, if any, reagents currently available to assess or monitor the occurrence of apoptosis in living tissue. TUNEL (t-UTP nick end labeling) has been arguably the most common label used for the identification of apoptotic cells, even though it has a relatively low level of specificity for apoptotic cells as nicked DNA can occur in cells dying by either necrosis or apoptosis (1-3). Another major drawback is that TUNEL cannot be used on living cells or animals since it requires permeabilization of the cell membrane. What has been attempted to image apoptotic activity in patients or animals

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has typically involved radioactive labeling with annexin V and autoradiography or other complex imaging strategies using MRI/MRS (4-8). Annexin V has a benefit as an early marker of programmed cell death, but it is also very short lived and difficult to reliably detect since the timepoint of assessment can be critical. In addition, Annexin V does not bind to all apoptotic tumor cells (9) and it also binds positively to normal and healthy bone marrow derived cells (10). Furthermore, the inversion of phosphatidyl-serine may not be exclusively related to apoptosis and this adds to the background issues (11).

We are currently studying the mechanism of action and therapeutic potential of agents directed towards the tumor vasculature. We discovered that Arsenic Trioxide (As_2O_3 , ATO), an FDA-approved leukemia drug, also has potent vascular damaging effects against multiple experimental tumor models and can sensitize tumors to radiation and thermal therapy (12). However, the specificity and mechanism with which ATO targets tumor parenchymal or stromal cells is still under study. Therefore, we sought a means to study the amount of apoptosis induction caused by an agent such as ATO in the live animal. We recently hypothesized that a cell permeable, specific probe for activated caspases tagged with a fluorescent molecule may be able to localize the extent of apoptosis in living tissue upon i.v. injection of the agent. As a means to address these questions, we have adopted a dorsal skin-fold chamber technique to observe individual FSaII murine fibrosarcoma tumors in real time *in vivo* before, during and after ATO treatment. We identified a poly-caspase binding reagent for detection of apoptosis not previously optimized *in vivo* (FLIVO, polycaspase inhibitor conjugated to carboxyfluorescein, Immunochemistry Technologies, LLC, Bloomington, MN). Since FLIVO is actually an inhibitor of activated caspases, and thus of the apoptotic process, it binds to cells undergoing caspase-dependent apoptosis and inhibits the progression of programmed cell death to some extent. The temporary hold put on apoptotic cells by FLIVO suggested that it may be a good reagent for live detection of apoptotic activity (13). Ultimately, our goals include further development of this reagent to assess the affects of therapies such as anti-angiogenesis strategies and radiation therapy in live animals or patients as a measure of therapeutic activity.

Materials and Methods

FSaII Model

This fibrosarcoma cell line from female C3H mice, originally obtained as a kind gift from Dr. Herman Suit at Massachusetts General Hospital, is grown in RPMI1640 medium supplemented with 10% calf serum. All studies were approved by the University of Minnesota institutional animal care and use committee.

Arsenic Trioxide

An injection of 8 mg/kg i.p. Arsenic trioxide (ATO or Trisenox, Celphalon Oncology, Inc., Frazer, PA) was performed by using a clinical grade 1 mg/ml stock solution for each mouse and imaging was performed at specific times after this injection. Control mice were injected with an equal volume of phosphate buffered saline, pH 7.4.

Green FLIVO™ Reagent

FLIVO (FAM-VAD-FMK, 50 µg per vial, Immunochemistry Technologies, LLC, Bloomington, MN) was first dissolved in 50 µL of DMSO. For injection it was then diluted by the addition of 200 µL of sterile PBS, pH 7.4. After an i.v. injection of 50 µL of FLIVO cell permeant probe *via* the lateral tail vein, the FLIVO reagent was allowed to circulate in the mouse for 30 minutes before analysis. Fluorescent images were captured at 20× using a Hamamatsu C2400 camera (Hamamatsu, Japan) and Broadway Imaging Software (Data Translation, Malboro, MA) on an Eclipse TE200 bench-top microscope (Nikon, Japan).

Window Chamber Tumor Growth and Intravital Microscopy

Skin-fold chambers made of anodized aluminum frames were surgically implanted into a fold of dorsal skin in female nu/nu mice. Briefly, the dorsal skin was sandwiched between two identical anodized round aluminum frames. The 19 mm × 22 mm chamber was held fixed on the mouse by three screws between the frames. The skin was also attached to the chamber with 4-O silk. The skin on both sides of the viewing region was removed, exposing the dermis containing the microvasculature. Excess fascia on the dermis was removed to assist in clear visualization of the microvasculature. Windows milled from quartz glass microslides (Chase Scientific Glass, Rockwood, TN) were used to cover the vascular area. The distance between the window and the opposing aluminum frame was maintained at 450 µm using spacers on the screws, leaving room for seeded tumor to grow. Tumor cells were added in 30 µL of matrigel just before placement of the glass windows. Treatments and imaging were performed over the course of tumor growth and treatment as described (14). The mouse was laid on a specially constructed microscope stage, which allows the window chamber to be held in place perpendicular to the light path, similar to a microscope slide.

Flow Cytometry Analysis

Tumor tissue was collected from the window chamber by scraping the tumor out of the chamber into a 0.25% trypsin solution in RPMI640 medium, stirring for 30 minutes with 10 µg/mL DNase and 5 µg/mL collagenase, and finally filtering the suspension using a 70 µm cell strainer. Flow cytometry was then performed using a FACS Caliber flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) for the analysis of apoptosis in the cell population obtained from the window chamber. The fluorescence derived from the intracellular bound FLICA inhibitor probes was monitored *via* argon laser driven 488 nm excitation/530 nm emissions filter-tandem setting. All data was acquired with an event acquisition set for 10,000 events. Data was analyzed using CellQuest Pro cytometer software.

