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Detergent Sclerosants Stimulate Leukocyte Apoptosis and Oncosis

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WHAT THIS PAPER ADDS

Excessive mixing of sclerosants with blood in the target vessels results in a fall in the final active concentration and a procoagulant profile. At such final low concentrations, sclerosants are not capable of inducing lysis of the circulating leukocytes and, to the contrary, can activate different pathways on these cells and induce inflammation, angiogenesis, apoptosis, and oncosis. The present findings may suggest a potential utility of detergent sclerosants as a novel therapeutic strategy to induce cell death.

Objective/Background: The objective was to investigate the effects of the detergent sclerosants sodium tetradecyl sulfate (STS) and polidocanol (POL) on human leukocytes at sublytic concentrations.
Methods: Leukocytes were isolated and labelled with antibodies to assess for apoptosis and examined with confocal microscopy and flow cytometry. Isolated leukocyte count and viability was assessed using trypan blue, and propidium iodide staining. Phosphatidylserine (PS) exposure, a universal hallmark to measure cell apoptosis, was identified by flow cytometry using lactadherin. Caspases 3, 8, and 9, and Bax activation, as well as inhibitory assays with pan-caspase (Z-VAD-FMK) and Bax (BI-6C9) were assessed to determine apoptotic pathways. Porimin activation was used to assess cell permeability.
Results: Up to 40% of leukocytes maintained membrane integrity at sublytic concentrations ($\leq$0.15%) of sclerosants. The remaining 60% did not maintain membrane integrity but were not completely lysed. PS exposure was increased with both STS and POL exhibiting a dose- and time-dependant trend. While activation of caspases 3, 8, and 9, as well as Bax activation, were increased in leukocytes stimulated with low concentrations of STS, only caspases 3 and 9 and Bax were increased with POL. Inhibitory assays demonstrated caspases 3, 8, and 9, and Bax inhibition at low concentrations with both STS and POL. Both agents increased the leukocyte activation of porimin at all concentrations. On confocal microscopy, stains for caspases 3, 8, and 9, and Bax were increased for both STS and POL. Porimin stain was markedly positive for both STS and POL.
Conclusion: Both sclerosants induced leukocyte apoptosis at sublytic concentrations. STS activated both extrinsic and intrinsic pathways of apoptosis, while POL stimulated the intrinsic pathway of apoptosis only. Both agents induced oncosis. Based on these results, STS appears to have a greater effect than POL.
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INTRODUCTION

Sodium tetradecyl sulfate (STS) and polidocanol (POL) are detergent sclerosants commonly used to induce endothelial lysis in the treatment of varicose veins and other vascular lesions. The lytic effect on the endothelial cells results in exposure of the underlying collagen leading to endovascular fibrosis and occlusion.[1] In a recent study, morphological evidence of sclerosant-induced nonlytic forms of cell death in vascular and circulating blood cells exposed to detergent sclerosants was observed.[2] The cytoplasmic and nuclear changes were consistent with typical morphological changes observed in cells undergoing apoptosis and oncosis. Prior to the present study there has been no attempt to investigate a possible apoptotic effect of sclerosant on cells exposed to these agents during sclerotherapy. Previous observations prompted the investigation of whether sclerosants are capable of inducing apoptosis and oncosis in leukocytes. An apoptotic effect on cells such as leukocytes may open up the possibility of new treatment indications for detergent sclerosants in diseases that have demonstrated an inappropriate activation of apoptosis such as cancers, autoimmune diseases, and neurodegenerative diseases.

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Apoptosis is a tightly regulated enzymatic process resulting in a "programmed cell death" characterised by the formation of apoptotic bodies that are subsequently removed by phagocytes.[3] Here, the cell contents are not released and there is no subsequent inflammatory response. Oncosis is a traumatic cell death that results from the failure of the ionic pumps in the plasma membrane and decreased levels of cellular adenosine triphosphate (ATP). This process involves loss of membrane integrity, cytoplasmic swelling, and release of cell contents, resulting in an inflammatory response. Similarly, lysis results in the rupture of cell contents and a subsequent inflammatory response. While apoptosis and oncosis are mechanisms of cellular death, necrosis is the final stage that cells undergo long after dying.

Apoptosis is mediated via two distinct pathways that ultimately lead to a common pathway of apoptosis. The extrinsic pathway is initiated by the activation of transmembrane death receptors such as Fas. Death receptors are stimulated by a diverse range of ligands such as tumour necrosis factor (TNF)-α, resulting in the activation of a cysteine protease, caspase-8. The intrinsic pathway of apoptosis is initiated by a wide range of signals such as cell stress, radiation, and cytotoxic drugs. This pathway is controlled and regulated by the B-cell lymphoma (Bcl)-2 family of proteins, which govern the permeability of the mitochondrial membrane. They act as proapoptotic (Bax and Bak) or antiapoptotic (Bcl-2 and Bcl-XL) regulators.[4] Subsequently, cytochrome C is released from the mitochondria to interact with Apaf-1, dATP, and procaspase-9 in a protein complex known as apoptosome.

