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Flow Cytometric Characterization of Accidental Cell Death Highlights Connections to Regulated Cell Death

Gary Warnes*

Flow Cytometry Core Facility, The Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary London University, 4 Newark Street, London E1 2AT, UK

*Correspondence should be addressed to Gary Warnes, g.warnes@qmul.ac.uk

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Damage-Associated Molecular Patterns (DAMPs) are known by their nature to cause inflammatory responses in numerous disease states from cancer, trauma to age related diseases (*e.g.* atherosclerosis, Alzheimer's and Parkinson's diseases), these molecules are released by cells undergoing cell death. The possibility of controlling their detrimental effects has consequently brought much attention to different forms of Regulated Cell Death (RCD) such as necroptosis which has been reported to release more DAMPs than apoptosis given the different nature of the breakdown of the cell [1-4]. Carcinomas have a high level of oncotic [5] or Accidental Cell Death (ACD) due to adverse changes in environmental conditions within the tumor (*e.g.* hypoxia, chemical, pH, temperature, mechanical) which also release a high level of DAMPs promoting a pro-inflammatory response [4]. The incomplete knowledge of potential signaling pathways involved in ACD however has not hindered drug development such as the preclinical use of PARP inhibitors in promoting DNA repair which in combination with chemotherapy switches cell death from oncotic to apoptotic (lower levels of inflammation) which resulted in anti-tumor activity in metastasis [1].

The incomplete knowledge of ACD signaling pathways is due in part to the broad breadth of how oncosis can be brought about (*e.g.* hypoxia, chemical, mechanical, temperature, pH, [5,6]. A necrotic cell resulting from a RCD process is identifiable but what of the oncotic cell given the lack of a biological marker(s) for ACD, this brings the question to how would an oncotic cell be identified [5,6]? All cells after losing plasma membrane permeability breakdown in a manner reflecting the induction route of the RCD or ACD then become necrotic with the final dissolution of the cell and its contents [7]. ACD or oncosis occurs after insult from the environment which causes the cell to swell and rupture its organelles and plasma membrane which then subsequently breaks down further by the process of necrosis [5]. Previously oncotic and apoptotic cells would be identified by electron microscopy, while fluorescence microscopy and immunoblotting has been used extensively to research RCD processes, with flow cytometry being used only to investigate mainly apoptotic cell death. Flow cytometry currently principally employs the Annexin V assay which binds to the phospholipid phosphatidylserine when externalized by cell undergoing apoptosis, while oncotic cells do not externalize this phospholipid but undergo plasma membrane permeability [5,8,9]. Cells undergoing apoptosis would also lose mitochondrial function gradually as detected flow cytometrically, while oncotic cells would lose this function very quickly [10,11]. So what phenotype would oncotic cells have given their death by swelling and rupture, the process can vary presumably in response time depending upon the type of insult inflicted upon the cell so there may be no marker when the process is very rapid. Longer term insults could result in signaling pathway activation particularly involving the mitochondria given their rapid decline in function [10,12].

Recently the development of a flow cytometric assay which identifies necroptosis and apoptosis as well as autophagy, RIP1-dependent apoptosis, ER stress, DNA Damage, cleaved PARP and γH2AX activation of cleaved PARP or parthanatos has allowed the same intracellular labeling of antigens to identify numerous subpopulations of oncotic cells [13-16]. Oncotic Jurkat cells undergoing ACD by sodium azide treatment lose the integrity of the plasma membrane and do not activate caspase-3 but express RIP3 (<20%) or not (80%) highlights a potential oncotic mechanism given the presence of phenotypically

different subpopulations of oncotic cells. This bodes the question to what other cell phenotypes would be induced by other forms of ACD (*e.g*. hypoxia, heat treatment, pH or mechanical) with a resultant variation in the type of DAMPs released. Interestingly we recently showed that etoposide induction of apoptosis gave rise to oncotic Jurkat cells with a similar distribution of cell viability^{+ve}/ RIP3$^{+/-ve}$ as induced by sodium azide [16]. However both types of etoposide derived oncotic (RIP3$^{+/-ve}$) cells expressed a high level of parthanatos while sodium azide cells (viability^{+ve}/RIP3^{-ve}) expressed more DNA Damage indicating that the mechanism of induction of ACD does result in phenotypically different forms of oncotic cells [16]. The release of different types of DAMPS from these different types of oncotic cells from carcinomas may cause changes in the inflammatory response with a differential effect on the clinical prognosis for the patient. This is further highlighted by the fact that sodium azide dead RIP3^{+ve} cells had more γH2AX, parthanatos and hence less negative (<30%, Quadruple Negative, QN) phenotype than the RIP3^{-ve} cells which have a higher incidence of the QN population (>60%) and thus are potentially less bioactive than other types of oncotic cells [16].

