PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 170, 373–377 (1982)

Aging and Hydrocortisone Effects on Transient Structures of Replicative DNA of Human Fibroblasts (41445)

C. ICARD-LIEPKALNS AND A. MACIEIRA-COELHO[1]

Département de Pathologie Cellulaire, Institut de Cancérologie et d'Immunogénétique, (INSERM U 50 et Association Claude-Bernard), 94800 Villejuif, France

Abstract. Alkali-labile sites accumulate in DNA during aging *in vitro* of human embryonic lung fibroblasts. The sedimentation velocity of newly synthesized DNA was analyzed in alkaline sucrose gradients in young and old cells. Although most of the old cells had gone through a whole S period, their DNA sedimented in a dispersed fashion. Hydrocortisone shortened the time of appearance of bulk DNA. The results are compatible with the slow-down of DNA chain elongation during aging and could explain to a certain extent the sustaining effect of hydrocortisone on the human fibroblast lifespan.

We have previously described the presence of alkali-labile sites in chromosomal DNA of human embryonic lung fibroblasts aged *in vitro* (1). These findings have been recently confirmed with a different methodology (2). The small molecular weight DNA found in alkaline sucrose gradients of old cells could be caused by apurinic sites, single-strand breaks, or replication intermediates either due to the presence of slow dividing cells (3) or to a decreased rate of chain elongation (4, 5). We have now followed the sedimentation velocity of newly synthesized DNA in order to distinguish between these possibilities.

Materials and Methods. *Cell culture.* Two human normal embryonic lung fibroblast cell lines were used: the MRC-5 obtained from Dr. Jacobs (Medical Research Council, U.K.), and the HEF-964, received from Dr. J. Pontén (University of Uppsala, Sweden). The cells were split at confluency at a 1:2 ratio in 75-cm^2 flasks and maintained in 30 ml of Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and gentamycin (16 μg/ml). Previous results had shown that these two cell lines accumulate DNA alkali-labile sites at the same rate during aging (1). Some cultures were cultivated for 10 passages in the presence of 12 μM hydrocortisone in the culture medium for which maximum growth response was obtained. Cells up to the 40th population doubling were considered as young and beyond that as old.

Single-stranded DNA sedimentation. Cells were grown in 60-mm-diameter petri dishes until confluency when no mitoses could be seen and stimulated to enter the S phase by medium change containing 20% FCS. Eight hours later, 1 μCi [^{14}C]thymidine/ml ([^{14}C]TdR) (sp act 50 μCi/mmole) was added to the nutrient medium and the cells were used at different times thereafter depending on the experiment. After a 30-min chase with unlabeled TdR (10 μg/ml), cells were lysed on top of a linear 5–20% alkaline sucrose gradient as described elsewhere (1). Centrifugation was carried out for 2 hr at 40,000 rpm in an SW_{41} Beckman rotor at 12°. Denatured λ phage DNA (40.5 S) was used as a standard.

Autoradiography. Cells identical to those used for gradient centrifugation but growing on coverslips were labeled with [^{3}H]TdR (0.01 μCi/ml, sp act 2 Ci/mmole). At the times the cells were used for gradient centrifugation, coverslips were washed with MEM without serum and fixed in acetic acid methanol (1:3). When dry, they were coated with Kodak NTB 3 liquid emulsion and the film was developed 7 days later. Grains above 100 interphases were counted on each of duplicate samples and plotted on histograms.

To calculate the length of the S period,

[1] To whom reprint requests should be addressed.

0037-9727/82/070373-05$01.00/0

actively growing cells were labeled with the same concentration of [^{3}H]TdR. Each hour after labeling, duplicate coverslips were fixed and prepared for autoradiography. The number of grains above 100 metaphases on each coverslip was determined and the cumulative percentages of the grain count distributions were plotted on probit paper. The plot gave straight lines and the intersection of the lines with the 50% line on the paper gave the peak values of the metaphase grain counts at each time after addition of [^{3}H]TdR. The time between the appearance of the first labeled metaphases and the time when the peak metaphase grain count saturated corresponds to an S period (6).

