Vol. 118, No. 3, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1984 Pages 730-735

AGE-RELATED CHANGES IN UNSCHEDULED DNA SYNTHESIS BY RAT HEPATOCYTES

Marguerite M. Plesko and Arlan Richardson

Department of Chemistry, Illinois State University
Normal, Illinois 61761

Received December 30, 1983

SUMMARY: Ultraviolet-induced unscheduled DNA synthesis was studied as a function of age in hepatocytes isolated from 6- to 32-months-old rats. Unscheduled DNA synthesis was measured by both DNA specific activity and autoradiography. Using both procedures, a significant decline in unscheduled DNA synthesis was observed after 14 months of age.

In 1959, Szilard (1) proposed that the elementary step in aging was the accumulation of DNA damage in somatic cells, which eventually resulted in the inactivation of chromosomes and the death of the cell. Although this hypothesis was quite popular, evidence in support of it was limited until Hart and Setlow (2) showed that a strong positive correlation existed between the ability of fibroblasts to repair UV-damage and species lifespan. This observation has been confirmed in a variety of species including closely related subspecies of the mouse (3,4).

Although DNA repair in relation to age, rather than lifespan, has also been studied in several laboratories, the effect of aging on the DNA repair capacity of a cell is still uncertain. DNA repair capacity declines only slightly during *in vitro* aging in fibroblasts, and this decline occurs only in very late passage cells (5). Lambert et al. (6) found a significant age-related decline in DNA repair after UV-irradiation in peripheral lymphocytes from human subjects. However, the decline was slight; individuals 90 years old had levels of repair 30% lower than individuals 20 years of age. In this study, suspensions of hepatocytes isolated from rats of various ages were used to study the effect of aging on DNA repair. A marked age-

Abbreviations: UDS, unscheduled DNA synthesis; UV, ultraviolet

0006-291X/84 $1.50
Copyright © 1984 by Academic Press, Inc.
All rights of reproduction in any form reserved.

related change in the ability of hepatocytes to repair DNA damage induced by UV radiation was observed as measured by DNA specific activity and autoradiography.

MATERIALS AND METHODS

The animals used in this study were male Fischer F344 rats, which were obtained from the National Institute of Aging animal colony maintained by Charles River Breeding Laboratories. Animals were fasted for 12 hours prior to sacrifice and hepatocytes were isolated by *in situ* collagenase perfusion as described by Engelmann et. al. (7). Hepatocyte preparations consisted almost entirely of liver parenchymal cells (7), and their viability ranged from 87 to 91% as determined by trypan blue exclusion.

Nine ml of hepatocytes (2×10^6 cells/ml) in Hanks solution (8) were placed in siliconized 150 ml beakers, which resulted in a depth of 5 mm, and incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 in a shaking water bath. The suspensions were irradiated using a General Electric GE 15T8 germicidal lamp with primary output at 254 nm and a dose rate of 1.4 W/m^2. The irradiated hepatocytes were divided into duplicate samples for the measurement of UDS. Hydroxyurea was added to a final concentration of 1 mM, which was found to completely inhibit DNA replication. Autoradiographic analysis of over 3,000 cells showed that 1 mM hydroxyurea completely blocked replication by hepatocytes but had no effect on UV-induced UDS. [^{3}H]-thymidine (6.7 Ci/mmole) was added to each flask at a final concentration of 10 μCi/ml. The hepatocytes were incubated for 60 min with the [^{3}H]-thymidine. The incorporation of [^{3}H]-thymidine into DNA was linear during this incubation period; therefore, the initial rates of UDS were measured in this study.

