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APOPTOSIS INDUCES TEMPORAL INCREASE IN ATTENUATION AS MEASURED BY OPTICAL COHERENCE TOMOGRAPHY

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Optical coherence tomography (OCT) was used to determine optical properties of pelleted human fibroblasts in which necrosis or apoptosis was induced. We analyzed the OCT data including both the scattering properties of the medium and the axial point spread function of the OCT system. We measured that the optical attenuation coefficient in necrotic cells decreased from $2.2 \pm 0.3 \text{ mm}^{-1}$ to $1.3 \pm 0.6 \text{ mm}^{-1}$, whereas with the apoptotic cells a clear increase (up to $6.4 \pm 1.7 \text{ mm}^{-1}$) in scattering was observed. The results on cultured cells presented in this study indicate the ability of OCT to detect and differentiate between viable, apoptotic and necrotic cells based on their backscatter properties. This functional supplement to high-resolution OCT imaging can be of great clinical benefit, enabling on line monitoring of tissues, e.g. for feedback in cancer treatment.

**INTRODUCTION**

In every multi cellular organism a delicate balance exists between cell division on one hand and cellular death on the other hand. Cellular death can be executed by two different pathways, necrosis and apoptosis. Necrosis, the pathological pathway, is also known as accidental cell death which is triggered by external disturbances e.g. physical trauma, chemical stress or hypoxia. Its morphology is characterized by slight initial cellular swelling, followed by cell lysis. In vivo, the resulting cell debris triggers an inflammatory response.

The physiological pathway of cell death is called apoptosis, also known as programmed cell death. It is a general mechanism for the clearance of cells that have become superfluous or show an aberrant function, without causing inflammation. The apoptotic pathway is conducted by a series of tightly regulated biochemical processes in which a cell, once triggered, goes through consecutive phases of cell shrinkage, chromatin condensation and breakdown,
nuclear disintegration, cell blebbing and the formation of so-called apoptotic bodies. These apoptotic bodies contain nuclear fragments and cell organelles (Figure 6-1). Under normal physiological conditions they are cleared by either macrophages or neighboring cells through phagocytosis.

As the physiological counterpart of cell growth, apoptosis plays an important role in the balance of tissue dynamics. Disturbances in this balance results in disease. If aberrant cells do not undergo apoptosis, a tumor can develop. On the other hand, excessive apoptosis can result in degenerative syndromes such as atrophy and cardiomyopathy. In clinical practice, treatment of these symptoms and diseases involves reduction or stimulation of apoptosis, respectively. Consequently, there is a strong need for methods that can detect and quantify apoptosis on a microscopic level. Currently, the standard methods of apoptosis detection in tissue are histological or biochemical, which are time consuming and require biopsies. In vivo detection using radionuclide imaging and magnetic resonance imaging are currently under investigation.

![Figure 6-1. A schematic representation of the different stages of apoptosis.](image)

Once apoptosis is triggered in a normal cell (A), some shrinkage will occur (B) and the nucleus condensates (B, C). The loss of cytoskeleton integrity induces blebbing of the cellular membrane (C). The condensed nucleus is fragmented (D) followed by disintegration of the whole cell into apoptotic bodies, containing remnants of the nucleus and other cell components (E). Apoptotic bodies are cleared by phagocytosis by macrophages or neighboring cells, or undergo secondary necrosis.

Recently, Czarnota and coworkers reported that apoptosis could be detected using high-frequency ultrasound. They observed an increase in backscattering of the ultrasound signal, which they attributed to the disintegration of the nucleus, a distinctive feature of apoptosis. Optical coherence tomography (OCT) imaging, the optical and high resolution equivalent of ultrasound imaging, is based on time of flight dependant intensity differences of backscattered light. In a previous publication, we used OCT to image porcine carotid arteries in an ex vivo tissue culture setup. We reported an increase in back scattering in the medial layer after balloon dilation. Since there is a rapid onset of apoptosis after balloon angioplasty, we hypothesized that the changes in OCT signal can be attributed to this characteristic form of cellular death. Functional OCT allows for quantitative measurement of tissue absorption and scattering properties, at the microscopic level. Because both apoptosis and necrosis directly influence the cell’s main scatterers (i.e. nucleus, membrane, mitochondria, and other cellular organelles) we hypothesized that the local attenuation
OCT imaging of apoptosis and necrosis

coefficient ($\mu_c$) could be used to assess cellular death. Moreover, due to the differences in the morphology of the two pathways to cell death, we hypothesized that differences could be observed in attenuation of light by necrotic and apoptotic cells as compared to normal cells, and that those changes could be monitored in time, after induction of cell death. In this paper, we present OCT measurements of the attenuation coefficient of pelleted human fibroblasts, in which necrosis or apoptosis was induced.

