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Phenotypic changes in mitochondrial membrane potential ($\Delta\psi_m$) during valinomycin-induced depolarisation and apoptosis

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Abstract. A large body of evidence has implicated mitochondria in control of cell death, where key apoptotic mechanisms involve change in mitochondrial membrane permeability and depolarisation of mitochondrial membrane potential ($\Delta\psi_m$). Assessment of $\Delta\psi_m$ is traditionally conducted using the lipophilic cation JC-1 on the flow cytometer or by fluorescent microscopy. Here we assess JC-1 aggregation using the novel tool of digital texture analysis to establish mitochondrial phenotypic changes induced by the K^+ ionophore, valinomycin in a unique model comprising SW480 and SW620 cell lines. This provides an opportunity to study these phenomena in the context of colorectal cancer. Valinomycin-induced apoptosis was detected using morphology and analysis of DNA content. Cells were treated with valinomycin, images digitally recorded on a calibrated video photometer and subjected to high resolution digital texture analysis. This demonstrated that the HARAM texture features (Mean of the Haralick texture features) were highly valuable in describing the transition of $\Delta\psi_m$ as the cell undergoes apoptosis. In conclusion this study illustrates the potential of texture analysis as a novel and additional technique for quantifying JC-1 aggregation and revealing the spectrum of collapse of $\Delta\psi_m$ during apoptosis.

Keywords: Mitochondrial membrane potential, apoptosis, colon cancer

1. Introduction

Apoptosis is well known as a specialised mode of programmed cell death, finely regulated at the molecular level. Recently a large body of evidence has implicated mitochondria as an intrinsic part of cell death control that inevitably plays a role in apoptosis [7,11, 13]. The molecular pathways leading to apoptosis can involve the formation of a proteinaceous pore at sites of contact between the inner and outer mitochondrial membranes [7]. This is accompanied by a decrease in mitochondrial membrane potential ($\Delta\psi_m$), normally 180–200 mV and heralds a transition in the permeability of the mitochondrial membrane (Permeability Transition – PT). The phenomenon culminates in the release of sequestered molecules such as cytochrome c and apoptosis inducing factor (AIF) that are involved in downstream control and formation of the apoptotic phenotype [13]. These processes are among the key mechanisms of apoptosis and analysis of $\Delta\psi_m$ is a reliable mirror of membrane permeability.

The cell lines SW480 and SW620 have recently been revalidated as an acceptable scientific model [9] and provide a unique opportunity to study these phenomena in the context of colorectal cancer progression. Membrane potential may be assessed using the fluorescent, lipophilic cation JC-1 (5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazaolcarbocyanineiodide) in living cells and is traditionally conducted on the flow cytometer, where JC-1 can detect variations in $\Delta\psi_m$ at both single cell and single organelle level [3]. Digital texture analysis is usually performed on nuclei [2,4]. We have extended this technique to assess the textural changes within the cytoplasm of the cell line model and in particular, to assess JC-1 aggregation and hence

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$\Delta\psi_m$ to establish mitochondrial phenotypic changes induced by the K^+ ionophore, Valinomycin [5].

2. Materials & methods

The K^+ ionophore valinomycin was obtained as a 25 mg solid (Sigma, UK), dissolved in 1 ml of dimethylsulphoxide (DMSO, Sigma) to form a 2.25×10^{-5} M stock solution and stored in a brown glass bottle in the dark at 4°C. The mitochondrial probe JC-1 was obtained as a 5 mg solid (Molecular Probes, The Netherlands) dissolved in 1 ml of DMSO to form a stock solution and stored at −20°C in glass bottles protected from light.

The SW480 and SW620 colorectal carcinoma cell lines were obtained from European Collection of Cell Cultures (ECACC, UK). They were maintained in 95% air/5% CO_2 at 37°C in 75 cm^3 flasks with 20 ml of Dulbecco's phenol free growth medium which had been supplemented with 10% foetal calf serum/500 ml and 2 mM L-glutamine/500 ml (Sigma). No antibiotics were used.

