Visualization of Apoptotic Cells using Scanning Acoustic Microscopy and high frequency ultrasound

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Abstract— The goal of this project is to investigate changes in the acoustical properties of cells undergoing cell death for the development of a method for tissue apoptosis detection using high frequency ultrasound (10-60 MHz). A scanning acoustic microscope (SAM) was used for visualization of individual cells undergoing apoptosis (SASAM, Fraunhofer IBMT, Germany). The use of the SAM offers high resolution (1 µm spot size) and therefore enables the exploration of acoustical properties of the cell nucleus. Cells were labeled with H33342 and DIOC 3(5) for visualizing condensed chromatin and membranes in fluorescence microscopy. In addition the same cell lines interrogated microscopically were investigated using high frequency ultrasound. Recorded radio frequency (rf) data were analyzed using ultrasound spectroscopy. Integrated backscatter coefficients and attenuation values were computed for two cell lines: HeLa and MDCK. Both cell lines responded to the applied chemotherapeutic agent by apoptosis, assessed by fluorescence microscopy. Acoustical and optical microscopy using the SASAM system clearly enabled a differentiation between apoptotic cells and cells not responding to the treatment. Apoptotic cells displayed a higher contrast in the acoustic images and were less regular in shape. Optical images of the same cells showed nuclear condensation and membrane disruption. Spectral parameters estimated from rf ultrasound showed a 100% increase in the integrated backscatter coefficients for HeLa and MDCK. Attenuation values were increased by 50% to 70% for both cell lines as a function of treatment time. The results of this investigation provide a better understanding of changes in the acoustical properties of cells with cell death and thus to the development of a non-invasive method for measuring the treatment response of tumors using acoustic waves. (Abstract)

Keywords: ultrasound spectroscopy; apoptosis; acoustic microscopy;

I. INTRODUCTION

During mitosis cells undergo the complex process of cell division. The new cell has to pass 4 checkpoints in the cell cycle. In cases where the proper function cannot be guaranteed, the cell undergoes cell death. This self-induced destruction is called apoptosis and plays an important role in tissue regulation and homeostasis. It was originally defined by Kerr et al. 1972 [2]. However sometimes cells mutate and loose the ability to die, when not passing the checkpoints. The cells are undergoing uncontrolled cell division and are likely to proliferate to cancerous tissue or tumors respectively. Most chemotherapeutic agents target the fast and uncontrolled cell division in order to force cells to induce apoptosis by destruction of important cell compartments like the nucleus or die by necrosis. In order to ascertain the success of anticancer treatments, a method for detecting apoptotic regions in cancerous tissue is desirable. Monitoring anticancer treatment responses would allow a fast, individual adaptation of the therapy. Different biochemical techniques have been developed to determine whether cells are undergoing apoptosis. However, these methods are invasive and time consuming. The most promising non-invasive technique uses SPECT (Single Photon Emission Computed Tomography) or PET (positron emission tomography) imaging, combined with specific radioligands. However, these methods need radioactive substances to be introduced into the body and therefore scans cannot be performed repetitively over a longer period of time (Lahorte et al. 2004). The use of bioluminescence markers combined with optical imaging methods for identifying apoptosis work non-invasively as well, but lack penetration depth. Kolios et al. 2002 and Czarnota et al. 1999 used high frequency ultrasound in the range of 20MHz to 60MHz for imaging apoptosis. Since ultrasound intensities used in diagnostics does not harm the human body like PET or X-ray, scanning procedures can be performed repetitively. An imaging method based on ultrasound measurements would offer the ability of monitoring treatment response over an expanded time period.

Kolios et al. 2002 observed increasing intensity in ultrasound backscatter when apoptosis occurred. The experiments included in-vitro studies investigating cancerous cell lines in culture and in-vivo experiments interrogating tumors grown in mice legs. However, regions of increased brightness in common ultrasound B-mode images can have a variety of reasons. Therefore a method for more accurate determination of apoptosis needs further specification from the ultrasound signals. Since regular ultrasonic imaging devices only use the intensity of the backscattered ultrasound, additional characteristics of the detected signals can be extracted from unprocessed rf data and used for determination of the tissue condition, especially apoptosis.

In this study optical and acoustical microscopy of cells undergoing apoptosis was performed to investigate characteristic changes in acoustical properties of cells

undergoing cell death. Apoptosis was verified by laser fluorescence microscopy. Conclusions obtained from microscopy investigations were related to observations from high frequency ultrasound experiments on the same cell lines but in pellets of cells. In acoustic microscopy cells undergoing apoptosis were found to be less regular in shape and higher in contrast in the images. When interrogating cells using high frequency ultrasound the intensity of ultrasound backscatter increased for progressing post-treatment time by 100% for HeLa and 25% for MDCK cells. It was found that acoustic attenuation increased by 50% to 80% as a function of post treatment time for both cell lines.

