# BIOGERONTOLOGY

# Poly(ADP-Ribose)-Polymerase-1 and Aging: Experimental Study of Possible Relationship on Stationary Cell Cultures

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Experiments on "stationary aging" cultures of B11dii-FAF28 Chinese hamster cells showed that contact inhibition of cell growth and further culturing of cells in the stationary phase led to continuous inhibition of enzymes realizing poly-ADP-ribosylation of chromatin proteins. Cell density in the monolayer and percentage of damaged cells detected by trypan blue staining decreases during this process.

**Key Words:** *poly(ADP-ribose)-polymerase-1; Chinese hamster cell culture; cell viability; aging*

Some kinds of DNA damage lead to activation of poly-ADP-ribosylation of chromatin proteins [6]. The main enzyme realizing this process is poly(ADP-ribose)-polymerase-1 (PARP-1, CF 2.4.2.30). The important role of poly-ADP-ribosylation in the maintenance of genetic stability and integrity of the genome has been shown [10]. On the other hand, a relationship between genetic stability of the genome and aging has been revealed [2]. The number of DNA injuries and other genetic disorders increases with age, while the efficiency of repair system decreases [1,8].

The interest of geronotologists to poly-ADP-ribosylation of chromatin proteins is primarily explained by detection of a correlation between poly-(ADP-ribose)-polymerase (PAR-polymerase) activity in leukocytes and the maximum species-specific life span of mammals [9,12]. The data on age-specific changes in PAR-polymerase activity are scanty. This parameter decreases during aging [9,13]. The cause-effect relationships between PAR-polymerase activity and aging process remain little studied.

Many parameters used in the studies of aging correlate with the age of the organism, but are not directly related to aging [14]. Therefore, longitudinal experiments or experiments on the so-called "essence" models, based on certain mechanisms of aging, advocated by the authors, are recommended for detecting the cause-effect relationships. One of these models is "stationary aging". It is based on a concept that limitation of cell proliferation during the ontogeny is the cause of accumulation of macromolecular injuries with age; these injuries promote dysfunctions of organs and tissues and increase the probability of death [3]. We propose stationary cell cultures as a model for studies of age-associated changes in the cells of an aging organism.

We studied changes in PAR-polymerase activity during "stationary aging" of a cell culture.

## MATERIALS AND METHODS

The study was carried out on transformed B11dii-FAF28 (clone 237) Chinese hamster ovary cell (CHO)

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culture, a gracious gift from Dr. G. B. Raevskaya from Medical Genetic Center of Russian Academy of Medical Sciences. The cells were cultured in closed rectangular glass flasks with 30 $cm^2$ surface in DMSI (Biolot) with 10% bovine serum (Biolot) and 80 mg/liter gentamicin sulfate (complete medium) at 37°C. After formation of a monolayer (on days 4-5 of culturing) the cells were re-inoculated in a 1:6-1:10 ratio. The cells were removed from the surface with EDTA (0.02%) and trypsin (0.25%) mixture (1:1).

Cell cultures of different "age" were obtained by one of the two methods. In "longitudinal" studies the cells from several flasks were pooled and inoculated in 10-20 flasks at a density of about $35\times10^3$ cell/$cm^2$. Further culturing was carried out at 37°C without replacing the medium. After certain periods the cells from 3-5 flasks were removed and their viability and PAR-polymerase activity were determined.

For cross-sectional studies cultured cells were re-inoculated every 3-4 days into flasks at a density of about $35\times10^3$ cell/$cm^2$ and cultured as described above. Thirteen-fourteen days after inoculation of the first cultures the cells were removed from all flasks and cell viability and PAR-polymerase activity were determined.

