Induction of morphological changes in death-induced cancer cells monitored by holographic microscopy

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Abstract

We are using the label-free technique of holographic microscopy to analyze cellular parameters including cell number, confluence, cellular volume and area directly in the cell culture environment. We show that death-induced cells can be distinguished from untreated counterparts by the use of holographic microscopy, and we demonstrate its capability for cell death assessment. Morphological analysis of two representative cell lines (L929 and DU145) was performed in the culture flasks without any prior cell detachment. The two cell lines were treated with the anti-tumour agent etoposide for one to three days. Measurements by holographic microscopy showed significant differences in average cell number, confluence, volume and area when comparing etoposide-treated with untreated cells. The cell volume of the treated cell lines was initially increased at early time-points. By time, cells decreased in volume, especially when treated with high doses of etoposide. In conclusion, we have shown that holographic microscopy allows label-free and completely non-invasive morphological measurements of cell growth, viability and death. Future applications could include real-time monitoring of these holographic microscopy parameters in cells in response to clinically relevant compounds.

Introduction

Cancer is the second leading cause of death both within the EU as well as in America and it is a remarkably heterogeneous disease that is characterised by unrestrained cell growth and division (Jemal et al., 2011). A key challenge for researchers is to unravel the full spectra of intricate mechanisms that drive cancer growth, in order to aid the development of highly efficacious cancer treatments that will improve prognosis and overall survival of the patient. The loss of balance between cell viability and cell death in cancer is a widely studied subject among cell biologists. There are two types of cell death, apoptosis and necrosis. Apoptosis is an orchestrated process of programmed cell death in vertebrates that plays a central role in development and homeostasis, whereas necrosis is the form of cell injury that results in the premature death of cells in living tissue. The mechanism of apoptosis is complex and involves many pathways (Wong 2011). Morphologically, dying cells differ vastly from viable cells in several aspects (Kroemer et al., 2005). Specific morphological features, in particular volume changes accompany cell death processes and are often used to define the different cell death pathways (Krysko et al., 2008).

Holographic microscopy is an approach for label-free non-invasive imaging of cultured cells, in order to monitor growth, viability and death. The technique is non-destructive and a non-phototoxic method, allowing the user to perform both qualitative and quantitative measurements of living cells over time. In comparison to techniques that measure extracellular release of markers, such as cell staining methods, and the western blot technique, holographic microscopy enable the users to non-invasively collect information on cellular area, confluence, shape, optical thickness and cell volume which can give an indirect measurement of cell volume (Ferraro et al., 2005; Charrière et al., 2006; Carl et al., 2004; Kemper et al., 2006; Rappaz et al., 2005; Mann et al., 2005; Chalut et al., 2012). Since the first studies on living cells, holographic microscopy has been used to study a wide range of different cell types, *e.g.* protozoa, bacteria and plant cells, mammalian cells such as nerve cells, stem cells, various tumor cells, bacterial-cell interactions, red blood cells and sperm cells (reviewed in Alm et al., 2013).

It is also possible to use holographic microscopy for counting of adherent cells (Mölder et al., 2008). Four different adherent human cell lines were investigated by analyzing cells daily for up to four days. By performing measurements over time with holographic microscopy,

changes in proliferation pattern were recorded showing high correlation with manual cell counting.

Recent holographic microscopy studies have shown that the technique can be used to monitor cell changes in PanC-1 pancreatic cancer cells, cell death in single DU145 prostate cancer cells (Alm et al., 2013) as well as in microgravity-induced cell cycle alterations in MLO-Y4 osteocytes (Pan et al., 2012). Moreover, holographic microscopy has been utilized for analysis of staurosporine-induced cell death in human epithelial KB cells (Khmaladze et al., 2012) and in studies of cell death triggered by excessive stimulation of neurotransmitters in primary cultures of mouse cortical neurons (Pavillon et al., 2012). Using cervical cancer cell line, HeLa and Chinese hamster ovary cell line, CHO-K1, Kuhn and co-workers described how the technique can be used for cytotoxic screening for high-throughput purposes (Kühn et al., 2013). Indeed, the use of holographic microscopy as a fast, automatic, and cost efficient evaluation tool for different cancer treatments is promising (Rappaz 2014).

Here we show that morphological cellular changes, such as volume and area, induced by the chemotherapeutic anti-cancer drug etoposide can be identified by monitoring with holographic microscopy. We present data on measurements of growing cells, proliferation, and dying cells, cell death induction, of L929 mouse fibroblast cells and DU145 human prostate cancer cells.

Materials and methods

Cell lines and culture

L929 mouse fibroblast and DU145 human prostate cancer cells were obtained from The American Type Culture Collection (ATCC/LGC Standards, Teddington, UK). L929 cells were cultured in Minimal Essential Medium (MEM) with 1 % Non-Essential amino acids, HEPES Buffer Solution 1 M, L-Glutamine 200 mM, 10% fetal calf serum (FCS) and 1% penicillin/streptomycin. DU145 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 1g/L glucose, 1 % sodium pyruvate, 1% glutamine supplemented and 10% FCS and incubated at 37°C in a humidified atmosphere containing 5% CO₂ (medium and supplements were obtained from Invitrogen, Carlsbad, CA, USA).