MATERIALS AND METHODS

Cells and reagentia

Human fibroblasts were maintained in DMEM (Gibco/BRL) supplemented with 10% fetal calf serum, streptomycin at 100 $\mu$g/ml, penicillin at 100 U/ml in a fully humidified atmosphere containing 5% CO$_2$ at 37 °C. Prior to the experiment, subconvolvent grown cells were trypsinized, and collected by centrifugation at 500g for 10 minutes. At t=0, the pelleted cells were resuspended in DMEM containing either 10% ethanol, to induce necrosis, 200 $\mu$M cytosine arabinoside (AraC, Sigma) to induce apoptosis, or 0.1 mg/ml colchicine (CX, Sigma) to induce a mitotic arrest. After 30 minutes incubation, the cells were pelleted and imaged with OCT for 6 hours. In a second set of experiments, the dose-dependency of apoptosis induction by AraC was studied. Culture flasks with subconvolvent grown cells were incubated with 50 nM, 100 nM or 200 nM AraC for two hours. Cells were trypsinized, and collected by centrifugation at 500g for 10 minutes. Pelleted cells were immersed in medium, and imaged with OCT at 3, 6, 9, 12 and 24 hours after the induction of apoptosis. Between and during measurements, the cells were kept at 37°C.

Verification of apoptosis

At the end of the OCT experiment, samples of pelleted cells were subjected to immunofluorescence analysis, using a commercially available apoptosis detection kit (Sigma). The double labeling assay with annexin-V-Cy3 (AnnV) and 6-carboxyfluorescein diacetate (CFDA) allows differentiation between apoptotic, necrotic and viable cells. The CFDA is processed in metabolic active cells into fluorescing 6-CF. The AnnV-Cy3 label binds to phosphatidylserine residues when they appear in the outer leaflet of the cellular membrane, which occurs in compromised cells, being apoptotic or necrotic. Therefore, cells, only labeled with 6-CF (green) are viable cells, cells only labeled with Ann-Cy3 (red) are necrotic cells, and cells staining with both are apoptotic. Labeling was performed conform the instructions, as provided by the manufacturer.

Flow cytometry

To measure the necrotic and apoptotic fraction after induction of cellular death, cells were harvested at different time points, and washed in ice-cold HEPES buffer (10 mM HEPES, 150 mM KCl, 1 mM MgCl$_2$ and 1.3 mM CaCl$_2$, pH 7.4) supplemented with 1 mg/ml glucose and 0.5% (w/v) BSA. Cells were then incubated with FITC-labeled
Annexin V (diluted 1:200 in HEPES buffer) for 15 min and washed again in HEPES buffer. Just before analysis of the samples by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA), PI was added (final concentration 5 μg/ml) to distinguish necrotic cells (Annexin V-/PI+) from early apoptotic cells (Annexin V+/PI−) and late apoptotic cells (Annexin V+/PI+). The samples were analyzed on a FACSCalibur (Becton Dickinson) equipped with CellQuest software. The cytometer was calibrated by eye with fluorochrome beads supplied by the manufacturer.

**OCT imaging**

OCT imaging of cells was performed with a high resolution OCT setup in which a Ti:Sapphire laser (FemtoSource, Vienna, Austria), operating at a central wavelength of 800 nm with a bandwidth of 125 nm, was used as light source. Depth scanning, by changing the (optical) path-length in the reference arm, was performed using a so-called rapid-scanning-optical-delay (RSOD) line. This allowed precise and constant (speed and intensity) depth ranging, and has the additional advantage to allow easy correction for dispersion mismatch between the reference arm and sample arm. In depth scanning was performed at 2 lines per second. For this system, we measured a dynamic range of 110 dB and a free space resolution of 5 μm. For each cell pellet and each time point 3 to 5 b-scans were made.

**Data analysis**

In each b-scan, the attenuation coefficient (μ) of the pelleted cells was obtained in a procedure as described previously. In short, the depth dependence of the amplitude of the OCT signal can be described as the product of the axial point spread function (PSF) of the optics used and the attenuation of the light by the tissue structures. An average

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**Figure 6-2.** Examples of OCT images of pelleted cells. Untreated control cells (A) remained unchanged during the entire experiment. Apoptotic AraC treated cells (B) showed an increase in scattering in the top layer, whereas necrosis (C) resulted in a decrease in signal.
signal of 50-100 adjacent A-scans as a function of depth was fitted to the model with $\mu_t$ as the fitting parameter. Using this simple analysis algorithm, which incorporates the position of the focus in the tissue and the depth of focus, the scattering coefficient $\mu_t$ for the ROI's was determined \textit{in situ}.

![Figure 6-3](image-url) The measured attenuation coefficient for 800 nm light in pelleted human fibroblasts, as a function of time. Time was measured in minutes from the point that the cells forced into necrosis (filled squares) or apoptosis (filled dots). Sham treated (control) cells show no change in scattering (circles).

