Abstract

Purpose/Aim: In this work, we examined the possible effects of ionizing radiation (IR) on biomechanical properties of the membrane–cytoskeleton of human erythrocytes, after X-ray irradiation. Materials and Methods: Whole human blood from three healthy middle-aged volunteers was drawn by venipuncture and stored in tubes containing anticoagulant. Six blood samples were collected for each volunteer. Five of them were irradiated in the range of 0.1 Gy–2.0 Gy doses and one was used as control. The morphology and the elastic modulus of the erythrocytes were examined using atomic force microscopy and just few drops of whole blood. Results: No morphological changes appeared according to the shape and the morphology of the erythrocytes. The elastic modulus of the irradiated samples was reduced with the increase of radiation dose. The findings indicate that X-ray irradiation affects the biomechanical properties of erythrocyte cytoskeleton. The mean value of Young’s modulus of all the irradiated blood samples was significant difference from the control at a level, $P < 0.01$. Conclusions: The elastic modulus of the erythrocytes could be an indicator of the adverse effect in the human blood generated by IR exposure through a radiotherapy treatment.

Keywords: Biomechanical properties, elastic modulus, erythrocyte cytoskeleton, ionizing radiation effects

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Introduction

The use of ionizing radiation (IR) as a diagnostic and/or radiotherapeutic tool is the subject of a strong and sometimes controversial study, regarding the radiation-induced harmful effects, as well as the clarification of the primary site of the radiobiological damage. In recent years, the number of diagnostic and therapeutic procedures using IR has been increased. Particularly, in pediatric patients which exhibit an increased radiosensitivity, in combination with high radiation doses received by patients of these procedures, advanced studies are required for the effects of IR at cellular level[1,2] to improve sensitive biosensors for radiation effect evaluation. It is well documented that IR can cause biological damage at the molecular level and cellular DNA decomposition, as single- or double-strand breaks.[3,4] However, the possible effects of IR on the biomechanical properties of cells should also be a very interest subject. It is recognized, in several biomedical disciplines, that the biomechanical properties (i.e., deformability, stiffness, elasticity) of cells can be an important predictor of circulation efficiency and cell health. According to the latest research studies, the biomechanical properties of individual living cells are closely related to the health and function of human body.[5-7] More precisely, morphological and cytoskeletal abnormalities of red blood cells (RBCs), namely erythrocytes, have been associated with important disorders such as spherocytosis,[8] systemic lupus erythematosus,[9] diabetes,[10] and oxidative disorders caused by pollution, radioactive radiation, toxics, and bad nutrition.[11] Changes in deformability of a single RBC are assessed through micropipette aspiration,[12] automated rheoscopes,[13] and microfluidics with optical traps using two beads fixed on either side of an RBC.[14]
Since it is worldwide accepted that IR causes biochemical damages at the molecular level and the cellular DNA, in this research work, we investigate how possible is a radiation-induced damage at cellular cytoskeleton level? We examined the effects of IR on the morphology and the biomechanical properties of RBCs after X-ray exposure. The blood samples were exposed to radiation doses in the range of doses delivered in typical daily radiotherapy treatments. Studying any possible effects of radiation on the deformability of RBC, which lack DNA, we separated the radiobiological effects from DNA damage or from the interference of intracellular membranes. RBCs are a suitable candidate for monitoring the radiation effects for one more reason: they are the “war correspondents” for the whole-body exposure, since they circulate all over the body.

The erythrocytes’ changes were probed using advanced microscopic techniques of atomic force microscopy (AFM) which is ideally suited for single cell measurements, providing simultaneously information about the mechanical properties. AFM is a scanning probe microscopy technique which can create three-dimensional micrographs with resolution down to the nanometer and Angstrom scales. AFM utilizes a nano-tip (probe) attached to a flexible cantilever of a specific spring constant. AFM can also provide force-distance curve measurements by force spectroscopy application. It can record the amount of force felt by the cantilever as the probe tip is brought close to and even indented into a sample surface and then pulled away. Hence, this technique can elucidate local chemical and mechanical properties.

**Materials and Methods**

**Blood sample selection**

Peripheral human blood from three healthy middle-aged volunteers was drawn by venipuncture and was temporarily stored in standard anticoagulant-containing ethylenediaminetetraacetic acid (EDTA) vacutainer test tubes. All the blood samples were selected voluntarily from the authors of this work. The irradiations were performed within 3 h. The AFM analysis was conducted for all the samples 1 day after irradiation. The blood samples were kept at 4°C before each treatment. Blood samples were subjected to the minimum possible treatment which could affect RBCs deformability (buffers, centrifugation, etc.).