In order to evaluate PAR-polymerase activity, the cells from 3-5 flasks with cultures of the same "age" were harvested, suspended in 30-50 ml (10 ml/flask) complete medium, and centrifuged for 10 min at 200*g* on a K23D centrifuge (VEB MLW). Precipitated cells were resuspended in cold (4°C) buffer for permeabilization (10 mM Tris-HCl buffer (pH 7.8) with 1 mM EDTA, 4 mM $MgCl_2$, and 30 mM β-mercaptoethanol), diluted to a concentration of $2\times10^6$ cell/ml, and left on an ice bath for 15 min. The cells were precipitated by centrifugation (15 min, 200*g*), resuspended in buffer for permeabilization, and the concentration was brought to $20\times10^6$ cell/ml. The resultant suspension was put into 4 plastic 1.5-ml tubes (50 μl PER TUBE) and after 3-min preincubation at 30°C the reaction mixture (50 μl) was added, of the following composition: 33 μl reagent for evaluation of PAR-polymerase activity (100 mM Tris-HCl buffer (pH 7.8) with 120 mM $MgCl_2$ and 1 mM $NAD^+$), 1 μl [adenine-$^3$H]NAD solution (1 μCi/μl), 7 μl palindromic CGGAATTCCG oligonucleotide (0.717 mg/ml) in 15 mM NaCl, and 9 μl $H_2O$. The tubes were incubated for 10 min at 30°C. The reaction was stopped by adding 1 ml 10% TCA, cooled on ice bath and containing sodium pyrophosphate (2%) and left overnight at 4°C. In control samples TCA with sodium pyrophosphate was added directly before adding the reaction mixture. The fraction insoluble in TCA and containing PAR with radioactive label was separated by filtration on a Brandel M-12S harvester (Brandel). The contents of the tubes was transferred onto GF/C filters (Whatman) and washed with ice-cold TCA with sodium pyrophosphate and then with ethanol. The filters were dried in a thermostat at 180°C for 1 h and placed into scintillation flasks with 5 ml toluene scintillator (2.5 g 2,5-diphenyl oxasone and 0.2 g 1,4-diphenyl oxasolylbenzene in 1 liter of toluene). Radioactivity of the filters was measured on an RZhS-20 scintillation counter (Inst. Mol. Genet., Russ. Acad. Sci.).

Viability of cultured cell was evaluated by trypan blue staining: 100 μl cell suspension and 100 μl 0.4% trypan blue were put in 1.5-ml plastic tubes. The percentage of damaged (intensively stained) cells was evaluated after 5-10 min under a microscope in a Goryaev chamber.