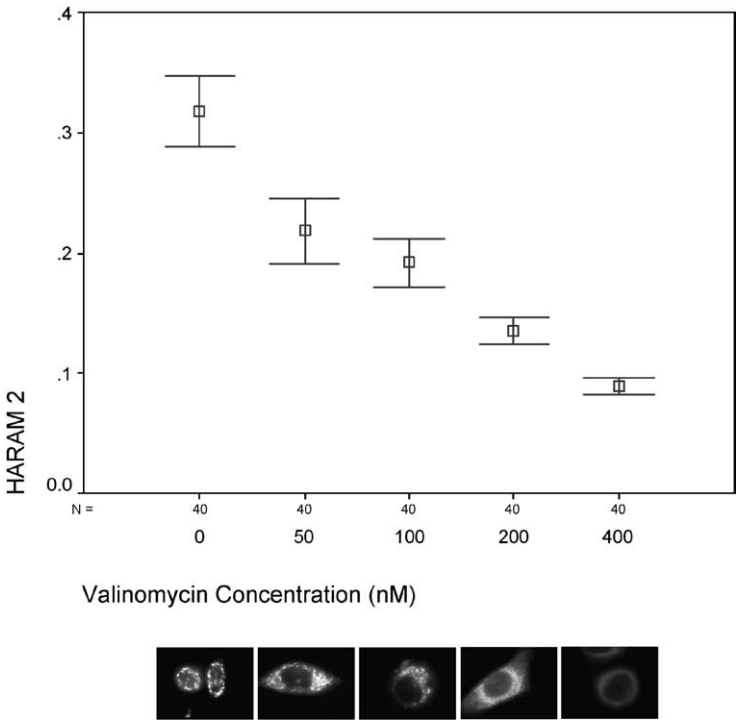
Cells were grown on coverslips and treated with 400 nM valinomycin (SW480) and 100 nM valinomycin (SW620) for 5 min. These concentrations were determined as optimal for each cell line. Controls consisted of medium containing the equivalent concentration of DMSO only. All cells were washed in growth medium, stained with JC-1 and examined by fluorescent microscopy (HBO 50W Mercury vapour burner, Leica N2.1 filter). HARAM texture features are the mean of each of Haralick's 14 features [4], averaged over the four directions from the grey level co-occurrence matrix (GLCM). For texture analysis, images were digitally recorded under oil immersion on a calibrated video photometer equipped with a Hamamatsu chilled CCD camera (640×480 pixels and 8-bit greyscale) and a Meteor PCI frame grabber graphics card. Resolution at the specimen level was 0.0196 μm^2 for the ×100 objective. Fluctuation of voltage supply to the microscope's light source may cause fluctuations of grey levels. The light source for texture analysis therefore, was stabilised using a regulated power supply so that voltage fluctuations were kept to a minimum. Images of 40 cells were collected for each concentration. This figure was determined by optimisation, where time taken to capture images, minimising fluorochrome fading together with the optimum number of cells were considered (data not shown). Background correction was performed. Each cell was then subjected to high resolution digital texture analysis using KS400 version 3.0 image analysis software (1997 Carl Zeiss Vision GmbH, Germany). This software permits interactive tracing of cells and runs a variety of texture measurements [8].

In order to determine if valinomycin treatment resulted in apoptosis, two methods were used. Cells were grown on glass coverslips, treated with the respective concentration of valinomycin to induce depolarisation and monitored at 1, 3, 5, 24, 48 & 72 hr intervals. Haematoxylin and Eosin staining was used to assess morphological changes associated with apoptosis by counting the number of apoptotic cells per mm^2. Further cells were harvested by scraping following their respective valinomycin treatments and the DNA content analysed using propidium iodide staining on a Coulter Epics Elite flow cytometer.