Results and Discussion. Figure 1 illustrates the sedimentation velocity of DNA at different times after adding [^{3}H]TdR to stimulated cultures of young and old cells. In both cultures 45 min after labeling the radioactivity is distributed in small peaks through the gradient. Then DNA progres-

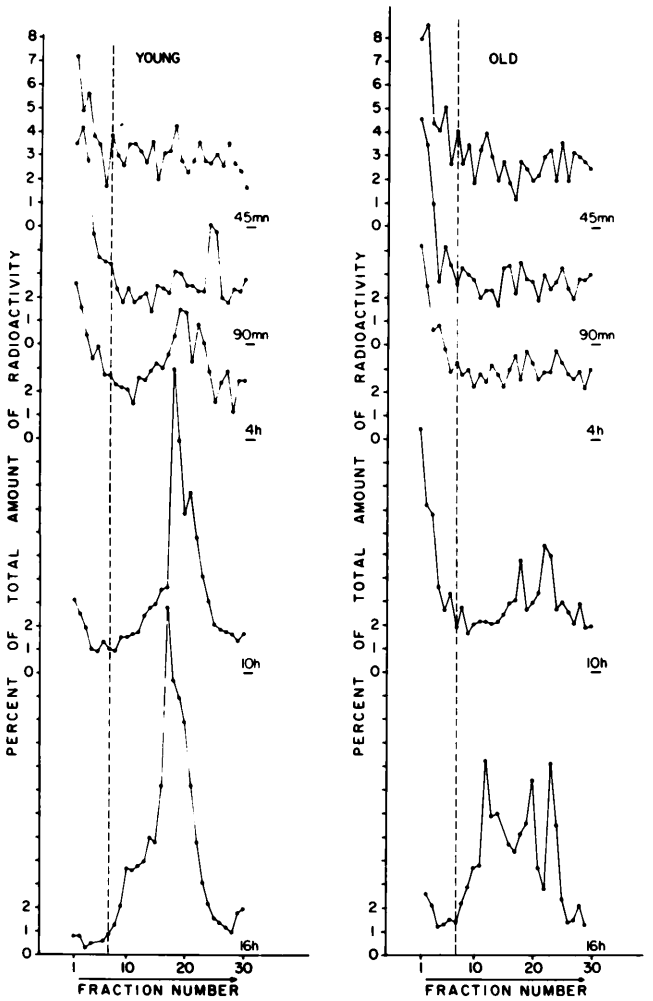

FIG. 1. The curves represent the radioactivity collected from the different fractions of the gradients expressed as percentage of the total amount of radioactivity of the sample. The dashed line indicates the fraction where denatured λ phage DNA (40.5 S) sedimented. The sedimentation direction from left to right is indicated by the arrow. Young cells were at the 27th population doubling level (PDL) and old cells at the 52nd PDL.

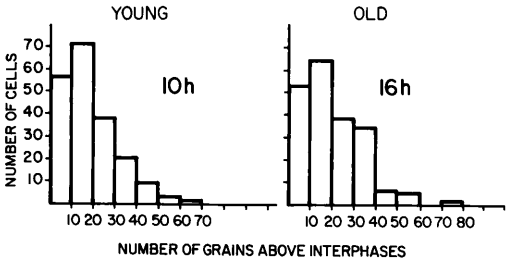

FIG. 2. Distribution of the number of grains above interphases in cultures identical to those used in the experiment illustrated in Fig. 1 and fixed 10 and 16 hr after labeling of young and old cultures, respectively.

sively sediments in young cells as a single peak which is completed 10 hr after labeling. In old cells, however, even at the 16th hr after addition of [^{3}H]TdR, DNA sediments in several peaks with different sedimentation velocities.