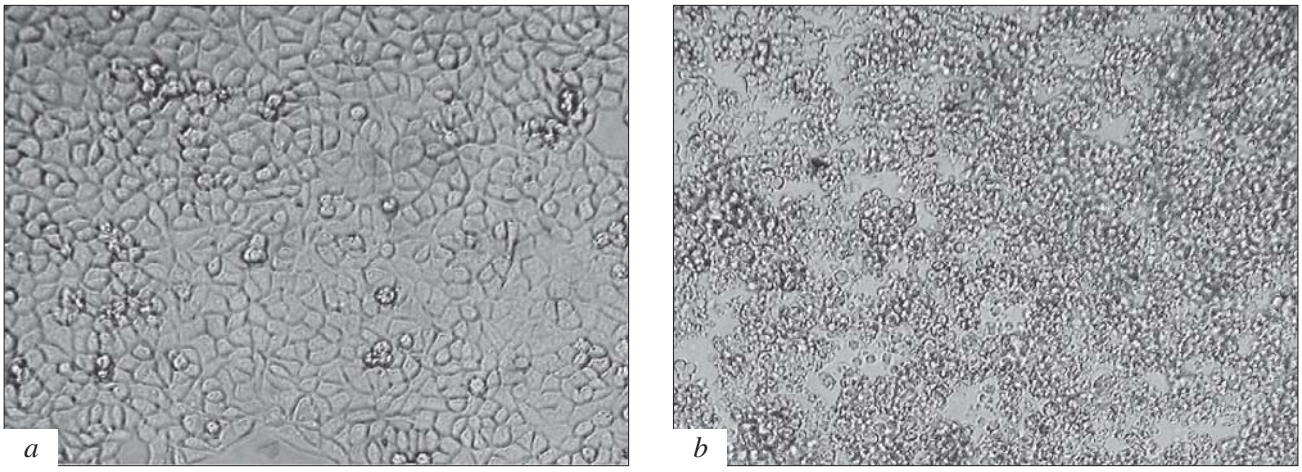


**Fig. 1.** Changes in Chinese hamster cell morphology with "aging" of the culture; ×160. *a*) "adult" cells (7 days after re-inoculation, beginning of stationary stage); *b*) "old" cells (24 days after re-inoculation, late stationary stage).

The results were statistically processed using SigmaPlot 8.0 software. The data were presented as means±standard deviations. The significance of differences between two samplings was evaluated using Student's *t* test.

## RESULTS

At the early stages of "stationary aging" CHO formed a monolayer of clearly contoured and greatly flattened cells (Fig. 1, *a*). At later stages the monolayer disappeared, many cells detached from the surface, while the rest cells shrink and acquire a round shape (Fig. 1, *b*).

The number of adherent cells in CHO culture decreased after attaining the stationary phase (Fig. 2, *a*). The percentage of damaged cells, detected by trypan blue staining among cells left on the growth surface, increased (Fig. 2, *b*). The greatest decrease in culture viability was observed after 7 days of culturing.

Longitudinal studies showed that stationary aging of CHO culture was associated with a decrease in PAR-polymerase activity (Fig. 2, *c*), manifesting after 5 days of culturing (after reaching the stationary growth phase). By day 10 of culturing PAR-polymerase activity decreased almost 10-fold, and by day 13 it was virtually undetectable (results are not presented).

Cross-sectional studies gave similar results (Fig. 3). Nine-day CHO culture was characterized by lower density in comparison with 5-day culture (Fig. 3, *a*), higher percentage of damaged cells (Fig. 3, *b*), and lower PAR-polymerase activity (Fig. 3, *c*).

The pattern of changes in the parameters did not alter with the culture "age" in both longitudinal and cross-sectional studies, this indicating that the methodology of experiments was inessential for the results. Destructive disorders in CHO culture started developing after it reached the stationary status. This manifested by changes in cell morphology, reduction of cell adhesion, and increased percentage of damaged cells.

The method for evaluation of cell viability used in our study (trypan blue staining) is based on detection of cells with damaged membrane. During culturing these injuries can emerge not only in primary necrosis, but also at the post-apoptotic stages, as a result of the so-called secondary necrosis.