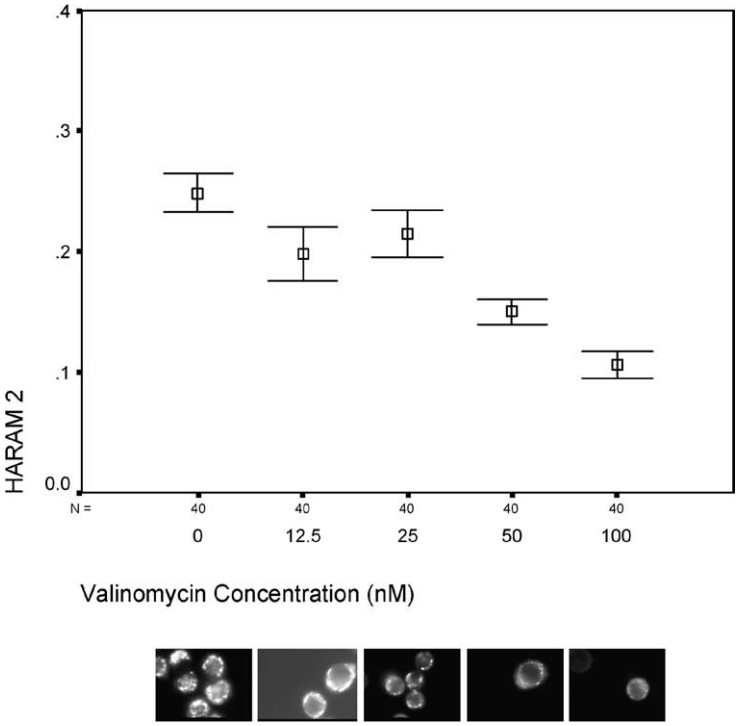
3. Results

Texture analysis revealed that the HARAM set of Haralick features was the most useful to describe this change for both SW480 and SW620 cell lines. All 11 features showed similar increasing or decreasing trends over the increase in valinomycin concentration. Figure 1 illustrates HARAM 2 (Mean of Contrast feature) for the SW480 and SW620 cell lines together with the visual appearance of JC-1 stained cells over the range of valinomycin concentration. This texture feature shows a clear decrease in value with increasing valinomycin concentration. The HARAM features clearly demonstrate a difference between the extremes of membrane potential in this model but they do not permit conclusive identification of cells that are incompletely polarised.

In this model, treatment with valinomycin induced apoptosis. H&E staining revealed an increase in the number of apoptotic cells per mm^2 in both SW480 and SW620 following treatment with valinomycin compared with untreated controls. This is illustrated in Fig. 2 for up to 5 hours after treatment. After 5 hours the number of cells detached from the coverslips had increased and apoptosis could not be reliably assessed. DNA analysis on both cell lines illustrated marked cell cycle perturbations on treatment with valinomycin and showed an overall increase in the percentage of apoptotic cells with time as shown in Figs 3a,b. The SW480 cell line required a much higher concentration of valinomycin (400 nM) for initial depolarisation of $\Delta\psi_m$. than SW620 cells. At later time points (24–48 hrs) the

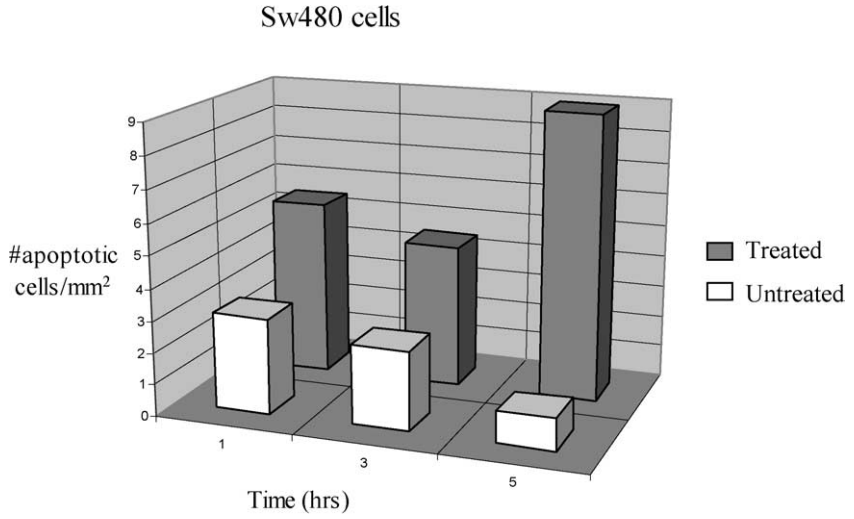


(a)

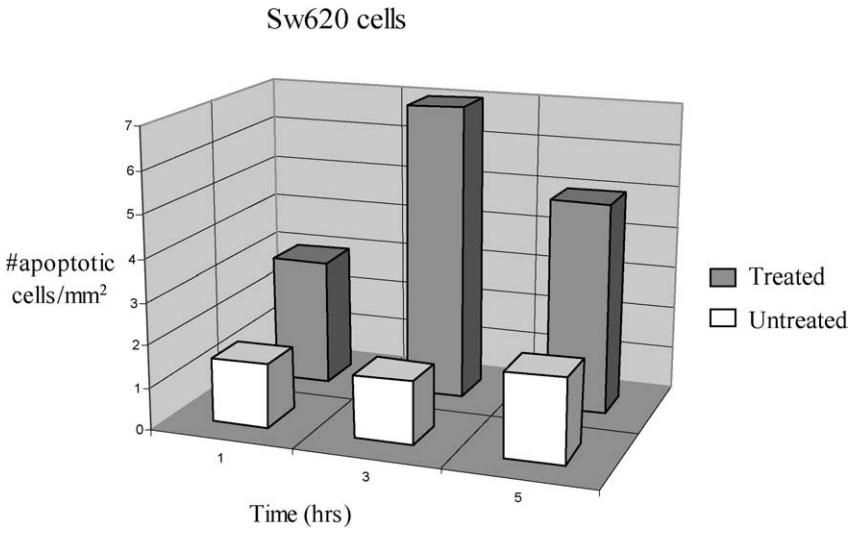


(b)

Fig. 1. HARAM 2 texture feature for (a) SW480 cells and (b) SW620 cells demonstrating decreasing trend in HARAM 2 value with depolarisation of $\Delta\psi_m$ and change in appearance of the cells.