Reagents

Etoposide was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Co., St. Louis, USA) and used at a final concentration of 1, 5 and 10 µM. 2×10^5 L929 cells and 2×10^3 cells were seeded into 25 cm² flasks or 96-well plates, respectively. After 24 h, cells were treated with etoposide and incubated for another 24, 48 and 72 h.

Cell viability measurements

Different concentrations of cells were seeded. L929 cells were seeded at 50,000; 100,000; 150,000 or 200,000 and DU145 were seeded at 150,000; 200,000; 250,000 or 300,000 into 25 cm² flasks and allowed to adhere for 24 h. Following adherence, cell growth was monitored with DHM over time, for 24, 48, 72 and 96 h. For cell death studies of L929 and DU145, 2,000 cells were seeded in 96-well plates with 100 µl of DMEM or MEM medium respectively, and incubated for 24 h. Cells were then treated with etoposide as described previously under section 2.2. Post-treatment, MTS-assay was performed by adding 20 µl of MTS/PMS solution CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega Corporation, Madison, USA) to the desired wells and plates were then incubated for 2 h. The absorbance was measured at 490 nm by using BIO-TEK® micro plate reader.

Digital holographic microscopy measurements

Following treatment with etoposide, holographic microscopy images were acquired using Holomonitor™ M3 (Phase Holographic Imaging AB, PHIAB, Lund, Sweden) equipped with a 0.8 mW HeNe laser (633 nm). The laser intensity for this system is approximately 10W/m^2 during imaging and the exposure time is less than 5 ms (Gustafsson et al., 2004; Gustafsson and Sebesta 2004). In the M3 Holomonitor system, the laser ray is divided into two beams, an object beam passing through the sample and a reference beam. By combining both object- and reference beams together on a CCD camera digital sensor, an interference pattern is created.

Computer software

The fully automated computer algorithm *Hstudio* (PHIAB) is established by a standard Fresnel approximation and reconstructs the interference shape (created of the reflected light of the object) into a holographic image (Cuche et al., 1999), with unwrapped focal plane which construct a planar background (Sebesta and Gustafsson, 2005).

The focal plane is automatically estimated by quadratic minimization of the variance of the amplitude image (Dubois et al, 2006). The phase image at the focal plane is unwrapped using Flynn's unwrapping algorithm (Ghiglia and Pritt, 1998) and fitted to a second-order polynomial to construct an approximately planar background (Sebesta and Gustafsson, 2005; Miccio, 2007).

Several were captured from each individual sample. Image areas covered with cells were then segmented using a watershed-based algorithm, yielding data on cell number, confluence, cell volume and cell area could be obtained (Mölder et. al., 2008). For absolute values of refraction indexes for cells and culture medium, assumptions were included in the automated computer algorithm *Hstudio*. As the laser intensity is only approximately 10W/m^2 during imaging, and the exposure time is less than 0.5 s, it is assumed that the laser irradiation has only minimal effects on the physiological functions of the cells (Mölder et. al., 2008; Hawkins and Abrahamse, 2006; Logg et al., 2009; Tinevez et al., 2012).

Statistical methods

Mean and standard deviation were used for statistical analysis of all calculations. For the cell proliferation and apoptosis studies, at least 20 images per sample were captured from at least

two individual experiments. The error bars shown in the images were calculated as the standard deviation between individual images.

Results

Measurements of cell growth curves using holographic microscopy

Different concentrations of L929 cells (50,000 - 200,000) and DU145 cells (150,000 - 300,000) were seeded and measured after 24 - 96 h. The number of cells per area unit (Fig. 1A-B) and the confluence in % per area unit (Fig. 1C-D) were calculated by digital holography. The number of cells and the confluence increased over time for L929 and DU145 cells.

Measurements of viability, cell number and confluence

Induction of cell death was analysed after 24, 48 and 72 h treatments with 1 μ M, 5 μ M and 10 μ M etoposide in L929 and DU145 cells. Concentrations of etoposide higher than 10 μ M killed both cell lines already after 24 h incubation. The number of control cells per area units increased over time, while for treated cells, the numbers of cells was unchanged over time (Fig. 2A-B). The confluence in percent per area units increased for L929 control cells over time, while a minor increase of the confluence was obtained over time in the treated cells (1 μ M and 5 μ M etoposide). For 10 μ M etoposide treated cells, the confluence was unchanged over time (Fig. 2C). For DU145 control cells, the confluence increased over time, while for cells treated with 1 μ M etoposide, the confluence remained unchanged over time. For cells treated with 5 μ M etoposide, the confluence increased after 48 h and decreased after 72 h. For 10 μ M etoposide treated cells, the confluence decreased at all time-points (Fig. 2D).