To see if the small-molecular-weight DNA in old cultures could be due to a larger fraction of cells that had not completed DNA synthesis due to a slow transit through the division cycle (3), identical cultures treated in the same way as those described in Fig. 1 were fixed for autoradiography 10 and 16 hr after labeling for young and old populations, respectively (Fig. 2). Grain counts above interphases showed that the distribution of radioactivity was the same in both cell populations. The length of the S period was also measured in young and old cultures as described under Methods. Results illustrated in Fig. 3 show that the peak metaphase grain count saturated 7 and 10 hr after labeling in young and old cells, respectively. Hence the results show that in old cells DNA sediments in alkaline sucrose gradients in a dispersed fashion even after most of the cells have gone through a whole S period. Identical results were obtained with both cell lines.

It was previously shown that hydrocortisone stimulates the growth of postnatal human fibroblasts (7) and that cortisone and hydrocortisone increase the long-term survival of fibroblasts obtained from human embryonic lung (8, 9). Thus we analyzed the sedimentation velocity of newly synthesized DNA in mid-passage control cells and identical cultures that had been carried for 10 doublings in hydrocortisone-supplemented medium (Fig. 4). In nontreated cultures from the 4th to the 10th hr after adding [^{3}H]TdR there was a progressive shift in the sedimentation of DNA from small to high molecular weight. In hydrocortisone-treated cells, however, high-molecular-weight DNA appeared earlier and the peak of radioactivity was maximal after 8 hr. Thus hydrocortisone, which stimulates the growth of cells and increases survival, accelerated the appearance of large-molecular-weight DNA. Identical results were obtained in three different experiments.

The dispersed sedimentation of the DNA from old cell populations when most of the cells have gone through a complete S period could be explained by the slowdown of DNA chain elongation which had been previously described during aging of fibroblasts (4, 5); the deficient synthesis of chromosomal proteins that occurs during human fibroblast senescence (10–15) could result in a defect of the gap-filling step during the rejoining of adjacent replicons (16). On the other hand steroid hormones stimulate acetylation and phosphorylation of histones and nonhistone chromosomal proteins (17), which might be important for the stabilization of the links between adjacent replicons; the sustained action of the hormone

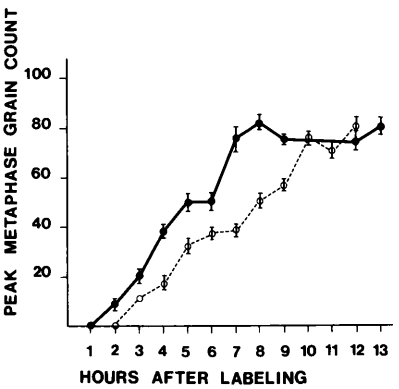

FIG. 3. Peak metaphase grain count determined in young (●) and old (○) cultures as described under Methods.