(a)



(b)

Fig. 2. Graphs showing an increase in the number of apoptotic cells per mm^2 in valinomycin treated samples compared with normal cells. These were assessed using morphology based on HE-stained cells. Counts were performed as described. After 5 hours detachment of cells from the coverslips made assessment unreliable.

numbers of cells lost had increased and the percentage of apoptotic cells could not be determined. These results indicate that at the concentrations used and for an extended period of time, valinomycin is inducing apoptosis.

4. Discussion

We have shown that over a set period of time and using a combination of fluorescent colour change and texture analysis it is possible to accurately distinguish extremes of membrane polarisation.

Texture is one of the important characteristics used in identifying objects or regions of interest within any image [8]. Texture analysis has come to the fore in recent years as a tool with which to obtain quantitative and reproducible information about digital images of nuclei or tissue sections that can be of diagnostic use. Digital images are composed of numerous small sampling units called pixels. Each pixel has a single grey value representing its intensity on the original im-

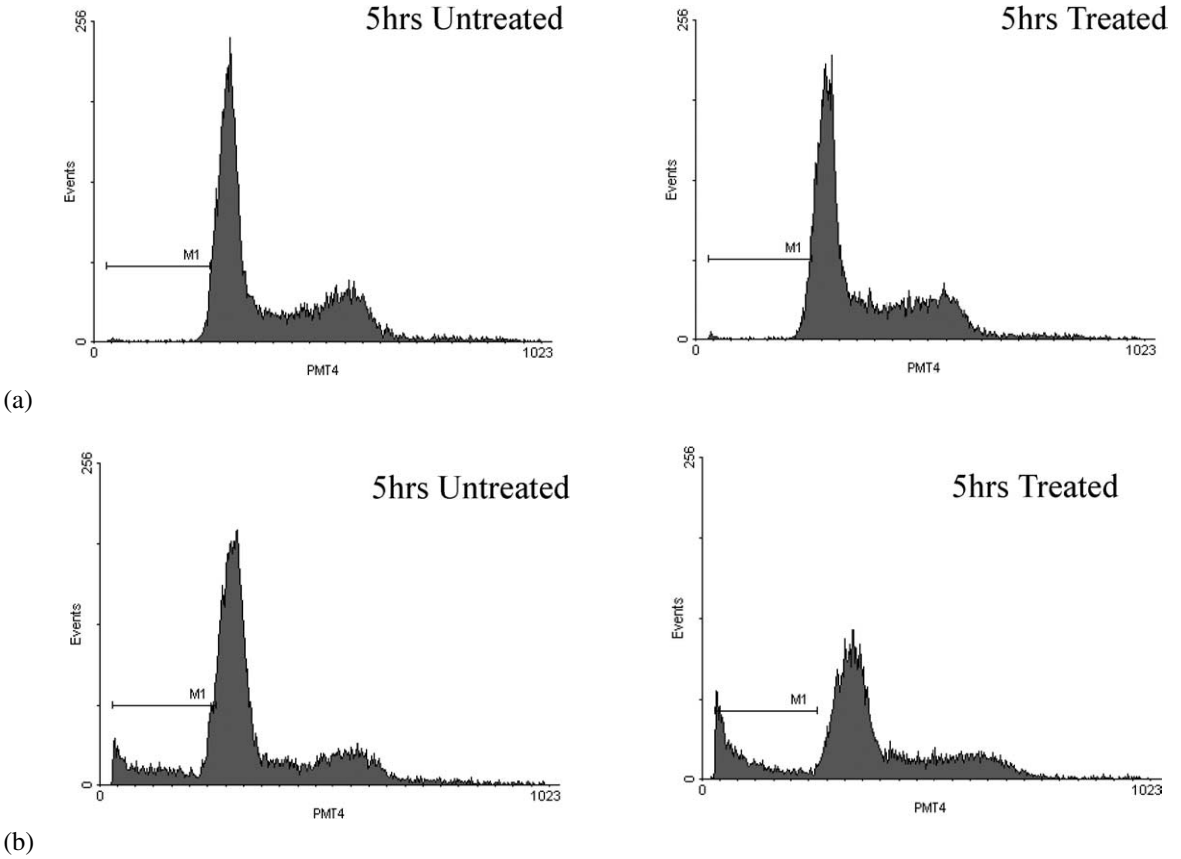


Fig. 3. (a) DNA histograms of Sw620 cells at 5 hrs showing the percentage of cells in the sub-G0/G1 gate, M1. This population increased from 3.7% in the untreated cells to 7.5% in treated cells. Also note widening of peak in the treated sample. (b) DNA histograms of Sw480 cells at 5 hrs showing the percentage of cells in the sub-G0/G1 gate, M1. This population increased from 18.3% in the untreated cells to 26% in treated cells.

