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STRENGTHENING OF THE DNA-PROTEIN COMPLEX DURING STATIONARY PHASE AGING OF CELL CULTURES

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UDC 612.67.014.2:577.213.7]-085.23

KEY WORDS: stationary phase aging; cultured cells; DNA–protein cross-linkage.

The writers previously postulated that limitation of proliferation of cells of the body is the main cause of accumulation of injuries to genetic material with age. It was accordingly suggested that stationary cell cultures (i.e., cultures consisting of undividing cells) be used to model processes of aging taking place at the molecular level [6-9]. Experiments showed that changes similar to those taking place in cells aging *in vivo* over a period of many years also take place in the cells of such cultures, but within a short time (not more than 2-3 weeks). In particular, accumulation of alkali-labile regions in DNA [9] and an increase in the number of spontaneous sister chromatid exchanges [6, 7] were found.

In the investigation described below the possibility of accumulation of cross-linkages in the DNA-protein complex was studied during stationary phase aging of cells in culture. Investigation of injuries of this type to genetic material was indicated for the following reasons. The theory according to which the primary cause of aging is progressive accumulation of cross-linkages between proteins, nucleic acids, and other macromolecules, was formulated by Bjorksten as long ago as in 1941-42 [11, 12] and was developed in his subsequent researches [13, 14] on the basis both of his own data and of results obtained by other investigators. Several papers have recently been published [1-3, 10, 15] in support of this concept. In particular, it is an interesting fact that cells of patients with progeria (a syndrome of premature aging) are unable to repair induced cross-linkages of the DNA–protein complex [10], although no other defect of the DNA repair system could be found in them [4].

EXPERIMENTAL METHOD

Experiments were carried out on cultures of Chinese hamster cells (line B 11 dii-FAF 28), normal human diploid embryonic fibroblasts (strain E2), and fibroblasts from a patient with xeroderma pigmentosum (strain IMG-667). The Chinese hamster cells were grown on Eagle's medium with glutamine, containing 10% bovine serum, the human fibroblasts on medium consisting of 80-85% of Eagle's medium with glutamine, 10-15% bovine serum, and 5% of human umbilical serum. Cells removed from the glass with trypsin solution were reseeded in several Carrell's flasks (1:6 in the case of Chinese hamster cells, 1:2 in the case of fibroblasts from the patient

Research Institute of Physicochemical Medicine, Ministry of Health of the RSFSR. Institute of Medical Genetics, Academy of Medical Sciences of the USSR, Moscow. Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 101, No. 4, pp. 416-418, April, 1986. Original article submitted May 11, 1985.

0007-4888/86/0004-0437$12.50

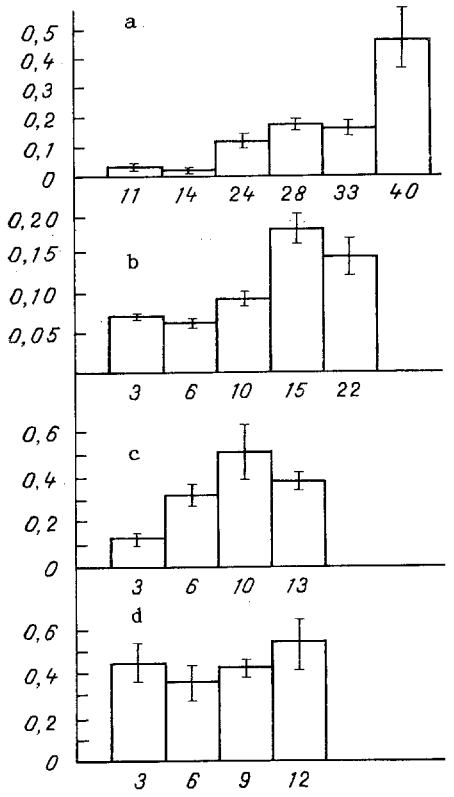

Fig. 1. Dependence of values of $^{14}C/^{3}H$ ratio on time elapsing after seeding of the cells. Abscissa, time of culture (in days); ordinate, ratio $^{14}C/^{3}H$. a, b) Chinese hamster cells, c) normal human embryonic fibroblasts, d) cells from patient with xeroderma pigmentosum. Vertical lines mark 95% confidence intervals.

with xeroderma pigmentosum, and 1:3 in the case of normal human embryonic fibroblasts). Under these circumstances the Chinese hamster cells were put into flasks in their usual growth medium, whereas both types of fibroblasts were put in medium consisting of 85% of Eagle's medium with glutamine, 10% of embryonic calf serum, and 5% of human umbilical serum. ^{3}H-thymidine (0.3 μCi/ml, specific activity 22 Ci/mmole), ^{14}C-valine (10 μCi/ml, 26 Ci/mole), and ^{14}C-leucine (2 μCi/ml, 32-58 Ci/mmole) were added to the medium. Having taken the method of determining the number of cross-linkages in the DNA–protein complex as the basis [5], we modified it considerably, and it will therefore be described in full.

