ATOMIC FORCE MICROSCOPY STUDY OF FIBROBLASTS OF FANCONI ANEMIA PATIENTS AFTER EXPOSURE TO Γ-RADIATION

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In this study the structural and surface morphological changes of Fanconi anemia patient's fibroblasts occurring after exposure to γ–radiation were investigated by atomic force microscopy and foci immunofluorescence staining. It was found that the reorganization of the actin cytoskeleton had occurred in Fanconi anemia patient's fibroblasts in 24 hours after irradiation, having resulted in reduction of the cell 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]. 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.

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 Fanconi anemia (FA) patients 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. Thereafter fibroblasts were washed five times in phosphate-buffered saline (PBS), dehydrated in a graded series of ethanol and air dried.

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 (Fig.1) of skin fibroblasts isolated from an FA patient exhibited the characteristic spindle shaped cells with irregularly shaped flat lamellipods. The AFM investigations revealed a considerable range of spreading and the lengthened shape of cells. For FA patient fibroblasts cellular length varied from 70 to 120 μm. For FA patient cells nuclear region height is about 600 – 950 nm with lamellipodia thickness from 80 to 380 nm. The nuclear height of the fibroblasts was around three times higher than that the lamellipodia height.

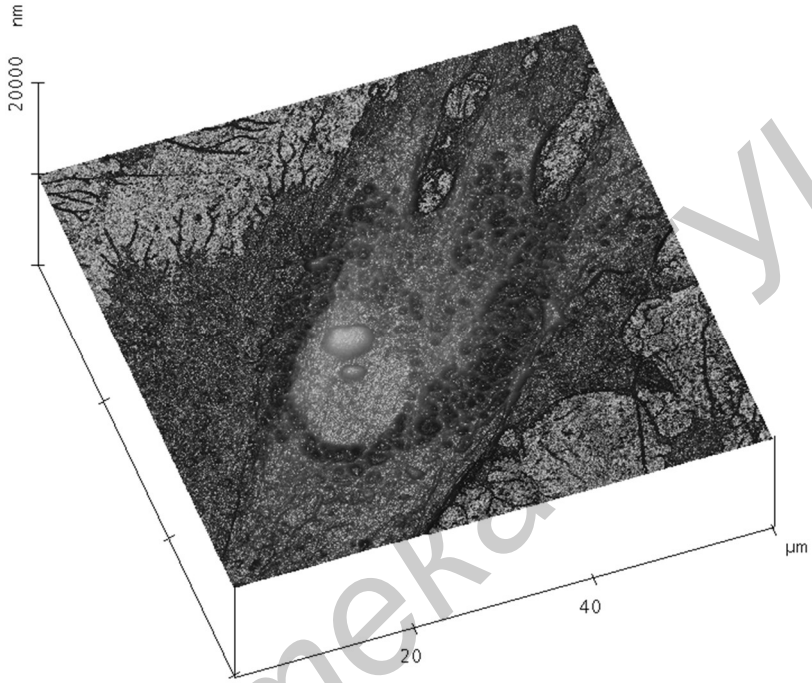

Figure 1 - AFM image of skin fibroblasts isolated from an FA patient

AFM images obtained on a smaller scan area (Fig. 2) present more detailed information of the organization, shape and dimensions of single actin fibers. The lateral size of densely packed parallel arrays of actin stress fibers varies from 30 to 200 nm for untreated FA fibroblasts.