To confirm the ability to use holographic microscopy for cell viability, an MTS-assay was performed. L929 and DU145 cells were treated and analysed after 24, 48 and 72 h. The L929 and DU145 cell viability increased over time for control cells and decreased for treated cells. For etoposide concentrations less than 1 μ M, the viability for DU145 decreased, whereas for L929 the viability increased but at a much lower rate, compared to the control (Fig. 2E-F). The addition of the drug vehicle DMSO had no effect on the parameters investigated in this study (data not shown).

Morphological changes in hologram images

The morphology of etoposide treated L929 and DU145 cells changed after 24-72 h compared to untreated cells. The cell area increased and the number of cells decreased compared to the

untreated cells (Fig. 3-4). As seen in the hologram images, the morphological changes were both time dependent and etoposide concentration dependent.

Measurements of the mean cell area and the mean cell volume with digital holography

The mean cell area and the mean cell volume were also measured with digital holography. While the cell volume decreased, there were no major changes of the L929 and DU145 cell area for untreated cells over time. However, L929 and DU145 cells treated with 1 and 5 μM etoposide showed an increased cell area (Fig. 5A-B) and cell volume (Fig. 5C-D) compared to untreated cells, while the cells treated with 10 μM etoposide showed decreased cell area and volume.

Discussion

In the current study investigating morphological changes in two cell lines using holographic microscopy, we undertook the same strategy as in a previous study when counting cells using the same method (Mölder et al., 2008). By first optimizing the cell growth curves for viable cells with holographic microscopy, we could determine the morphological changes after etoposide-treatment over time.

Here we show the results of careful measurements on cell populations, analyzed with holographic microscopy and the commonly used MTS viability assay. The growth curves obtained with holographic microscopy and MTS assay were very similar. With holographic microscopy, we are able to show that untreated control cells could be distinguished from etoposide-treated death-induced cells by determining cell number, confluence and morphological alterations such as cell area and volume. When treating the cells with the lowest dose of etoposide, 1 μM , the number of both L929 and DU145 cells decreased already at 24 h, which was the first time point analyzed. The overall decrease in cell number was both time dependent and concentration dependent, and was more prominent when analyzing cell confluence, especially at the lower doses of etoposide. Indeed, this is due to the initial swelling of the dying cells, resulting in an increase of both the cell area and volume of the L929 and DU145 cells at etoposide concentrations of 1 and 5 μM , respectively. Only the high dose of etoposide (10 μM) induced an initial decrease in the cell area and volume.

The results shown in this study are on cell populations, but single cells can also be analyzed. We have previously analyzed and monitored individual cells treated with etoposide at early time-points by holographic microscopy. DU145 cells that contracted became dense and rounded up after approximately 4 h of treatment (Alm et al., 2013). Thereafter, the cells became uneven and 5.5 hours after the beginning of etoposide treatment, the cells fragmented. Interestingly, the remnants of the cell body contracted into a smaller cell-like structure that resembled an apoptotic body. The fragmentation of the cell is a classic hallmark of apoptosis.

Cell volume regulation of neuronal cells has been monitored by holographic microscopy phase response, which allowed estimations in a very short time-frame (Pavillon et al., 2012; Rappaz et al., 2014). By varying the concentration and exposure time of L-glutamate to primary cultures of mouse cortical neurons, Pavillon et al could identify reversible phase responses corresponding to phase recovery through an efficient ionic homeostasis. By

monitoring the phase shift, nuclear condensation and “blebbing” induced by treatment was distinguished, which could indicate that cells were apoptotic rather than necrotic. Importantly, cells recognized within minutes by their holographic microscopy phase signal as unable to regulate their ionic homeostasis, were only several hours later identified as dead by trypan blue staining. Interestingly, Rappaz et al., compared holographic microscopy with both differential interference contrast (DIC) and transport of intensity equation (TIE) and showed the advantages with holographic microscopy for automated phenotypic drug screening (Rappaz et al., 2014). Moreover, Khmaladze and colleagues used holographic microscopy to measure early stage morphological features of apoptosis in cells, which were associated with a marked decrease in cell volume (Khmaladze et al., 2012). Monitoring drug effects can help avoid cancer cells from spreading or developing drug resistance as a result of an ineffective treatment approach. Importantly, we have also recently shown that holographic microscopy has the capacity to identify specific changes in cell phase volume that correlate to either a G1 or a G2/M arrest (Falck Miniatis et al., 2014). In that study, holographic microscopy analysis of average cell phase volume was of comparable accuracy to flow cytometric measurement of cell cycle phase distribution as recorded following dose-dependent treatment with etoposide. We believe that holographic microscopy is an important tool for future image-based analyses of cell volume changes.

Conclusion

We demonstrate that morphological changes in cancer cell cultures can be monitored by holographic microscopy. The cell-death induction provides data on decreased cell volume. This can open a new window for personalized medicine investigations, determining the optimal therapeutic concentration for both individual treatment and for different cancer types.

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