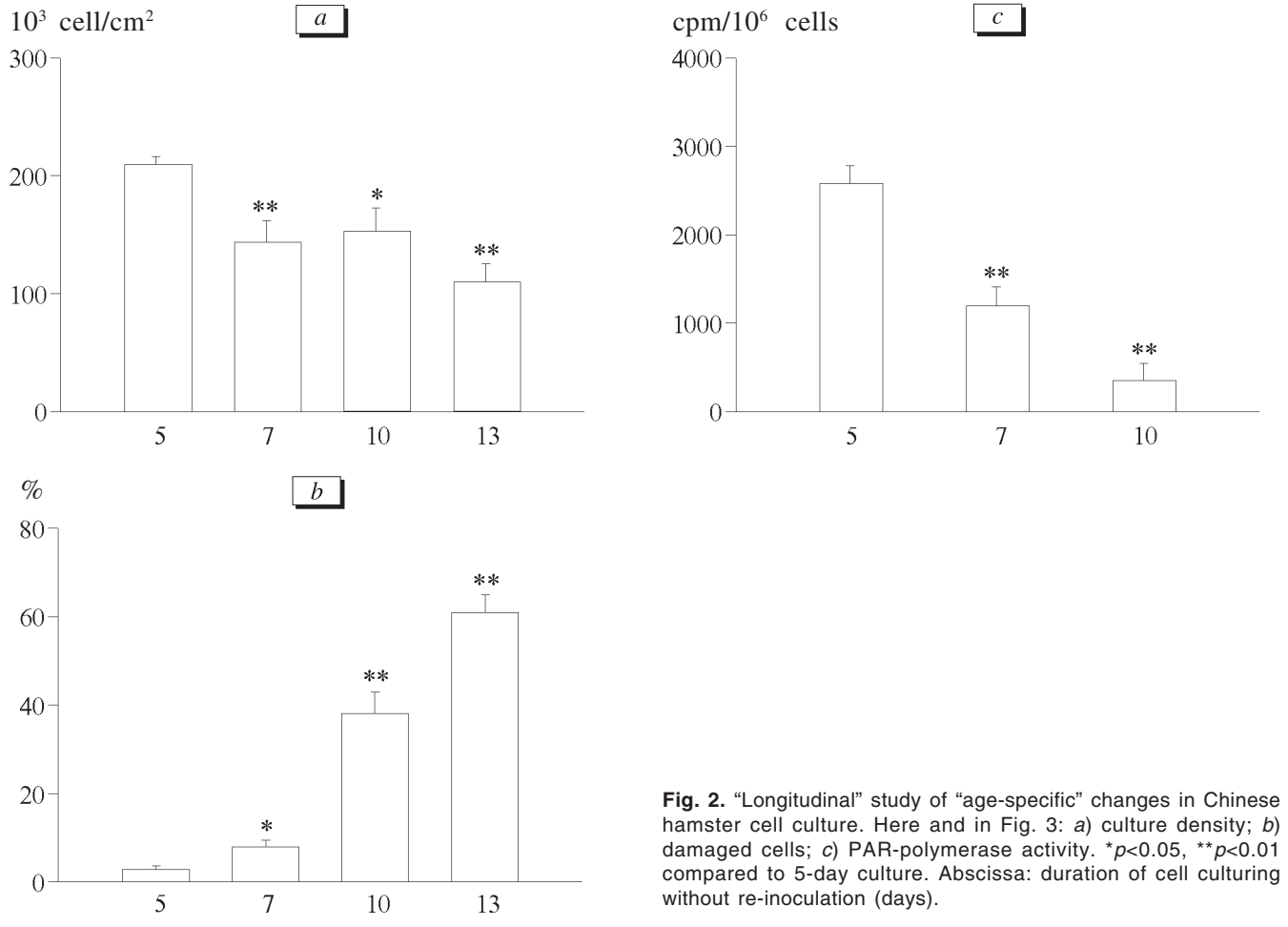


**Fig. 2.** "Longitudinal" study of "age-specific" changes in Chinese hamster cell culture. Here and in Fig. 3: *a*) culture density; *b*) damaged cells; *c*) PAR-polymerase activity. **p*<0.05, ***p*<0.01 compared to 5-day culture. Abscissa: duration of cell culturing without re-inoculation (days).