age, values can range from 0 = black to 255 = white. Therefore the image can be represented as matrix of numbers. Texture features are a series of measurements which reflect the distribution and spatial relationships between the pixel grey level values of a digital image. They are quantitative, reproducible and have previously been used to describe changes in organisation of nuclear chromatin [4]. The mean (HARAM) of each of Haralick's 14 features is averaged over the four directions from the grey level co-occurrence matrix (GLCM). There is a strong degree of correlation between many of these features as they measure subtle variations on the same thing and generally only a subset of Haralick's features are employed for image analysis. Texture analysis of fluorescent images is a new and evolving area. It has been shown to have use in confocal images of prostatic lesions and fluorescent images of colonic tissue [2]. We have shown that while texture analysis cannot be used to identify individual cells at individual points of membrane collapse it may be employed to better illustrate the spectrum of $\Delta\psi_{\mathrm{m}}$ collapse than fluorescence alone. The technique could potentially provide valuable information for categorisation of fluorescent staining.

Ionophores are antibiotics that increase the permeability of living and model membranes to specific inorganic ions [5]. They differ in specificity, mode of action and fall into two classes of molecule known as mobile ion carriers or channel formers. All ionophores shield the charge of their ion so that it may be transported across the hydrophobic interior of the membrane. They are not coupled to energy sources therefore only permit movement of ions down existing electrochemical gradients [1].

Valinomycin is a cyclic polymer and one of the most widely studied ionophores. It is a mobile ion carrier that transports potassium (K^+) down its electrochemical gradient and into the organelle [1]. In low doses valinomycin uncouples oxidative phosphorylation and causes dissipation of the mitochondrial membrane po-

tential without affecting the pH gradient. We have demonstrated that doses of the K^+ ionophore, Valinomycin depolarises $\Delta\psi_m$ and go on to trigger apoptosis in this cell line model. SW480 and SW620 cell lines have a monoclonal origin [6]. They were established from the same patient, a 50 year old male, at the Scott & White Clinic, Temple, Texas and first classified by Leibovitz et al., 1976 [10]. SW480 was obtained from surgical biopsy of the primary tumour, a moderately differentiated colon adenocarcinoma Dukes' stage B. SW620 was established several months later from the same patient who showed metastasis of the disease to liver and mesenteric lymph nodes [10]. However these cell lines show a distinct difference in their response to valinomycin. During experiments to optimise collapse of $\Delta\psi_m$ it was discovered that SW620 were more sensitive to valinomycin than SW480. Consequently as demonstrated during texture analysis, SW620 cells required 100 nM and SW480 400 nM to completely depolarise $\Delta\psi_m$. This implies cell line dependence of $\Delta\psi_m$ and warrants further investigation.

Valinomycin at the concentrations used was sufficient to depolarise $\Delta\psi_m$ after 5 min but required at least 1 hr before inducing an apoptotic response in the cell lines. Mitochondrial permeabilization generally precedes the signs of apoptosis. It is possible therefore, that the delay between depolarisation and apoptosis could facilitate the 'post-mitochondrial' phase of the cell death process during which factors released from the organelle cause activation of caspases and other endonucleases to form the apoptotic phenotype [11]. Given the relatively short time scale involved however, it is likely that valinomycin initiated apoptosis through non-specific Ca^{2+} release due to hyperpolarisation of the plasma membrane or other phenomena involving changes in ion osmolarity [12].

In conclusion, the role of mitochondria in cell death is a well described paradigm. Loss of $\Delta\psi_m$ inevitably plays a part in the apoptotic process at some stage, even as an ancillary event [13]. In this study we demonstrated how in the model system of SW480/SW620 cells the K^+ ionophore valinomycin depolarises $\Delta\psi_m$ and goes on to trigger cell death. We have also described a novel approach for quantifying JC-1 aggregation using digital texture analysis and illustrated how this novel tool may be useful for cataloguing change in membrane potential induced by valinomycin and other agents.

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