Valencia, Spain, [2]INCLIVA-UCIM, Universidad de Valencia, Valencia, Spain

Background: Detection of human immunotoxicity is relevant to predictive and regulatory toxicology but is usually complex, while nodal steps may provide simple and suitable endpoints. NF-kB activation is both a promising therapeutic target and an important immunotoxicity endpoint. Conventional methods employed to determine nuclear translocation of NF-kB lack statistical robustness (microscopy) or the ability to discern heterogeneity within the sampled populations (Western blotting and gel shift assays). Previously, we developed an in vitro assay of immunotoxicity based on quantitative measurement by Multispectral Image-in-Flow Cytometry (MsIFC) of NF-kB translocation in a monocytic cell line. MsIFC combines the high image content information of microscopy with the high throughput and multiparametric analysis of flow cytometry which overcomes the aforementioned limitations of conventional assays. In this study, we have adapted and validated this assay for human peripheral whole-blood samples. Materials and Methods: The degree of NF-kB nuclear translocation was quantified in U937 cells and in peripheral blood samples after treating samples with different compounds affecting in vitro NF-kB-dependent immune functions, including activators (LPS and PMA), inhibitors (PDTC and Wedelolactone) and six test immunotoxicants. Negative controls were cell cultures treated with appropriate solution vehicles. To analyze the effects of NF-kB inhibitors, samples were pre-incubated with or without them for 1h and then treated with activating or pro-oxidant agents. After treatment, surface staining was performed for 100 µl whole blood samples using PC5 anti-CD14, erythrocytes were lysed by addition of 2 ml BD FACS Lysing Solution 1X (Becton Dickinson). Then cells were fixed and permeabilized with 0.1 % Triton-4% PFA and stained with anti-NF-kB p50 conjugated to Alexa Fluor 488 (Biolegend). Afterwards, cells were washed and counterstained with 7-AAD (Molecular Probes). MsIFC data were obtained for at least 15,000 events per sample using an ImageStream100 system (Amnis). Results: LPS- or PMA-induced NF-kB nuclear translocation was quantified in U937 cells and peripheral blood samples after 2 and 24 h of treatment with different compounds affecting in vitro NF-kB-dependent immune functions, including test immunotoxicants such as lindane, diazepam, hexachlorobenzene, t-butylhydroperoxide, verapamil and mercury chloride. Our results show that MsIFC allows to quantify the effect of xenobiotics and biological regulators on NF-kB nuclear translocation in peripheral bood samples. Automated image processing algorithms allowed to calculate the percentage of lymphocytes and monocytes showing NF-kB nuclear translocation in each condition. IC50 values could be derived, thus classifying the immunotoxic potency of test compounds and allowing comparison with general cytotoxicity, as measured by PI assay or Microplate Alamar Blue assay. Conclusions: This assay is a fast and simple method for detecting immunotoxicity on processes associated with this molecular target. This approach is not limited to NF-kB translocation and may be used for any transcription factor with action related to its intracellular location. Supported by Ministerio de Ciencia e Innovacion (BIO2010-19870) and Conselleria de Educacion de la Generalitat Valenciana (ACOMP2013/102).

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Resistance to Intrinsic Apoptosis Stimuli in Lymphocytes: A Role for Mitochondrial Dysfunction

Carl Bortner, Alyson Scoltock, John Cidlowski
Signal Transduction Laboratory, NIEHS/NIH, Research Triangle Park, NC, United States

Apoptosis is a physiological mode of cell death that removes unwanted cells at a specific time or in response to a given cell death signal. Apoptotic resistance is a common feature in many disease states that impedes the treatment of these conditions. We have developed a model system to investigate mechanisms of apoptotic resistance in T-cells using osmotic stress to drive selection of death resistant cells. Exposure of S49 (Neo) cells to multiple rounds of osmotic stress and viable cell recovery resulted in a stable population of T-cells (S49 (OS 4-25)), resistant to multiple apoptotic stimuli, including acute osmotic stress that utilizes the mitochondrial or intrinsic apoptotic pathway. However, S49 (OS 4-25) cells remain sensitive to FasL, or extrinsic apoptotic cell death, suggesting the cellular apoptotic machinery remains intact. The S49 (OS 4-25) cells undergo aerobic glycolysis, characteristic of the Warburg effect, and have a complete dependence on glucose, whereupon glucose withdrawal results in apoptosis. Microarray analysis of S49 (OS 4-25) versus the parent S49 (Neo) cells revealed over 8,500 differentially regulated genes with almost 90% of the genes having repressed expression. Pathway analysis indicates apoptosis and mitochondrial dysfunction as major variances in gene expression. Interestingly, gene expression of pro- and/or anti-apoptotic Bcl-2 family members were either repressed or unchanged. Using both flow cytometric and metabolic assays, our studies show mitochondrial respiration, specifically the ability of the S49 (OS 4-25) cells to undergo oxidative phosphorylation, as a major defect in signaling the apoptotic program. Altogether, our findings implicate stimulus-specific recognition defects and altered signaling mechanisms that are independent of specific pro/anti-apoptotic Bcl-2 proteins as critical determinants of apoptotic resistance in lymphoid cells.