Because hepatocytes incorporated over 50% of the radioactivity from [^{3}H]-thymidine into RNA and protein, total acid precipitable material could not be used to measure UDS. Rather than using CsCl-gradient centrifugation to purify DNA to measure UDS (9), which is time consuming , we devised a relatively simple and rapid procedure that allows an investigator to measure the amount of radioactivity incorporated into nuclear DNA. UDS was measured as DNA specific activity and determined as follows. Hepatoctyes were lysed by resuspending them in calcium-free Hanks solution containing 2% (w/v) Triton X-100. Nuclei were recovered by centrifugation at 600 x g. The nuclear pellet was treated with 1 N $HClO_4$ and the precipitate collected by centrifugation. The RNA in the precipitate was hydrolyzed by suspending the pellet in 0.33 N NaOH and incubating at 37°C for 60 min. The DNA and protein in the NaOH suspension was precipitated with 1 N $HClO_4$ and the DNA in the precipitate hydrolyzed by incubating the precipitate in 1 N $HClO_4$ for 10 min at 70°C. The radioactivity in the hydrolysate was determined using a scintillation counter and the DNA content was determined by measuring the absorbance of each sample at 260 nm.

Autoradiography was also carried out on portions of each sample, using standard procedures (10). Hepatocytes were stained with acridine orange and examined under the light microscope at 100 X magnification. One hundred cells on each slide were scored for grains over the nucleus. Grain counts were corrected for background grains by subtracting the average number of grains in several nuclear sized areas outside the cells on each slide.

RESULTS

Fig. 1 shows the level of UDS by hepatocytes isolated from rats of various ages over a range of UV doses (0 to 2,100 J/m^2). Yager and Miller (11)

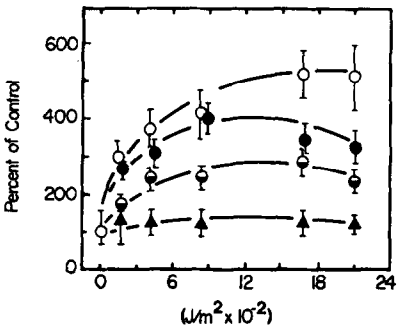

Figure 1. Effect of age on UV-induced UDS. Hepatocytes were isolated from 6(○), 14(○), 20(●), and 32(▲) month old rats and exposed to the indicated doses of UV. Each point is the mean ± SEM of data from 3 animals.

reported a similar UV dose-dependency of UDS in primary hepatocyte cultures. The viability of hepatocytes isolated from the different aged animals was not significantly different at any point during the experimental manipulations (data not shown). A significant increase ($p < 0.01$) in UDS was observed with increasing UV dose for each age except the oldest animals, in which no significant stimulation of UDS was observed at any dose of UV. The general trend observed in Fig. 1 is an increase in UDS capacity from 6 to 14 months of age, followed by a dramatic decline (80%) between 14 to 32 months of age. Statistically significant ($p < 0.001$) age-related differences in UDS were observed at all UV doses studied. The age-related changes appeared greatest at higher doses of UV, e.g., 840 J/m^2 and greater. Analysis of variance revealed highly significant ($p < 0.001$) age effects on UDS and a highly significant ($p < 0.001$) interaction was observed between the effects of age and UV dose on the UDS.

UDS was also monitored by autoradiography. Fig. 2 shows the frequency distribution of grains per nucleus in hepatocytes isolated from 6- to 32-month-old rats before and after a UV dose of 1,680 J/m^2 (this dose of UV is well into the plateau region of the dose-response curve for UDS). The grains per nucleus for unirradiated hepatocytes ranged from 0 to 20 (12.5 to 39.5% of the cells had no grains per nucleus). No significant age-related difference in the grains per nucleus for unirradiated hepatocytes were observed when the data were analyzed using contingency tables. It is evident from Fig. 2 that UV