A certain time after seeding cells in one flask were removed from the glass with a cold solution of versene, and the cells were sedimented by centrifugation (250g, 5 min, room temperature). The residue was suspended in 3-9 ml (depending on the number of cells growing in the flask) of cold 0.01 M phosphate buffer, pH 7.0, containing GM-urea and 2.5 M KCl. The resulting suspension, in a volume of 3 ml, was transferred into a conical glass test tube in an ice bath and irradiated in an MSE ultrasonic disintegrator, working under "amplitude-4, power stand-high" conditions (twice, 30 sec each time, with an interval of 30 sec). Samples of 0.3 ml of the homogenate were applied to 5 columns (from Pasteur pipettes) containing hydroxyapatite (volume 0.1 ml) from Biorad (Bio-Gel^{R} HTR). The following solutions were then passed through each column: 0.4 ml of 0.01 M phosphate buffer twice, 0.4 ml of 0.2 M phosphate buffer 5 times, and 0.4 ml of 0.4 M phosphate buffer once. All the solutions contained 6 M urea and 2.5 M KCl and their temperature was about 4°C. It was shown that the main mass of DNA (together with covalently bound proteins) was eluted in the first 0.4 ml of 0.4 M phosphate buffer. This eluate was applied seven drops at a time to Whatman 3MM filters. The dried filters were placed for 2 h in cold 10% TCA, and then for 30 min in absolute ethanol. Radioactivity of the redried filters was determined on a Mark III scintillation counter (model 6880, from Searle Analytic Inc.), working on program 5 (double labeling with ^{3}H and ^{14}C). The quantity of protein firmly bound with DNA was judged from the value of the coefficient $^{14}C/^{3}H$ (mean for 5 columns), determined with allowance for penetration of counting from the ^{14}C-channel into the ^{3}H-channel.

EXPERIMENTAL RESULTS

In the first experiment, conducted on Chinese hamster cells, the coefficient $^{14}C/^{3}H$ was determined for 11-, 14-, 24-, 28-, 33-, and 40-day cultures, and in the second experiment, for 3-, 6-, 10-, 15-, and 22-day cultures. In both cases (Fig. 1a, b) an increase in the parameter measured was observed with an increase in age of the cells, i.e., the time after seeding. Similar results also were obtained on normal human embryonic fibroblasts (Fig. 1c). In the case of cells from the patient with xeroderma pigmentosum (Fig. 1d), however, the value of the coefficient $^{14}C/^{3}H$, within the limits of experimental error, did not change from the age of 3 to 12 days. It was concluded from these results that the number of spontaneous cross-linkages of the DNA–protein complex increases during "stationary phase aging" of both transformed (Chinese hamster cells) and normal (human diploid embryonic fibroblasts) cells, i.e., cells possessing the restricted mitotic potential *in vitro*. The absence of any such increase in the case of cells from the patient with xeroderma pigmentosum cannot yet be fully explained, especially in connection with data showing normal repair of DNA–protein cross linkages in these cells [10]. The following point may be made: the coefficient $^{14}C/^{3}H$ cannot, of course, serve as an accurate indicator of the absolute number of cross-linkages in the DNA–protein complex in the cells studied, for it is largely dependent on the concrete experimental conditions. Only an increase or decrease in its value can enable the conclusion to be drawn that covalent bonds between DNA and protein molecules have appeared or disappeared. Nevertheless, if equal concentrations of labeled precursors are maintained, with equal volumes of growth medium and equal numbers of cells seeded in the flask, a comparative estimate can be made of the number of spontaneous cross-linkages in the DNA–protein complex in cells of the different cultures. Since experiments on normal embryonic fibroblasts and those on fibroblasts from the patient with xeroderma pigmentosum were conducted virtually identically, estimation of values of the $^{14}C/^{3}H$ ratio (the ordinate in Fig. 1c, d) suggested that in the second case this parameter was higher from the beginning and remained at that level throughout the period of culture. The possibility likewise cannot be ruled out that with lengthening of the period of stationary phase aging, an increase in the number of DNA–protein cross-linkages would also take place in cells from the patient with xeroderma pigmentosum. Undoubtedly this phenomenon requires further study.