II. Methods and Materials

A. Biological samples

In this study HeLa and MDCK-I cells were investigated. HeLa is cancerous cervical carcinoma cell line. MDCK (Madin-Darby-Canine-Kidney) is an epithelial non-cancerous cell line of the kidney, derived from a cocker spaniel. Both cell lines are adherent and were studied for their response to the chemotherapeutic agent cisplatin. Cells were incubated in minimum essential media (MEM) supplemented with 10% of heat-inactivated fetal bovine serum (FBS) and 0.1% gentamycin (antibiotics) in a humidified atmosphere at 37°C containing 5% CO_2. For the high frequency ultrasound investigation cells were thawed from frozen stock and grown in cell culture flasks for 10 days. Cells were exposed to 10 µg/ml cisplatin for 0h, 8h, 12h, 18h, 24h and 32h before harvesting. Trypsin was used to detach the adherent cells from the flasks. The cell suspension was then centrifuged at 216g and the medium was removed. Then cells were resuspended in phosphate buffered saline (PBS) and centrifuged to the final pellet at 1942g in a custom-made two-chamber holder, shown in Figure 1. In preparation for microscopic investigations, cells were grown in tissue culture cover-glass systems containing the described medium. For fluorescence microscopy cells were labelled with DIOC 3(5) and H33342 to visualize membranes and condensed chromatin.

B. Acoustic and light microscopy

The SASAM combines a scanning acoustic microscope, a phase contrast microscope and a laser fluorescence microscope in one device. The optical unit was an Olympus IX 81 microscope (Olympus, Japan). After performing acoustic microscopy, fluorescence makers were added to the medium. Hoechst–33342 was used for visualization of condensed chromatin in laser fluorescence. Cell membranes were visualized using the fluorescence marker DIOC3(5). In addition phase contrast imaging of the investigated cells was performed. A CCD camera with 672x512 elements and a resolution of 0.32µm was built in the microscope.

Scanning acoustic microscopy was performed with a highly focussed ultrasound transducer at 0.9 GHz centre frequency. During the scanning process the transducer unit was moved in a plane parallel to the cover-glass. For each acoustic scan 350x350 measurements were performed to create the image. The spacing between the transducer positions was 0.16µm and the dimensions of the resulting images were 56µm x56µm. At each position an ultrasonic pulse (centre frequency 0.9GHz, bandwidth 250MHz) was transmitted and the received echoes were digitized using a Acquireis A/D-converter. The sampling frequency was 4GHz. Received signals were windowed at the position of the glass plate, using a rectangular window, in order to estimate the reflection of the cover-glass. The resulting acoustic image shows the intensity of the reflected pulse. Scans were repeated 5 times in order to estimate averaged intensity values. The time duration of each scanning process was approximately 10 minutes.

C. High frequency ultrasound

Ultrasonic imaging and rf data acquisition was performed with an ultrasound biomicroscope (UBM) (VisualSonics, Toronto, Canada). The transducers used for the experiments were a 20 MHz (100% bandwidth, 8.5mm aperture diameter) and a 40 MHz (98% bandwidth, 3mm aperture diameter) transducer. The f-numbers were 2.35 and 3 respectively. The use of two transducers allows verifying the independence of the normalized rf-data from the system transfer characteristics. The UBM enabled real time B-mode imaging of the interrogated specimen. The sampling frequency of the A/D-converter unit was 500 MHz. From each cell pellet 255 rf lines were recorded at three different scan-planes. The length of each scan line was 3-4mm around the transducer focus. Signal analysis was performed off-line using a custom made MATLAB (Mathworks, Natick, USA) based program. It allowed reconstructing the B-scan images from the unprocessed rf-data and selecting a region of interest (ROI) enclosing only signals from inside the cell pellets. Horizontally, ROI's were placed inside the cell pellet and contained 200 RF-lines. For estimating averaged parameter values, 3 ROI's were chosen within each investigated cell pellet. All ROI's were centred vertically at the transducers focus and were approximately 1mm in height. A Fourier transform was applied to each rf-line within the ROI and power spectra were obtained by averaging the results of the independent scan lines. For calibration purposes all power spectra were normalized to the reflection of a flat quartz cylinder, placed at the focus of the transducer, which was used for normalization (Lizzi et al. 1983). Linear regression analysis was applied to the normalized power spectra. The integrated backscatter coefficients were also estimated from normalized spectra (Worthington et al. 2001). For estimating backscatter coefficients it was assumed that the attenuation along the path of sound propagation was 0.02 dB/(MHz mm) and therefore was close to the attenuation of distilled water (Duck 1990). Cell pellets were prepared in a custom made two-chamber holder with a quartz plate bottom. One of the chambers contained the cell pellet and the other one was filled with PBS, in order to estimate acoustic attenuation, using the spectral substitution technique (Kuc et al. 1985). The two-chamber holder, shown in Figure 1, enable the data

