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The use of atomic force microscopy for structural and surface morphological analysis of Fanconi anemia patient fibroblasts before and after exposure to γ-radiation

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The surface morphological changes of Fanconi anemia patient fibroblasts after exposure to γ–radiation were investigated by AFM and foci immunofluorescence staining. The reorganization of the actin cytoskeleton was found, having resulted in reduction of the membrane stiffness and increase of adhesion in nuclear and lamellipodial regions of the cell.

Atomic force microscopy (AFM) has proven to be a powerful tool for fibroblasts study. In addition to high resolution visualization, elastic properties of fibroblasts can be detected with the AFM [1, 2]. In this work the reorganization of cytoskeleton structure of Fanconi anemia (FA) patient fibroblasts and the change in the mechanical properties (stiffness, hardness, elasticity) of cell membrane after exposure to γ–radiation were studied by AFM and fluorescence microscopy.

Two strains of skin fibroblasts isolated from an FA patient were evaluated for their in vitro radiosensitivity using AFM and foci immunofluorescence staining. While one set of cells left untreated (control cells), the other one was exposed to γ–radiation at 5 Gy.

Primary skin fibroblasts were obtained from FA patient by minimal invasive 3-mm punch biopsy. Small pieces of skin were incubated in the appropriate medium (Dulbecco's Modified Earle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic in 6-well plates under the cover slides at 37˚C. Fibroblast's growth was observed after 10-14 days of cultivation. To expose FA fibroblasts to ionizing radiation 50,000 cells were transferred into the culture dishes with glass slides at the bottom and incubated 24 hours at 37˚C. Then several plates containing attached fibroblasts were exposed to γ radiation at 5 Gy, then incubated 24 hours at 37˚C. For AFM investigation cells were fixed with 2% glutaraldehyde for 30 min.

All data were obtained on a Nanoscope (R) IIIa MultiMode AFM (Digital Instruments/Veeco). Force modulation mode (FMM) was used to study mechanical properties (local stiffness and adhesion) of the fibroblasts. The AFM capabilities can be extended by using FMM, which enables to obtain information about relative difference in cell surface elasticity with nanometer-scale resolution. The AFM images were acquired by using silicon nitride cantilevers (NSC12/50) with a nominal force constant of 0.65 N/m (NT-MDT, Zelenograd, Russia). The measurements were performed in air at room temperature. AFM images were processed with the Nanoscope software (Digital Instruments/Veeco).

To stain actin and tubulin fibroblasts were grown into the chambers on slides. After exposure cells to irradiation slides were washed and fixed in 4 % paraformaldehyde solution for 30 minutes at +4˚C. Then samples were washed twice and permeabilized in 0.1% Triton X-100 for 15 minutes. Then samples were incubated with Alexa Fluor 488 anti-α-tubulin antibody (1:1000) and Alexa Fluor 633-Phalloidin (1:500) (Molecular Probes) for 1 hour at 37˚C, washed twice in PBS. Slides were additionally stained with propidium iodide to detect nucleus. Fluorescence was analyzed by confocal laser scanning microscope Leica TCS SPE.

AFM images of skin fibroblasts isolated from an FA patient exhibited the characteristic spindle shaped cells with irregularly shaped flat lamellipods. Fibroblasts cellular length varied from 70 to 120 μm. Nuclear region height was about 600 – 950 nm with lamellipodia thickness

from 80 to 380 nm. The AFM images of untreated FA fibroblasts demonstrate that actin stress fibers form densely packed parallel arrays with lateral size from 30 to 200 nm traversing the nucleus area. The structure of actin stress fibers appears better defined in the error signal image. Zooming in on the nucleus the granular structure of elongated bundles of actin filament with minimum measured granule size of 30 nm was visualized.

Irradiated FA fibroblasts revealed densely packed parallel long, straight actin stress fibers with average fiber diameter in the range of 30-70 nm. Thick parallel actin stress fibers with the lateral size from 100 to 320 nm extending throughout the nucleus were also visualized for FA fibroblasts in 24 hours after exposure to γ–radiation. The AFM study also showed a decreased height of nucleoli in the nucleus of irradiated FA fibroblasts as compared to nucleus of untreated fibroblasts. Disruptions of actin filaments were visualized in irradiated FA fibroblasts.