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Nuclear Apoptotic Volume Decrease in Individual Cells: Time-Lapse Confocal Microscopy Imaging and Molecular-Kinetic Modeling

Anastasiya Konokhova[1], Andrei Chernyshev[1,2], Darya Orlova[3,4], Maxim Yurkin[1,2], Irina Khalo[1], Valeri Maltsev[1,2]
[1]Institute of Chemical Kinetics and Combustion, Novosibirsk, Russia, [2]Novosibirsk State University, Novosibirsk, Russia, [3]Department of Genetics, Stanford University, Stanford, CA, United States, [4]Institute of Biophysics, Brno, Czech Republic

Background: There is a frequently expressed view that the reduction of cellular volume by loss of water also known as "apoptotic volume decrease" (AVD) is one of the earliest detectable apoptotic phenotypic features and events. However the particular mechanism underlying this process is not well established. One of the hypotheses likely to explain AVD is based on the assumption of intracellular osmolarity change due to partial proteolysis or protein destabilization. However methods of quantitative evaluation of AVD are lacking. To analyze this hypothesis we developed a method for detailed study of early nuclear morphological changes including chromatin condensation and nuclear AVD, that is also apoptotic hallmark, assuming herewith that nuclear AVD is mainly caused by water loss due intranuclear osmolarity changes caused by chromatin condensation.

Methods: Time-lapse microscopy imaging of HepG2 cells stably expressing H4-Dendra2 was performed using confocal laser scanning microscope (Leica TSC SP5 X). To induce apoptosis cells were treated with Etoposide. To analyze the dynamics of nuclear and chromatin changes 3D scanning of cells was carried out with an average time lapse of 8.8 min for a period of 9 h from the moment of apoptosis induction. The freely available ImageJ software was used for image analysis and processing, reconstruction of a region, occupied by chromatin, and volumetric estimation of region, occupied by chromatin particularly in condensed state. 3D reconstruction of a whole nucleus was performed with 3D alpha-shape algorithm. For the quantitative characterization of observed nuclear AVD dynamics and formation of the apoptotic ring of condensed chromatin inside the nucleus we developed a kinetic mathematical model describing nuclear shrinkage driven by intranuclear osmolarity change due to chromatin restructuring.

Results: Based on microscopy observations dynamics of apoptotic nuclear morphological changes in individually tracked HepG2 cells was characterized by three distinct phases: lag-phase with a constant nuclear volume, volume depletion and early chromatin condensation phase, and a phase with a final constant volume. The developed mathematical model was fitted to the observed kinetics of nuclear AVD and early chromatin

condensation in single cells. This allowed us to quantitatively characterize the process by lag-phase duration, AVD rate, nuclear volume shrinkage degree, and degree of chromatin condensation.

Conclusion: We present a method based on time-resolved confocal microscopic measurements and a mathematical model, describing measured nuclear AVD dynamics accompanied by chromatin condensation and formation of apoptotic ring, for quantitative characterization of an early nuclear morphological changes during apoptosis.

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G2 Transit Times in Pancreatic Cancer, Their Relationship to Genomic Instability, and the Therapeutic Potential of G2 Checkpoint Inhibitors

David Hedley, Sue Chow
Princess Margaret Hospital, Toronto, ON, Canada

Genomic instability is a hallmark feature of cancer. Pancreatic cancers are highly malignant, and show early onset of metastases and drug resistance that are likely due in part to genomic instability and high mutation rates. The G2 phase of the cell cycle allows proof reading for errors, and the repair of DNA damage prior to entry into mitosis. We hypothesize that pancreatic cancers showing high genomic instability require an extended G2 to resolve post-replication errors, and that agents that disrupt the G2 checkpoint might show selective toxicity towards these aggressive cancers.
The cytokinetics of solid tumours are of fundamental importance to cancer treatment, but in recent years this subject has been largely ignored by the flow cytometry community and the field has not kept pace with developments in basic science. Our experimental approach uses a large series of early passage xenografts derived from pancreatic cancer patients and maintained in the pancreas of immune-deficient mice. These tumours closely recapitulate the features of their corresponding patient donor, and their availability substantially overcomes the difficulty obtaining patient samples for flow cytometry-based experiments. Following disaggregation into single cells, combined staining of DNA content, Cyclin A2, and serine 10 phosphorylation of histone H3 allows assignment of cells in the G2/M peak to the sequence G2 → prophase → metaphase/anaphase → late mitosis. Applying this technique to pancreatic cancers we have successfully tracked the kinetics of G2 arrest in response to a single treatment of tumour-bearing mice with 8Gy radiation, and abrogation of the G2 checkpoint by treatment with a wee-1 inhibitor. Interestingly, in the absence of radiation wee-1 inhibition results in rapid exit from G2 into the metaphase/anaphase population, which is consistent with the idea that an extended G2 protects genomically unstable cancers from the effects of unresolved post-replication damage.
Our ongoing work involves the addition of EdU pulse/chase to allow estimation of the actual G2 transit times in vivo, and then to measure these in a series of pancreatic cancers selected based on cytogenetic complexity. This will allow testing of the hypothesis that increasing genomic instability results in prolongation of G2. We plan to use this information and our sophisticated flow cytometry platform to assess the potential for targeting post-replicative checkpoints in these highly aggressive cancers.