Both pathways ultimately lead to the executioner pathway, which involves activation of further members of the caspase family of enzymes (2, 3, and 7).[4]

The aim of this study was to confirm the morphological findings and to investigate the mechanism of sclerosant-induced nonlytic cell death.

MATERIALS AND METHODS

Materials

The following were used in this study: STS obtained as FIBRO-VEIN 3% (w/v), 47.4 mM (Australian Medical and Scientific, Chatswood, NSW, Australia); POL as AETHOXYSKLEROL 3% (w/v), 51.5 mM (Kreussler, Wiesbaden, Germany); paraformaldehyde (PFA) (ProSciTech, Townsville City, QLD, Australia); bovine serum albumin (BSA), poly-L-lysine solution 0.1% (w/v), and Triton-X100, BID inhibitor BI-6C9 (all Sigma Aldrich, St. Louis, MO, USA). Antibodies used were as follows: Alexa Fluor 647 phalloidin, propidium iodide (PI), Hoechst, Bax mouse mAb (92d2) primary antibodies, and Alexa fluor 488 goat anti-mouse IgG, IgM (H + L) secondary antibody (all Invitrogen, CA, USA); Alexa Fluor 594 goat antirabbit IgG (H + L) (Molecular Probes, Eugene, OR, USA); cleaved caspase-3 (Asp175) antibody, caspase-8 (1C12) mouse monoclonal antibody, and caspase-9 antibody (all Cell Signalling Technology, Arundel, QLD, Australia); human porimin antibody monoclonal mouse IgG (Mab3010) and general caspase inhibitor Z-VAD-FMK (both R&D Systems, Minneapolis, MN, USA); and Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). CD45 APC antimouse antibody, flow cytometry tubes, BD FACS lysing solution and Triton-X-100 were all from Becton Dickinson (Franklin Lakes, NJ, USA). Lactadherin was from Haematologic Technologies (Essex Junction, VT, USA) and ABT 737 was from Navitoxclax (Houston, TX, USA).

Methods

Sample collection. Whole blood (WB) samples were collected in 10-mL ethylenediaminetetraacetic acid (EDTA) tubes (BD Vacutainer, K2E 18.0 mg; Becton Dickinson) following informed consent from healthy volunteers taking no medications or supplements.

Leukocyte isolation. Red cell lysis buffer (2 mL; 8.3 g NH_4Cl, 1.0 g $KHCO_3$, and 1.8 mL EDTA disodium salt) was added per 100 µl WB, incubated at room temperature, and protected from light for 10 minutes. Samples were then centrifuged at 400 *g* for 10 minutes, supernatant was discarded, and cell concentration was adjusted to 1×10^6 cells/mL using a haemocytometer. White cell pellets were resuspended in 5% BSA and divided into different treatment groups. Five percent BSA was used as a negative control, ABT-737 was used as a positive control and increasing concentrations of sclerosants (0.075%, 0.15%, 0.30%, 0.60%, and 1.2%) were added to treatment groups.

Fluorescence immunostaining. Leukocytes were incubated with STS and POL at 0.15%, 0.25%, and 0.30% for 5 minutes. Cells were fixed with 2% PFA/phosphate buffered saline (PBS) for 10 minutes and blocked with 3% BSA/PBS for 30 minutes at room temperature. For intracellular antibodies, a permeabilisation step with 1% BSA, 0.5% Triton-X100 on PBS was added for 10 minutes before blocking the cells. Samples were incubated for 45 minutes with the appropriate primary antibody for caspase-3, caspase-8, caspase-9, Bax, or porimin, rinsed, and secondary antibody (Alexa Fluor 488 or Alexa Fluor 594 [1:250]), Hoechst DNA binding dye, Alexa Fluor 647 phalloidin or PI were added for 45 minutes in the dark. Cells were then cytospinned for 4 minutes and coverslips mounted using VectaShield mounting media. Samples were visualized using a Leica DM 6000 confocal microscope. Each channel was recorded independently and pseudocolour images were generated and superimposed. Maximum projections were performed with Image J software (National Institutes of Health, Bethesda, MD, USA).

Live cell imaging. Cells were placed on 12-well plates precoated with poly-L-lysine and placed in an incubator for 30 minutes to allow adherence. Cells were stained with fluorescein isothiocyanate (FITC)-conjugated lactadherin for 10 minutes and visualized on a live cell imaging (Leica DM 6000 [Power Mosaic]) microscope adjusted to a physiological environment (37 °C and 5% CO_2). Samples were then stimulated with STS or POL (0.15%), and series of images was captured every 8 seconds for 30 minutes. Pseudocolour

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