**Irradiation procedure**

For each volunteer, six EDTA tubes with blood were collected to irradiate at doses 0.1, 0.2, 0.5, 1, and 2 Gy. One EDTA tube was kept as control (0 Gy). Two samples were analyzed for each condition. The irradiation took place at the Radiation Therapy Unit of 2nd Department of Radiology of National and Kapodistrian University of Athens at University General Hospital “Attikon.” The procedure was done on linear accelerator Varian Clinac 2100C. Dose uniformity was ensured by irradiating the samples with two parallel-opposing anterior posterior fields 6 MV photon fields with a dose rate of 240 cGy/min. Dose calculations were done in Eclipse treatment planning system (version 15.1, Varian). Normal nonirradiated erythrocytes from the same volunteers were used as controls.

**Atomic force microscopy analysis**

The morphology and the elastic modulus of the erythrocytes were examined in comparison with nonirradiated erythrocytes using AFM (Nanoscope dilNovo, Veeco metrology, Santa Barbara, CA, USA) with an Innova scanner possessing a maximum range of 100 µm × 100 µm × 7.6 µm. Just few drops of whole blood were deposited on glass slides without any special preparation which could modify the membrane, at least at the nanometer level. Silicon nitride cantilevers with spring constant of 0.02 N/m were used. AFM tips were imaged utilizing scanning electron microscopy to have a reference value of their radii which were ranged to 40 ± 10 nm.

**Statistical analysis**

All the statistical comparisons for the distribution of Young’s modulus values for each irradiation condition were performed using analysis of variance (ANOVA) with Bonferroni correction. P <0.01 was considered statistically significant.

**Results**

**Atomic force microscopy topographic imaging**

Atomic force topography of the RBCs was recorded using the contact mode of AFM. In contact mode, also known as repulsive mode, the tip was in close contact with the surface of the sample constantly, which makes soft “physical contact” during the scanning process. No morphological changes were observed according to the shape of the erythrocytes for this range of doses; as shown in Figure 1a and b, both control and irradiated erythrocytes appeared donut-shaped. However, a rise of the membrane roughness was observed as the dose was increased. The roughness was expressed by the root-mean-square (RMS) roughness parameter estimated by the AFM apparatus software for all doses [Figure 2]. RMS is defined as the square root of the mean value of the squares of the profile height deviations.

**Elastic modulus determination using atomic force microscopy spectroscopy**

AFM appears as a powerful tool to provide information about micromechanical properties of cells. AFM spectroscopy is based on the detection of repulsive or attractive surface forces between the atoms of the sample and those of the tip (probe), which scans the surface of the sample. Using an atomic force spectroscopy application, the amount of forces felt by the cantilever can be recorded as the tip is brought close to and indented into the liposome surface and then pulled away. The plot of cell pushback on the tip versus the probe depth yields to a force curve that provides information about the cells elastic properties.
Force–distance curves were produced by recording the erythrocytes pushback on the tip (deflection) versus vertical Z position of the tip probe. Analysis was performed using selected force curves in SPMLab analysis software ver. 7.0.0.1 (Veeco, Metrology, Santa Barbara, CA, USA). The difference in the slope of the force curves represents the difference in stiffness of the samples. To obtain quantitative differences in the elasticity of the erythrocytes, the tip indentation \( dz \) into the soft sample was calculated as the difference of the respective Z positions relative to the ideal hard (glass) surface. The force curves were collected from the central part of the erythrocytes.

Figure 3 presents force curves recorded on erythrocyte as a function of the tip indentation for two different doses 0 and 1 Gy. According to Hertz–Sneddon model\(^{[19]}\) the applied force as a function with the indentation depth of AFM tip is given by the equation 1.

\[
F = \frac{4}{3} \frac{E \sqrt{R}}{(1 - \nu^2)} dz^\frac{3}{2}
\]

where \( F \) is the loading force, \( E \) is the Young’s modulus, \( \nu \) is the Poisson ratio, \( R \) is the radius of the AFM tip, and \( dz \) is the indentation of the AFM tip into the erythrocyte. The Young’s modulus can be calculated from the slope of the linear curve, which fits the plot of force versus indentation \( \frac{3}{2} \), according to the equation 2:

\[
E = \frac{3}{4} \frac{1 - \nu^2}{\sqrt{R}} \frac{dF}{dz^\frac{3}{2}}
\]

Cell membranes are generally assumed to be quasi-incompressible, characterized by a Poisson ratio of 0.50. A tip radius of 40 nm diameter and cantilever spring constant of 0.02 N/m were used for all calculations.