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Characterising the Heterogeneity of Inheritance across Mitosis

James Tonkin[1], Rowan Brown[1], Huw Summers[1], Paul Rees[1], Rachel Errington[2], Marie Wiltshire[2], Michelle Parker[1]
[1]Engineering, Swansea University, Swansea, United Kingdom, [2]School of Medicine, Cardiff University, Cardiff, United Kingdom

Understanding how individual cells behave as a collective population in both localised clusters and tissue structure environments is essential to our understanding of biological systems. With knowledge of the individual cell increasing rapidly alongside improved methods of high resolution imaging and targeted fluorescence tagging, the next big challenge is in scaling this knowledge up to accurately model cell populations and tissue formations. Whilst flow cytometry has provided large sample distributions of various morphological and fluorescence targeted aspects of such cell populations, it is intrinsically unable to relate the associated spatiotemporal aspects. Through the mining of high-throughput fluorescence microscopy data we can acquire these population wide statistics in parallel with their respective spatiotemporal dynamics. This unification of intrinsic cell features and spatiotemporal variables across large populations can provide the basis for more accurate models of the biological system.

The results presented here track internalized quantum dot fluorescence through 200 mitotic events to investigate inheritance heterogeneity between progenitor-daughter cells in human osteosarcoma cell-lines. Quantum dots here act as a representative for internal cellular material through localisation in the late endosomes. With respect to these internal fluorescence markers, the translation between progenitor and daughter fluorescence distributions is shown to be the result of an asymmetric binomial process with a population optimised value of $p = 0.1$. This asymmetry is in-keeping with previous analyses on flow cytometry data that also suggested a non-uniform distribution of internalised particles from progenitor to daughter cells. Here, however, for the first time the translation relates directly to traceable parent-daughter pairings detailing the highly asymmetric nature of single cell progenitor-daughter inheritance across mitosis events. Quantifying populous scale properties in this manner allows us to accurately model the evolution of internal quantities through division events in cell populations. Knowledge of the exact contribution made by each cell to the global distributions on both sides of a mitotic event also allows for investigation into whether the cross mitotic translation is better described with a probability dependence on n (namely, are low object numbers passed onto daughter cells in the same manner as high object numbers). Accurate modelling of variables across division events is essential in understanding the pharmacodynamic effects of drug dosing over multiple generations and enables more targeted developments in cancer therapies including improved longitudinal dosage strategies.

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Subcellular Cytometry – Quantitative 3-Dimensional Analysis of Discrete Subcellular Structures and Events Applied to Studies of DNA Damage and Repair

Jurek Dobrucki[1], Zbigniew Darzynkiewicz[2]
[1]Cell Biophysics, Jagiellonian University, Krakow, Poland, [2]New York Med Col, Valhalla, NY, United States

Many cellular processes are confined to small-size foci, regions or structures in the nucleus or cytoplasm. Examples include DNA replication, transcription, splicing and repair, exocytosis, endocytosis as well as oxidative phoshorylation, proteolytic degradation, etc. Such small foci can be imaged and analysed using concepts and principles similar to the ones that have been developed and are now applied successfully in the realm of flow cytometry and laser scanning cytometry. New optical microscopy methods, including super-resolution imaging techniques, open even more innovative possibilities in cytometric studies of various subcellular structures and processes.
We have developed new analytical and mathematical approaches aimed at measuring and analysing the numbers and volumes of subcellular microfoci, their fluorescence intensities, distances between barycentres of nearest neighbours of microfoci of various types in 3-D space, and spatial correlations and interactions between them. Such analyses were supplemented by the complementary methods of multicolor flow- and laser scanning-cytometry to provide information covering the range from a molecular- to a cellular- and a cell population level. We applied these techniques in studies of the processes of replication and endogenous DNA damage in human cells, as well as damage induced by oxidative stress and by several DNA damaging drugs including DNA topoisomerase I and II inhibitors. DNA damage sites were visualised by detection of phosphorylated histone