Pathways in ACD are to date still poorly understood but using this flow cytometric approach some possible signaling routes in ACD were highlighted [16]. Interestingly sodium azide besides inducing oncosis (<20%) caused a degree of caspase-3 dependent apoptosis (<30%) which was abrogated by pan caspase inhibitor zVAD, while necrostatin-1 an inhibitor of necroptosis caused an increase in apoptosis [16]. Given that sodium azide is a metabolic poison and directly inhibits oxidative phosphorylation on the inner mitochondrial membrane this mechanism appears to cause the activation of the RCD process of apoptosis as well as inducing oncosis without activating necroptosis which highlights a possible link of ACD and RCD implying the two processes are not completely independent [10,16]. Sodium azide treated resting live and oncotic dead RIP3^{+ve}Jurkat cells showed little change in γH2AX (DNA Damage), cleaved PARP and parthanatos to untreated cells [16]. However the more copious resting live and oncotic dead RIP3^{-ve} cells displayed higher levels of parthanatos and γH2AX than untreated Jurkat cells [16]. The presence of a significant proportion of live cells undergoing parthanatos a form of PCD after treatment with sodium azide is an intriguing observation as was the raised levels of DNA Damage in dead oncotic cells which was abrogated by necroptosis inhibitor necrostatin-1 [16]. These observations highlight potential signaling pathways involved in sodium azide induced ACD via its action as a metabolic poison with links to apoptosis and parthanatos. Thus it may be possible to regulate to a degree the phenotypic distribution of cells undergoing oncosis by the use of molecular inhibitors of RCDs. Carcinoma cells undergoing oncosis in parts of the tumor due to adverse changes in environmental conditions (*e.g*. hypoxia) may result in a variable release of DAMPs by these newly identified types of oncotic cells undergoing DNA Damage and parthanatos given the nature of their cellular breakdown will differ. So modulation of the incidence of these different types of oncotic cells could reduce the degree of inflammation resulting in an improved prognosis for the patient.

Recent unpublished work in this laboratory has investigated the role of mitochondrial functionality and the generation of Reactive Oxygen Species (ROS) within the oncotic cell population. Given that sodium azide is a metabolic poison and directly affects the functionality of mitochondria even after long term treatment (24 h) the remaining live Jurkat cells still displayed functioning mitochondria that were in a hyper-polarized state (>20%) with a 3-4 fold increase in cellular ROS levels [10]. Such live cells were comprised of mainly resting RIP3^{+ve}/ Caspase-3^{-ve}cells (80%, Double Negative, DN 20%) which expressed high levels of γH2AX (50%). Interestingly the oncotic phenotype (cell viability^{+ve}/Caaspe-3^{-ve}) were mainly DN (95%) for RIP3/Caspase-3 a high incidence of which had hyper-polarized functioning mitochondria (>15%, by 3 fold) and a very high level of cellular ROS (5 fold increase) [11]. These so called oncotic 'dead' DN cells with functioning mitochondria expressed low levels of parthanatos and γH2AX, while the remaining 5% of dead resting oncotic cells displayed increased cleaved PARP (in the absence of caspase-3).

This intracellular antigen flow cytometric approach to the identification of cell death processes identifies many sub-populations of cells and highlights the complexities and interconnections of ACD and RCD. This technique would allow the *ex vivo* analysis of isolated heterogeneous cells and tissues given that this technique could easily be transferred to Hyperion Image CyTOF technology permitting the analysis of cells undergoing cell death in three dimensions within diseased tissues. This approach would be a considerably improvement upon the homogenous cell immunoblotting approach in the detailed analyze heterogeneous cell populations.

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