The results described in this paper provide further evidence, in our opinion, of the value of stationary phase cell cultures for the study of the mechanisms of aging and also of some of the general principles underlying hereditary pathology.

The authors are grateful to V. V. Viktorov and A. N. Chebotarev for help with mathematical analysis of the results on the Hewlett-Packard 980B computer.

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EFFECT OF TRIHYDROXYOCTADECADIENOIC ACIDS ON BLOOD LEVELS OF PROSTAGLANDINS E_2 AND $F_{2\alpha}$ AND OF 5-HYDROXYEICOSATETRAENOIC ACID IN RATS WITH ALLOXAN DIABETES

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UDC 616.379-008.64-092.9-085.322:582.982.]-07:[616.154.577.175.859

KEY WORDS: diabetes; prostaglandin E_2; prostaglandin $F_{2\alpha}$; 5-hydroxyeicosatetraenoic acid; trihydroxyoctadecadienoic acids.

It was shown previously that the fraction of trihydroxyoctadecadienoic acids (THODA), isolated from the roots of *Bryonia alba* L. (Cucurbitaceae), causes the blood glucose level to fall in rats with alloxan diabetes [2]. C_{20}-homologs of the THODA of *Bryonia* are formed in platelets from 12-hydroperoxyeicosatetraenoic acid (12-HPETE), a product of lipoxygenase oxidation of arachidonic acid (AA). This process is observed in the presence of high concentrations of AA and subsides in the presence of glucose, for activation of the hexose monophosphate shunt leads to the formation of reduced glutathione, and then to reduction of 12-HPETE into 12-hydroxyeicosatetraenoic acid [5, 6].

The eicosanoids, as we know, are modulators of secretion of the hormones involved in regulation of the blood glucose level [4, 9]. In particular, leukotrienes B_4, C_4, and E_4 [11], 5-hydroxyeicosatetraenoic acid (5-HETE) [15], and 12-HPETE [9] are stimulators of insulin secretion. We also know that when the blood glucose concentration falls, there is a sharp increase in synthesis of both thromboxane A_2 and prostacycline [12], whereas when the glucose concentration in the medium rises, biosynthesis of prostaglandins E_2 and $F_{2\alpha}$ (PGE_2 and $PGF_{2\alpha}$, respectively) is inhibited [14]. Meanwhile, in diabetes biosynthesis of prostacycline [12] is stimulated and synthesis of leukotriene B_4 is depressed [8]. The formation of these various eicosanoids, in turn, has a decisive role in the vascular disturbances accompanying diabetes [7, 13].

In connection with the facts described above, the effect of the THODA fraction on blood levels of some eicosanoids and, in particular, of PGE_2, $PGF_{2\alpha}$, and 5-HETE in rats with alloxan diabetes was studied.

EXPERIMENTAL METHOD

The THODA fraction was isolated from *Bryonia* roots by the method described previously [3]. Experiments were carried out on noninbred albino rats weighing 170-220 g. Diabetes was induced by a single intraperitoneal injection of alloxan (150 mg/kg). Starting from the 7th day after injection of alloxan, when the group of animals had a blood glucose concentration of not less than 10 mM, an aqueous solution of the sodium salt of THODA was injected intramuscularly in a dose of 0.05 mg/kg daily. Animals of the control group received injections of the corresponding volumes of isotonic NaCl solution. The hungry animals were decapitated on the 21st day after injection of alloxan, under superficial ether anesthesia.

Concentrations of PGE_2, $PGF_{2\alpha}$, and 5-HETE in peripheral blood plasma were determined by radioimmunoassay, using kits from the firms Seragen and Clinical Assays (USA). To assess the loss of substances during extraction and chromatography, $^3H_8$-PGE_2 (140-170 Ci/mmole), $^3H_8$-$PGF_{2\alpha}$ (160-180 Ci/mmole), from Amersham Corporation (England), and $^3H_8$-5-HETE (from Seragen) were used. The yield of PGE_2, $PGF_{2\alpha}$, and 5-HETE was 80, 72, and 85%, respectively.

Research Sector of Radiobiology, Ministry of Health of the Armenian SSR. Institute of Biochemistry, Academy of Sciences of the Armenian SSR, Erevan. (Presented by Academician of the Academy of Medical Sciences of the USSR A. V. Val'dman.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 101, No. 4, pp. 418-419, April, 1986. Original article submitted May 20, 1985.

 0007-4888/86/0004-0440$12.50