Rear-limb Tumor Growth and Histological Analysis

A subcutaneous injection of 2×10^5 FSaII cells was performed in the right-rear limb of the mouse and tumors averaging 8 mm × 10 mm grew by 8-10 days after injection. The mouse was sacrificed and the tumor was removed at the specified timepoint after ATO treatment and 30 min after FLIVO injection and snap frozen in liquid nitrogen. Cryosections at 5 µm were prepared and the fluorescent signal was imaged using an Olympus BX40 fluorescent microscope at 20× magnification using the FITC filter.

Results and Discussion

In our studies, 10 µg of FLIVO was administered *via* the tail vein in 50 µl of a 20% DMSO/80% PBS solution at various times after treatment with the vascular damaging agent ATO and 30 min later, fluorescent imaging was performed. At each timepoint, brightfield imaging was performed to assess visible damage to the tumor vessel bed. Figure 1A summarizes the tissue damage and vascular effects of ATO treatment observed within the window chamber tumor growth model. Observation of tumor tissue within the window chamber validated our previous work with ATO in hind-limb tumor models that suggested vascular damage as a primary cause of the anti-tumor effects of ATO (1-3, 12). Total clearance of unbound FLIVO has been found to occur by 30-50 min in mice and, therefore, we chose 30 min after injection to assess binding of the reagent in the tumor (unpublished observation).

As the images in Figure 1B demonstrate, 3 h and 24 h after a single injection of 8 mg/kg ATO there was substantially more green fluorescence generated in the tumor after i.v. injection of the FLIVO reagent than in a sham treated control tumor growing in the window

chamber. Two individual tumors were imaged for each condition and representative fluorescent signal fields are shown. The apoptosis signal also correlated with the extent of visible vascular damage after ATO treatment (Figure 1A), peaking in overall cell specific signal at 3 h and decreasing at 24 h after drug injection. To confirm the cellularity of these results, the window chamber tumor tissue was collected, cells were dissociated by enzymatic and magnetic stirring methods (15), in order to remove any unbound intra or extracellular signal, and resulting single cells were analyzed by flow cytometry. The results revealed significant cellular binding of the FLIVO probe at 3 h after ATO injection, which decreased but remained elevated at 24 h after injection, agreeing with our visual observations of apoptotic signal in the window chamber (Figure 1C). However, as might be expected from an acute vascular event, the numbers of cells staining positive for FLICA were reduced at 24 h after drug injection, suggesting that apoptosis may have occurred at earlier points and those cells had subsequently been removed from the tissue, along with the probe. Indeed, in Figure 1B, the signal detected was less identifiable with cellular structures and instead may have been partially due to increased extravasation or trapping of the probe *via* the damaged blood vessels.

Finally, we treated mice bearing FSaII tumors in the rear limb with 8 mg/kg ATO or left them untreated, injected the FLIVO reagent i.v. 3h later, waited 30 min, sacrificed the animal, and snap-froze the tumor for histological sectioning. We were able to identify significantly more regions of cellular binding of the poly-caspase probe in the ATO treated mouse than in control, suggesting that the FLIVO poly-caspase probe did label cells undergoing caspase-dependent apoptosis *in vivo* (Figure 1D) for subsequent analysis after histological preparation.

The small oligomer sequences (such as FLIVO) that bind to activated caspases have been intensely studied for about the last ten years almost exclusively *in vitro* as markers or blockers of apoptotic activity in cells (13,16). The general solubility, cell permeability and ability to form covalent bonds with the active site of the enzyme are useful for studies employing microscopy, flow cytometry, and microplate based assays (17,18). The free or unbound probe is readily removed from cell samples with simple washing as the first studies using these compounds demonstrated (17,18). We undertook the current study to develop these markers as potential *in vivo* markers of apoptosis in living animals since we are interested in monitoring the effects of experimental therapies on solid tumors. A reagent such as FLIVO, that we describe here, appears to have great potential to detect immediate tumor cell death characteristics in response to various interventions and also as a predictive or prognostic tool for individualized targeted therapy of cancer or other diseases. There were no indications of toxicity of the FLIVO reagent, even after multiple injections. We interpret our results as a first step towards validating the probe as an *in vivo* apoptosis-tracer, as it has already been validated as an *ex vivo* caspase-tracer (16-18). Whether caspase-independent programmed cell death will play a significant role in the accurate use of this probe in various systems remains to be studied as well. We are currently carrying out studies for further development and validation of poly-caspase inhibitors as a method for non-invasive detection of apoptosis *in vivo*; ultimately with labeling strategies amenable to PET or MRI. If successful, these probes may assist numerous areas of the biomedical research community in basic and translational research to do longitudinal and minimally invasive studies in animal models or patients. In view of our initial experience, further studies to optimize and confirm the pharmacokinetics and binding characteristics of FLIVO or similar molecules in various conditions and living systems are highly warranted.

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Figure 1.

(A) Tiled 4× magnification images of an FSaII tumor grown in the window chamber before and at timepoints after injection of the anti-vascular agent arsenic trioxide (ATO). (B) Subsequently, we studied the possible induction of apoptosis and the dose/time response of this mode of cell death after treatment in two individual FSaII tumors grown in the window chamber for each condition. Each panel was taken from a separate window chamber tumor. (C) In order to confirm that the signal was specific labeling of the activated caspases in apoptotic cells, we removed the chamber, excised the tumor tissue, dissociated the cells in medium to allow clearance of unbound probe from the intracellular or extracellular environment and subjected them to flow cytometry for Fluorescein signal. (D) Typical field imaged at 10× magnification from histological sections of FSaII tumor from an untreated mouse or a tumor-bearing mouse that was treated with 8 mg/kg ATO, 3 h prior to injection of FLIVO, and removal of the tumor 30 min later.