The topographic, adhesion and stiffness images of the FA fibroblasts in 24 hours after exposure to γ–radiation at 5 Gy are given in Figure 1. Darker parts in the adhesion and stiffness images correspond to low adhesion and stiffness value on fibroblast membrane.

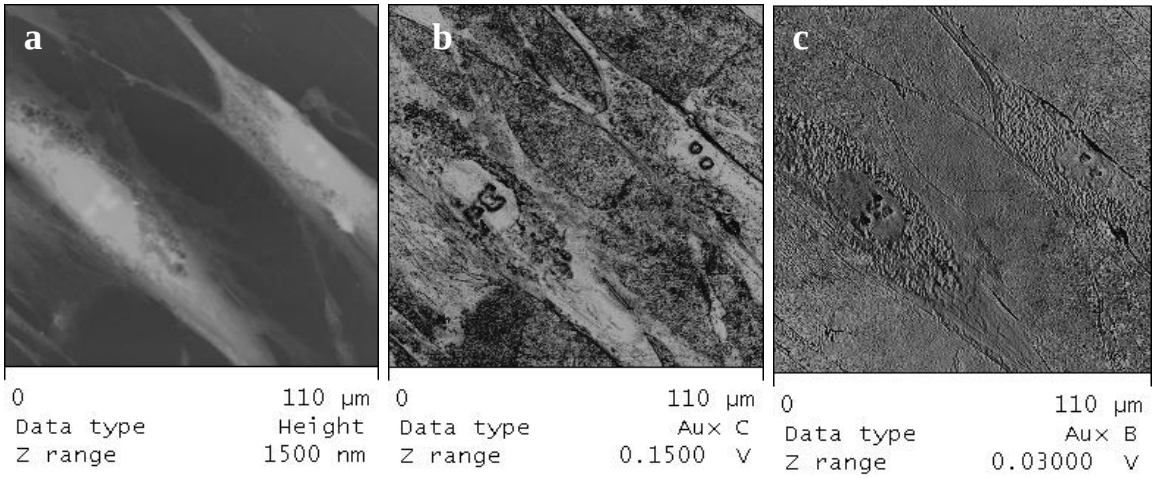

Figure 1. AFM images of FA fibroblasts (a- height, b- adhesion, c- stiffness) in 24 hours after exposure to γ–radiation at 5 Gy.

The reorganization of the actin cytoskeleton occurs in irradiated FA fibroblasts, resulting in reduction of the cell membrane stiffness and adhesion increase in nuclear and lamellipodial regions of the cell. As seen from the AFM images (Fig.1) the FA fibroblasts appear less stiff even in thinner lamellipodial regions.

The mechanical properties of fibroblasts most likely are regulated by the actin cytoskeleton structure. According to the fluorescent images of irradiated FA fibroblasts microtubules originated from the center and formed a radiating network near the nucleus. Moreover actin stress fibers traversing the nucleus area were less evident and the stress fibers were not as well stretched as in FA fibroblasts before irradiation. Fluorescent images revealed that actin stress fibers appeared more concentrated at the periphery of irradiated FA fibroblasts. Disruption of actin filaments and change of spatial organization of the actin cytoskeleton in 24 hours after exposure to γ–radiation lead to a softening of the FA fibroblasts' membrane. The pulsed force mode of AFM revealed that nuclei of untreated FA fibroblasts are more adhesive and less rigid than the surrounding nucleus region and the peripheral (lamellipodial) regions. The stiffest part of untreated fibroblasts corresponds to the lamellipodial region of the cell. The reorganization of the fibroblasts cytoskeleton structure after exposure to γ–radiation leads to change in the mechanical properties of cells, so it is possible to use the cell mechanical parameters as certain markers of the pathology.

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2. Sh. Hiratsuka, Yu. Mizutani, M.Tsuchiya et al, *J. Ultramicroscopy* **109**, 937 (2009).