For each measurement, 20 force curves were recorded, and the results are shown in histograms in Figure 4. According to the ANOVA results, the means for all doses were different to the control (0 Gy) at a level \( P < 0.01 \). However, no statistically significant difference was observed between the doses 0.1 and 0.2 Gy as well as between 0.5 and 0.2 Gy. The means for doses 1 and 2 Gy were significantly different to the means for doses 0.1, 0.2, and 0.5 Gy.

Figure 5 shows the change of mean elastic moduli of erythrocytes after the X-ray radiation as a function of dose.
the radiation dose. The mean values and the standard deviation of the elastic modulus were obtained by fitting the Gaussian distribution to the histograms after performing the Shapiro–Wilk normality. According to the literature, the values of Young’s modulus for human healthy erythrocytes lie in the range of 1–26 KPa.\textsuperscript{20,21} The elastic modulus of the erythrocytes is reduced nonlinearly as the X-ray dose increases and tends to reach a plateau for doses above 1 Gy.

**DISCUSSION**

A majority of publications are concerned with radiosterilization of blood as from the very beginning of IR biomedical applications. The potential use of this technology was for the sterilization and inactivation of pathogenic microbes in contaminated blood products. Irradiation of blood and blood components was practiced in developed and in a few developing countries, mainly for the prevention of graft versus host disease in immunodeficient patients by the abrogation of T-lymphocytes (IAEA, 1997).\textsuperscript{[22]} In this survey, the IAEA contributors contacted a literature review on the effects of IR (essentially gamma and X-rays) on blood cells. X-irradiation of erythrocytes at 200 Gy failed to alter their morphology, osmotic and mechanical fragility, or glycolytic activity. However, incubation at 37°C for 24 h slightly increased their fragility.\textsuperscript{[23]} In another study, Lessler\textsuperscript{[24]} showed that a lower level of X-rays (1 Gy) was sufficient to cause cytological abnormalities to bullfrog erythrocytes. A decade after the IAEA’s survey, Reverberi et al.\textsuperscript{[25]} tried to study the reasons of the posttransfusion loss of viability in irradiated erythrocytes. They reported that the erythrocyte deformability was the only parameter related to viability to show sufficiently precocious and important changes although the mechanism by which irradiation influenced viability remained unclear.

Erythrocytes’ membrane consists of 52% proteins, 40% lipids, and 8% carbohydrates.\textsuperscript{[26]} Changes in erythrocyte cell cytoskeleton properties have been strongly correlated with the distribution of lipids, fatty acids, and spectrin-a1 protein in the membrane–skeleton system.\textsuperscript{[27,28]} According to our AFM results, IR affects the biomechanical properties of the membrane–cytoskeleton of erythrocytes, which lack DNA. The erythrocytes can become less stiff as they are exposed to X-ray radiation through radiotherapy treatments. However, at high doses (≥1 Gy), a plateau is observed to the values of Young’s modulus, indicating that there is no linear dependence between irradiation dose and elasticity.

Comparative results have been established after Syrian golden Hamsters’ blood irradiation with X-ray at doses of 2, 4, 6, and 12 Gy.\textsuperscript{[28]} Erythrocytes became less stiff as they were exposed to ionizing irradiation and adverse effects in RBC’s deformability were observed at higher doses. The mechanical properties of the erythrocytes were also correlated with the cytoskeletal protein spectrin-a1 rearrangement depending on the radiation dose.\textsuperscript{[29]} Increased erythrocytes deformability has been observed in aplastic anemia\textsuperscript{[30]} and myelodysplastic syndromes,\textsuperscript{[29]} which have been associated with radiotherapy treatments.\textsuperscript{[31]}
The findings of this research work indicate that the IR has not only radiobiological effects on cells but also can influence the mechanical properties of the membrane–cytoskeleton. This may bridge the gap between the biological processes that take place inside the cells and the mechanical behavior of the cells. Biochemical changes can be transduced into mechanical deformations, and vice versa. Further work needs to be conducted to understand such mechanisms and behaviors. In future, biomechanical biomarkers can be used to assess the adverse effects on cells induced by radiation.

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Conflicts of interest

There are no conflicts of interest.

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