acquisition of both reflections at the same scan. The power spectra of the reflections were calculated and averaged from 25 scan lines in each chamber. Following this, power spectra were subtracted and the attenuation was estimated in dB/MHz from the slope of the resulting spectrum. By normalizing these attenuation values to the thickness of the cell pellet, the attenuation in dB/(MHz cm) was derived.

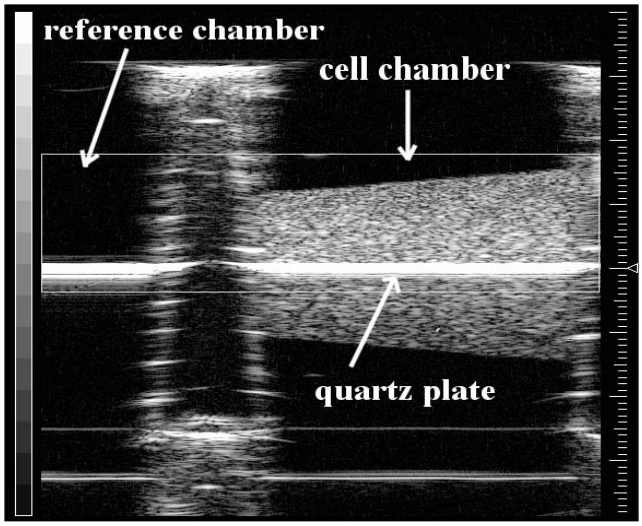

Figure 1: Two chamber holder; left) reference chamber, containing PBS; right) cell chamber, containing cell pellet

III. Results and Discussion

A. *Acoustic and light microscopy*

Figure 2 shows results of the acoustical and optical microscopy. Laser fluorescence images of H3332 and DIOC3(5) labeled cells clearly indicate cells undergoing apoptosis in which nuclear fragmentation occurs. Condensed chromatin can be seen as intense blue in the images. DIOC3(5) labeled membranes are stained green, showing the disrupted membranes, which is also an indication of apoptosis. In the images obtained by acoustic microscopy, cells undergoing apoptosis are significantly darker than normal cells. The brightness in these images is directly related to the ultrasound intensity reflected from the glass plate. Thus, darker regions indicate lower intensity of the reflected pulse. The darker regions seen in the acoustic images can be interpreted differently. In the case of a density alteration within the cell interior, the acoustic impedance of the cell would change the reflectivity of the boundary cell/glass-substrate. Also an increased acoustic attenuation of the cell interior, when undergoing apoptosis, would result in a decreased intensity of the reflected signal. Since the acoustic images are currently qualitative, it is not clear what these changes represent. However, fragmentation of the nucleus will most likely result in higher scattering and thus in higher attenuation. Due to high acoustic attenuation in the 1GHz frequency range, scattering from the cell interior could not be detected. A measurement of the intensity, scattered from the interior structure of the cell would provide further information and modifications to the system are made to attempt to measure these signals with a sufficient signal to noise ratio.

B. *High frequency ultrasound*

In Figure 3 the intensity of backscattered ultrasound as a function of treatment time is shown. Backsctatter intensity increases with exposure to cisplatin. Figure 3 also shows that backscatter coefficients are higher for measuring with the 40 MHz transducer compared with the 20 MHz transducer. Scattering will reduce the energy from the incident wave and thus, would lead to a lower detected intensity, when measuring with the setup used for acoustic microscopy. Acoustic attenuation was also estimated from high frequency ultrasound signals using spectral subtraction method. Attenuation values for both cell lines, MDCK and HeLa are shown in Figure 4. The attenuation values increased as a function of treatment time. These observations help in the interpretation of the data acquired with acoustic microscopy.