**RESULTS**

In the experiments described in this study, cells were pelleted and were proven to be viable (data not shown), similar to other studies. When compared to untreated cells (figure 6-2A), the treatment of the cells with 200 $\mu$M AraC resulted in a temporary increase in OCT signal and a decrease of imaging depth (figure 6-2B). On the contrary, cells treated with 10% ethanol showed an increase in imaging depth (figure 6-2C). The measurements of the $\mu_t$ of the samples in time are presented in figure 6-3. The AraC treated cells show an initial increase in $\mu_t$, followed by a decrease, ending up below the level of the control cells. Treatment with 10% EtOH results in an immediate decrease in backscatter, compared to untreated control cells.

As shown in the fluorescence images (figure 6-4), apoptosis was induced in the AraC-treated cells. Panels 6-4A and 6-4B show untreated control cells, and panels 6-4C and 6-4D show AraC treated cells. The green fluorescence (fig 6-4A and 6-4C) is a marker for metabolic active cells (i.e. either viable or apoptotic cells), whereas the red fluorescence marks the compromised cells (i.e. necrotic or apoptotic cells). Therefore, the double labeled cells can be discerned between viable (green), apoptotic (green and red), and necrotic (red) cells.
Figure 6-4. Images of immunofluorescence labeling of control cells (A,B) and cells treated with 200 mM AraC (C,D) at 24 hours. Using the green fluorescing label CFDA (A,C), viable cells can be identified, whereas the red fluorescing label AnnV (B,D) is specific for apoptotic cells. At 24 hours 5% of the control cells has become apoptotic, while after treatment with 200 mM AraC 61% of cells is apoptotic.

Figure 6-5. After induction with 10% ethanol (t = 0), the number of viable cells (gray line, open dots) decrease, and the number of necrotic cells (black line, filled dots) increases. The total number of cells (gray dotted line) decreases, due to loss of necrotic cells. The decrease in $\mu_1$ (black dotted line) coincides with the increase in necrosis.
Figure 6-6. Dose-dependency curves of the increase in $\mu$ after treatment with 50 $\mu$M (A), 100 $\mu$M (B), and 200 $\mu$M (C) AraC. The higher the dose, the earlier is the onset of the $\mu$ increase. The black lines depict the untreated control cells, and the dotted lines are AraC treated apoptotic cells.

Since the necrotic process rapidly results in clearance of cells by rupture of the cell membranes, the EtOH- treated cells were not visualized using fluorescence microscopy. FACS analysis can detect necrotic cells based on the scattering of remnants. The result of the cell counting of is presented in figure 6-5. The decrease in average attenuation (dotted line) coincides with a decrease in viable cells (gray line), and a synchronous increase in necrotic cells. No apoptotic cells were detected, therefore the decreasing total cell count has to be contributed to the total clearance of necrotic cells.

The induction of apoptosis by AraC is known to be dependent on the concentration. In figure 6-6, the results are plotted of the attenuation measurement of cells treated with 50 $\mu$M (6-6A), 100 $\mu$M (6-6B), and 200 $\mu$M (6-6C). The higher the concentration of AraC, the earlier the rise in attenuation is observed.

To study the effect of nuclear condensation on the attenuation, cells were treated with colchicine (figure 6-7). The resulting increase in $\mu$ mimicked the apoptosis curves.
DISCUSSION

In the experiments presented in this paper, an increase in $\mu_\text{r}$ is observed in pelleted cells, after treatment with AraC. AraC is known to induce apoptosis via incorporation into DNA during replication, acting as a chain terminator. However, AraC can also induce apoptosis directly through oxidative stress, with an increase in the generation of reactive oxygen species and p53-dependent cytotoxicity. This latter, rapid pathway has been shown to induce apoptosis in lymphoid cells within 3 hours. Apart from the use in leukemia treatment, is known to induce apoptosis in neuronal cells and fibroblasts.

Apoptosis was indeed detected using a commercially available viability assay, combining 6-CFDA with an annexin-V-Cy3 label. Annexin-V binds to the phosphatidylserine that redistributes from the inner to the outer leaflet of the plasma membrane as an early event in the apoptotic program. Binding of annexin V to externalized phosphatidylserine has formed the basis for widely used optical methods (fluorescence microscopy and flow cytometry) for detecting apoptosis. Furthermore, AraC is known to induce apoptosis in a dose-dependent fashion. A similar dependency is observed in the changes in $\mu_\text{r}$ in our experiments.

There is a great variety in apoptosis inducers. In an experimental setup similar to the one described here, we induced apoptosis in mouse fibroblasts (MF) with staurosporine, and subjected human lung carcinoma cell line (SW 1573) to ala-PDT. The preliminary results indicate that in both cell lines, an increase in $\mu_\text{r}$ is observed (data not shown).