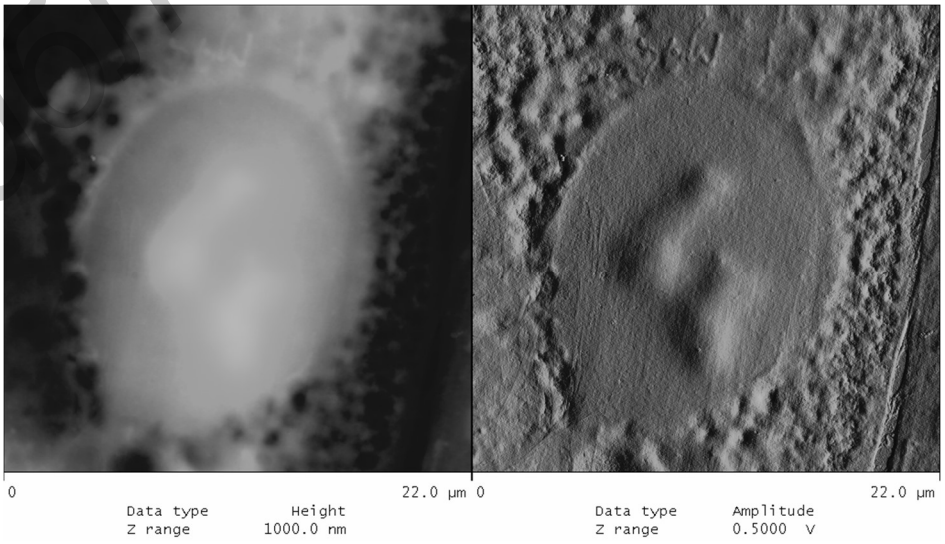

Figure 2 - AFM images of nucleus of untreated FA fibroblast

Zooming in on the nucleus the granular structure of elongated bundles of actin filament with minimum measured granule size of 30 nm is visualized. The structure of actin stress fibers appears better defined in the error signal image.

Fig. 3 presents the AFM images of zoomed area of the nucleus of FA fibroblast in 24 hours after exposure to γ–radiation at 5 Gy. 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. Disruption of actin filaments was visualized in irradiated FA fibroblasts.

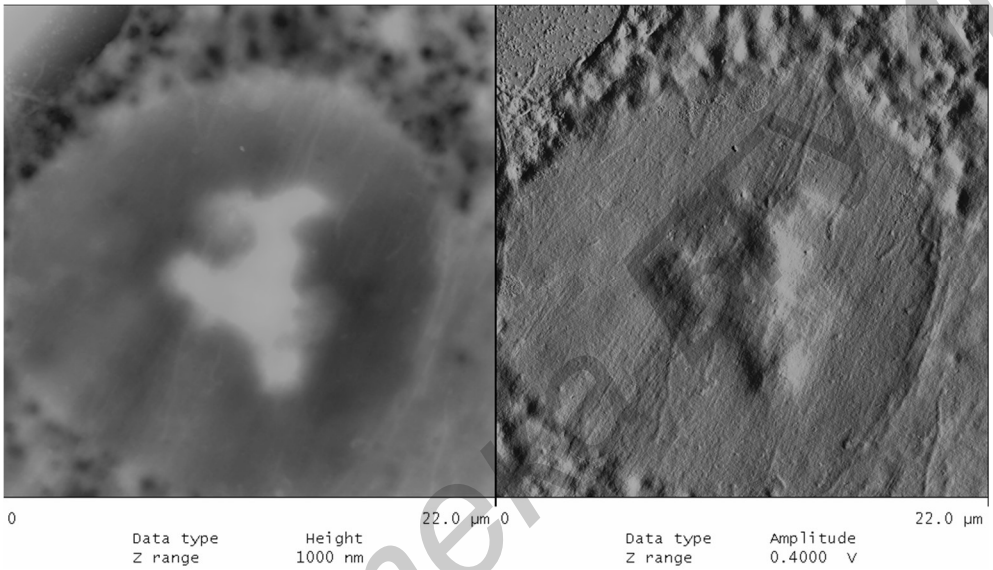

Figure 3 - AFM images of FA fibroblast nucleus in 24 hours after exposure to γ–radiation at 5 Gy

The mechanical properties of fibroblasts most likely are regulated by the actin cytoskeleton structure. Immunofluorescence staining was carried out to show cytoskeleton changes in FA fibroblasts in 24 hours after irradiation. 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. Using the pulsed force mode adhesion and stiffness images of fibroblasts were obtained simultaneously with its topographic image. 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 actin cytoskeleton occurs in FA fibroblasts in 24 hours after irradiation, resulting in reduction of the cell membrane stiffness and adhesion increase in nuclear and lamellipodial regions of the cell.

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