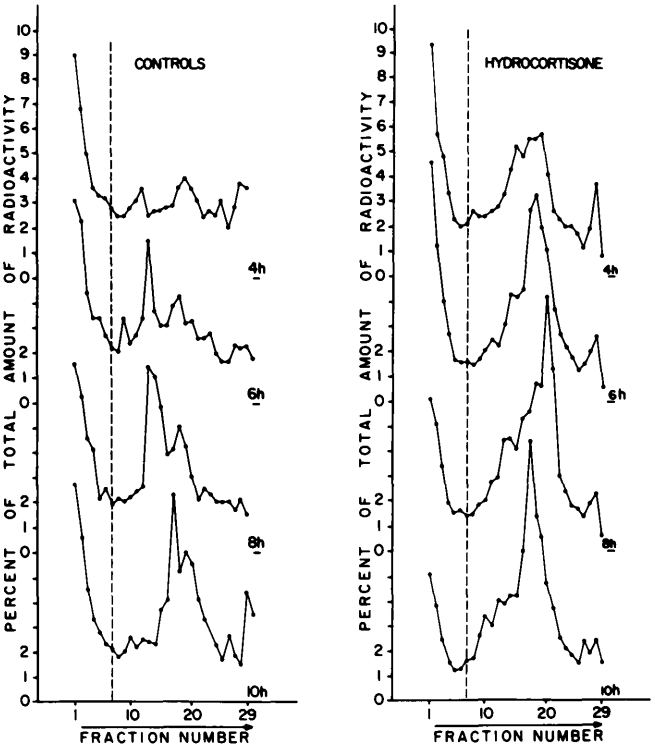

FIG. 4. Radioactivity found in alkaline sucrose gradients with chromosomal DNA from cells at the 37th PDL carried without and with hydrocortisone during the 10 preceding population doublings. The medium change in confluent cultures was made without and with the hormone in the fresh medium, respectively. The cultures were lysed at the indicated times after adding [^{3}H]TdR. The dashed line indicates the fraction where denatured λ phage DNA (40.5 S) sedimented. The sedimentation direction from left to right is indicated by the arrow.

on the division of human fibroblasts could be mediated through a faster maturation of these links in addition to the previously reported stimulatory effect on ribosomal RNA synthesis (18).

This work was supported by contract CRL 80 2 030 from the INSERM.

1. Icard C, Beaupain R, Diatloff C, Macieira-Coelho A. Effect of low dose rate irradiation on the division potential of cells in vitro. VI. Changes in DNA and radiosensitivity during aging of human fibroblasts. Mech Age Dev 11:269–278, 1979.
2. Suzuki F, Watanabe E, Horikawa M. Repair of X-ray-induced DNA damage in aging human diploid cells. Exp Cell Res 127:299–308, 1980.
3. Macieira-Coelho A. Kinetics of the proliferation of human fibroblasts during their lifespan in vitro. Mech Age Dev 6:341–343, 1977.
4. Petes TD, Farber RA, Tarrant GM, Holliday R. Altered rate of DNA replication in aging human fibroblast cultures. Nature 251:434–436, 1974.
5. Fujiwara Y, Higashikawa T, Tatsumi M. A retarded rate of DNA replication and normal level of DNA repair in Werner's syndrome fibroblasts in culture. J Cell Physiol 92:365–374, 1977.
6. Stanners CP, Till JE. DNA synthesis in individual L-strain mouse cells. Biochim Biophys Acta 37:406–419, 1960.
7. Castor W. The effects of chronic glucocorticoid excess on human connective tissue cells in vitro. J Lab Clin Med 65:490–499, 1965.
8. Macieira-Coelho A. Action of cortisone on human fibroblasts in vitro. Experientia 22:390–391, 1966.
9. Cristofalo VJ. Metabolic aspects of aging in diploid human cells. In: Holeckova E, Cristofalo VJ, eds. Aging in Cell and Tissue Culture. New York, Plenum, pp83–119, 1970.
10. Ryan JM, Cristofalo VJ. Histone acetylation dur-

ing aging of human cells in culture. Biochem Biophys Res Commun **48**:735–742, 1972.
11. Srivastava BIS. Changes in enzymic activity during cultivation of human cells in vitro. Exp Cell Res **80**:305–312, 1973.
12. Stein GH. DNA-binding proteins in young and senescent normal human fibroblasts. Exp Cell Res **90**:237–248, 1975.
13. Maizel A, Nicolini C, Baserga R. Structural alterations of chromatin in phase-III WI-38 human diploid fibroblasts. Exp Cell Res **96**:351–359, 1975.
14. Pochron SF, O'Meara AR, Kurtz MJ. Control of transcription in aging WI-38 cells stimulated by serum to divide. Exp Cell Res **116**:63–74, 1978.
15. Mitsui Y, Sakagami H, Murota SI, Yamada MA. Age-related decline in histone H1 fraction in human diploid fibroblast cultures. Exp Cell Res **126**:289–298, 1980.
16. Van't Hof J. PEA (Pisum sativum) cells arrested in G2 have nascent DNA with breaks between replicons and replication clusters. Exp Cell Res **129**:231–237, 1980.
17. Kanungo MS. Biochemistry of Aging. New York, Academic Press, p38, 1980.
18. Macieira-Coelho A, Loria E. Stimulation of ribosome synthesis during retarded aging of human fibroblasts by hydrocortisone. Nature (London) **251**:67–69, 1974.

Received January 6, 1982. P.S.E.B.M. 1982, Vol. 170.