![Figure 6-7](image-url)  
**Figure 6-7** Colchicine treated cells (black dotted line) mimic the $\mu_\text{r}$ curve of AraC treated cells (gray line). However, the maximum values of $\mu_\text{r}$ are higher, and secondary necrosis is not significantly detected. Untreated control cells are represented by the black line.
The origin of light scattering from cells is still subject of studies. Scattering occurs due to the mismatch in indices of refraction between these different cellular compartments and is also dependent on the size and shape of the scatterer. In the case of necrosis, the cell and its organelles disintegrate, resulting in an amorphous mass. The decrease in scattering from necrotic cells can therefore probably be explained by a removal of potential scatterers, resulting in less scattering events. Although, due to the cellular swelling prior to cell lysis, one could expect an initial increase in scattering, this was not observed in our experiments. Valenzeno et al. proposed a technique for measurement of the blood hematocrit based on the decrease of forward scattering during erythrocyte lysis. The loss of refractive index mismatches reduced the number of scattering events. The degree of hemolysis is inversely related to the intensity of small angle scattering. Furthermore, in experiments monitoring the optical density (OD) of cell suspensions, Kravtsov et al. reported a clear decrease in OD after the induction of necrosis.

The apoptotic process involves a series of morphologic changes, in which many potential scatterers are involved. It has been reported that the initial increase in scattering could be due to cellular shrinkage, to chromatin condensation, or nuclear fragmentation. Kravtsov and coworkers designed an assay in which they monitored the optical density (OD) of cells in suspension. When inducing apoptosis, they observed a temporary increase in OD, comparable to the increase in μ, in this study. Morphological analysis of their samples revealed that the increase in OD coincided with the blebbing of the apoptotic cell membrane. The initial OD increase is followed by a decrease when the majority of the cells pass the blebbing-stage. Considering the similarities with the results presented here, especially the transient tendency of the μ increase, it is likely that membrane blebbing is responsible for the results of our study.

In flow cytometry, apoptotic cells are also identified based on changes in scattering. Whereas this study focuses on back-scattered light, cytometry registers forward and side (90° angle) scattering of single cells. Forward scatter is related to the cell size, and side scattering is affected by the cells refractive and reflective properties and reveals optical inhomogeneity, such as resulting from condensation of cytoplasm or nucleus and granularity. During the apoptotic process, there is a slight initial decrease in forward scatter (cell shrinkage), followed by an increase in side scatter (nuclear condensation and fragmentation). The formation of apoptotic bodies is characterized by a decrease in both forward and side scatter.

Czarnota et al. used colchicine to image mitotic arrested cells, which would be normal cells with a condensed nucleus. Whereas the initial goal was to image cells with mere condensed nuclei, apparently also apoptosis was induced in the colchicine treated cells. Colchicine is described as an apoptosis inducer.

Clinical implications and study limitations.

Monitoring of apoptosis could play an important role in the clinic. Apart from an apparent diagnostic value, online monitoring of apoptosis in response to treatment could
greatly improve therapeutic efficacy in e.g. skin cancer treatment. 

Apoptosis also plays a role in cardiovascular disease. Apoptosis of vascular smooth muscle cells and macrophages localizes in so called ‘vulnerable’ lesions, i.e. those lesions most likely to rupture, and at sites of actual ruptured plaques. In *in vivo* studies, induction of apoptosis in endothelial cells as well as smooth muscle cells has been shown to result in thrombosis and plaque rupture respectively. However, before OCT can be used to detect vascular apoptosis, the specificity and sensitivity of the detection of cell death has to be investigated.

Although the loss of cell integrity during necrosis could be determined using flow cytometry, the identification of apoptosis in fibroblast met with insurmountable difficulties. The flow cytometry technique was developed for the analysis of single cells that grow in suspension. Adherent cells, like the fibroblasts used in this study, are very difficult to analyze with cytometry due to clumping of the cells, and rupturing of cells when resuspending. Furthermore, it should be stressed that because the cells detach during late stages of apoptosis, many apoptotic cells may be selectively lost if the analysis is limited to the attached cells only.

Further research has to be done to elucidate the origin of light scattering by cells, in order to address the question of the reason of light scattering changes in dying cells. Conversely, OCT measurements as presented in this study could be helpful in identifying the scatterers in cells.

**CONCLUSION**

The results on cultured cells a presented in this study indicate the ability of OCT to detect and differentiate between viable, apoptotic and necrotic cells, based on their optical properties. This functional supplement to high-resolution OCT imaging can be of great clinical benefit, enabling on line monitoring of tissues, feedback in cancer treatment. Furthermore, OCT imaging of apoptosis in the vascular wall might enhance the ability of OCT to detect the vulnerable plaque.

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