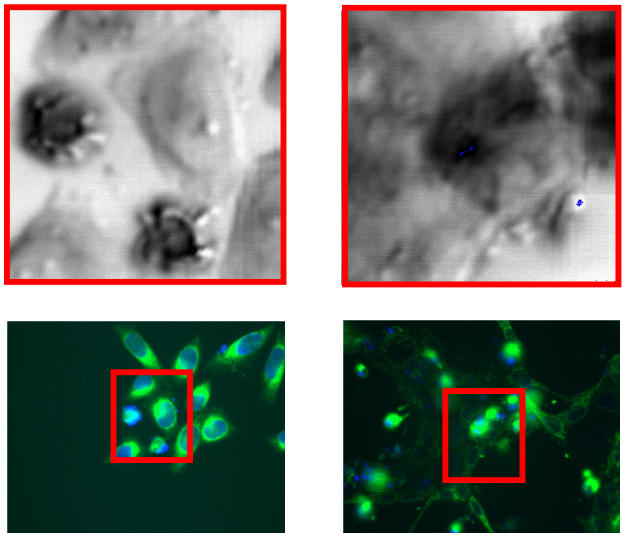

Figure 2: Microscopic images, acoustic top, laser fluorescence bottom -- left) HeLa, right) MDCK. Blue stained regions indicate condensed chromatin, green membranes.

IV. Conclusions

Acoustic and light microscopy was used to investigate physical changes during apoptosis on a cellular scale. Apoptotic cells shrank in size and showed an increase in attenuation when using acoustic microscopy in the 1GHz frequency range. Additionally, high frequency ultrasound measurements were performed on cell pellets subjecting the same cell lines. The treatment procedure was the same for both investigation methods. It was found that the intensity of backscattered ultrasound varies as a function of treatment time. Acoustic attenuation was also found to increase as a function of time in apparent agreement with the results of the acoustic microscopy. The combination of acoustic microscopy and high frequency ultrasound estimates of

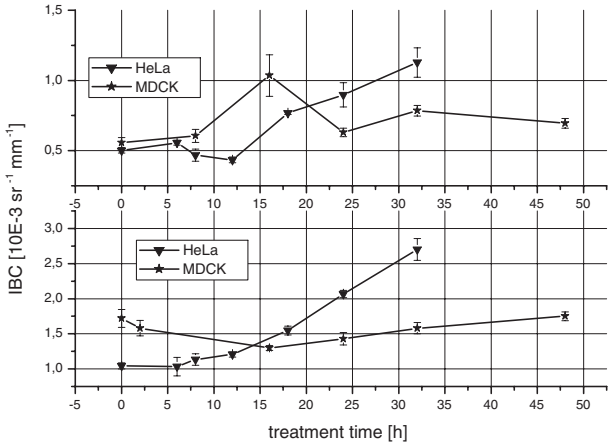

Figure 3: Integrated backscatter coefficients estimated as a function of treatment time top) 20MHz transducer, bottom) 40MHz transducer. Errorbars represent standard deviations between independent scan planes.

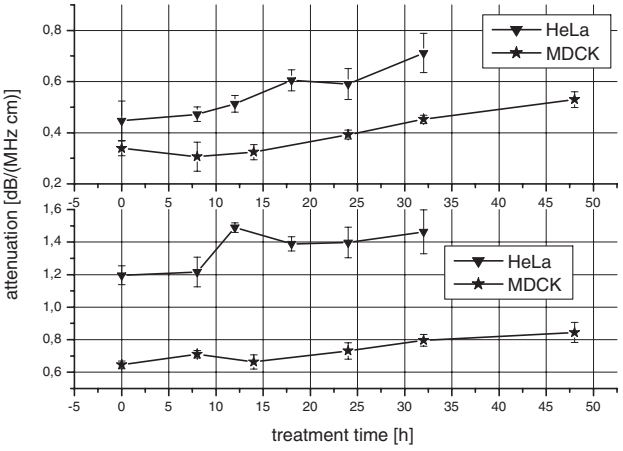

Figure 4: Attenuation estimated as a function of treatment time top) 20MHz transducer, bottom) 40MHz transducer. Errorbars represent standard deviations between independent scan planes.

acoustic attenuation provide further understanding in the high frequency ultrasound backscatter analysis. It shows that the entire cell during apoptosis changes its acoustical properties. Increasing attenuation measured in reflection is in agreement with the observed increase in backscatter intensity. Future work will focus on improvements of the system so as to extract quantitative acoustic properties to gain further understanding of the scatter origin on a cellular level as well as the influence of the spatial distribution of cells on high frequency ultrasound backscatter of cell pellets.

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