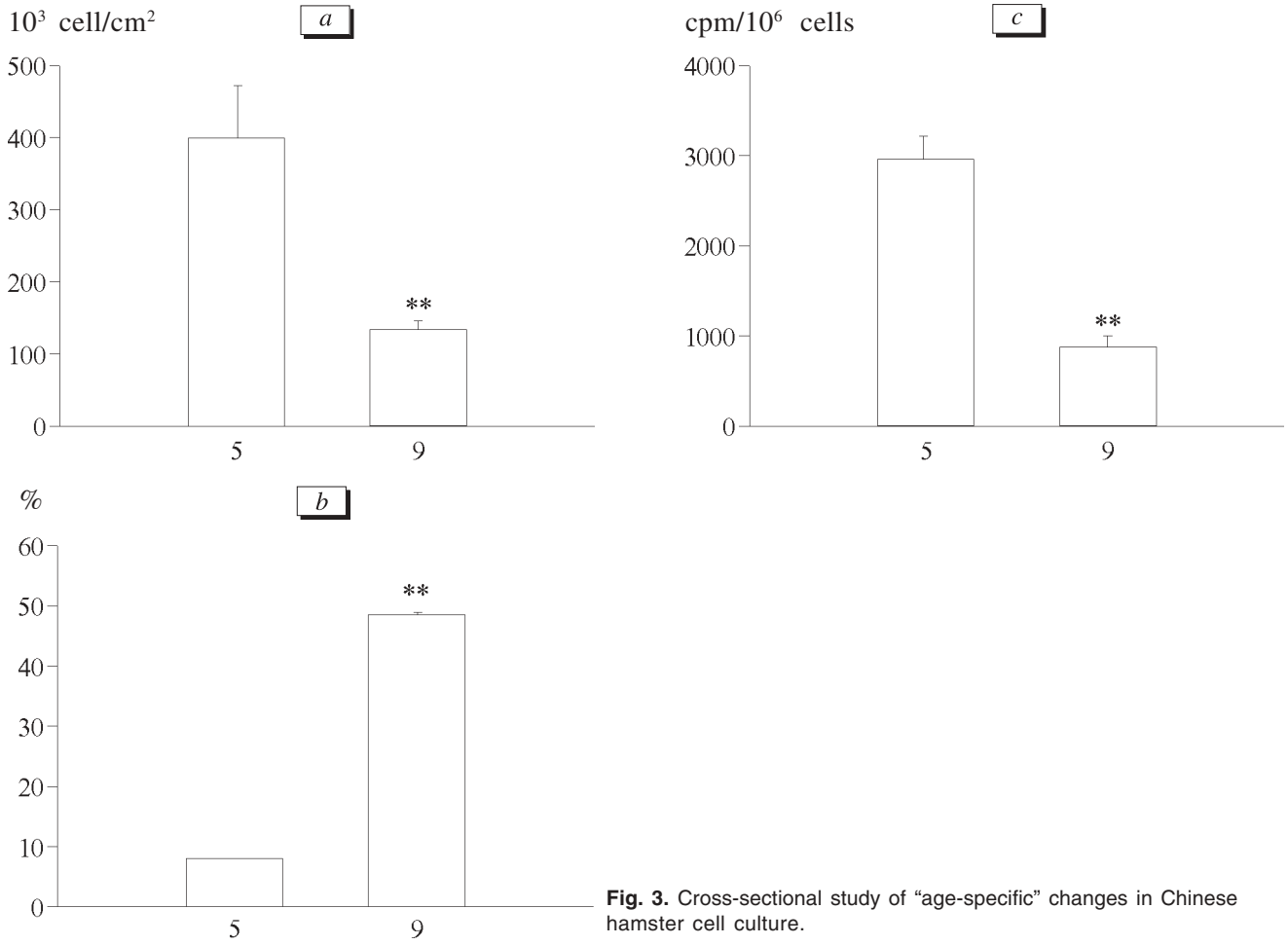


**Fig. 3.** Cross-sectional study of "age-specific" changes in Chinese hamster cell culture.

It is known that both necrosis and apoptosis lead to activation of proteolytic enzymes causing destruction of PARP-1 molecule [7]. This can account for reduction of PAR-polymerase activity in the course of stationary aging observed in our experiments.

Other causes of the observed reduction of PAR-polymerase activity are possible. Limitation of the proliferation rate of cultured human diploid WI-38 fibroblasts leads to reduction of PARP-1 gene expression [14]. Automodification of PARP-1 is another possible cause. It is known that intensification of PARP-1 poly-ADP-ribosylation leads to reduction of its catalytic activity [15]. Activation of PARP-1, induced by DNA damage, can serve as a signal triggering cell death mechanisms [11].

Hence, it seems that PAR-polymerase activity and cell culture viability are closely related. Reduction of cell culture viability with "age" is paralleled by activation of cell death processes, which can cause reduction of PAR-polymerase activity.

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