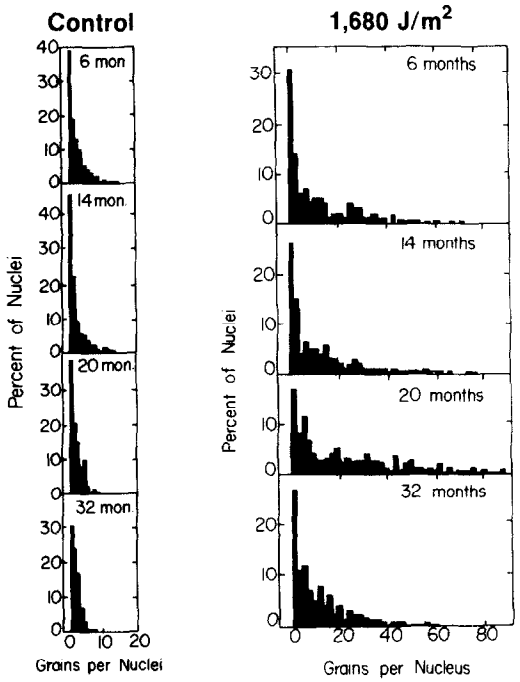

Figure 2. Effect of age on UDS as assayed by autoradiography. Hepatocytes were isolated from rats of various ages and exposed to 1,680 J/m^2 of UV radiation. Data are plotted as percent of the total number of cells that showed a particular grain count over the nucleus. Each graph represents data from 3 animals.

irradiation resulted in an increase in the grains per nucleus. The percent of cells with no grains per nucleus (12.5 to 27.0%) did not change significantly with age. Because the number of grains per nucleus in unirradiated hepatocytes ranged from 0 to 20, we defined UDS as a grains per nucleus greater than 20. Defined in this manner, UDS as measured by autoradiography showed the same general non-linear change with age as when UDS was measured by DNA specific activity (Fig. 1). Analysis of the data using contingency tables revealed a statistically significant age effect on UDS ($p < 0.01$). Close examination of the data in Fig. 2 also shows that the major age-related differences in the frequency distributions appear to be in the number of cells exhibiting the highest grain counts and not in the number of cells able to undergo UDS.

DISCUSSION

The effect of *in vivo* aging on DNA repair has been studied in a variety of systems with no clear relationship between age and repair capacity (for review,

see 12). Freshly isolated liver parenchymal cells represent a useful system to study the effect of aging on cellular metabolism. As a post-mitotic cell, the isolated hepatocyte is of the same age as the donor organism (13), and the age-related changes in several biochemical processes that occur in vivo have been observed in suspensions of hepatocytes (13,14).

In this study, UV radiation was used to damage DNA because UV-induced DNA damage has been well studied and because it is a direct-acting agent. Use of agents requiring metabolic activation was avoided because the activation of xenobiotics by liver has been reported to change with age (15). A significant age-related change in UV induced UDS, as measured by [^{3}H]-thymidine incorporation by hepatocytes was observed when evaluated by either DNA specific activity (Fig. 1) or autoradiography (Fig. 2). It is unlikely that the observed variations in [^{3}H]-thymidine incorporation were due to changes in dTTP pool sizes for the following reasons: (a) the concentration and specific activity of [^{3}H]-thymidine in the incubation media were at levels reported by Russell and Partick (16) to eliminate age effects on these parameters and (6) no significant age-related change in [^{3}H]-thymidine incorporation was observed in unirradiated hepatocytes (Fig. 2). The observed changes in UDS were also not due to age-related variations in DNA replication because no S-phase cells were observed by autoradiography using the conditions used to measure UDS. Therefore, it appears that the age-related changes in [^{3}H]-thymidine incorporation truly reflect changes in the initial rate of UDS.

Age-related variations in repair capacity could be due to changes in the levels or efficiencies of the enzymes involved in DNA repair or in the accessibility of the DNA to the repair enzymes. Several investigators have reported changes in liver chromatin with increasing age (17,18). Interestingly, the age-related change in UDS, which we observed, paralleled the age-related change in the transcriptional activity of the liver (Fig. 3). Therefore, it is possible that the mechanism responsible for the age-related decline in transcription is at least partially responsible for the decline in DNA repair.

Figure 3. Effect of age on DNA repair and transcription in liver. The open circles represent UDS induced by 1,680 J/m^2 of UV radiation (data from Fig. 1). The closed circles represent the total transcriptional activity of liver nuclei isolated from male Fisher F344 rats (data from Castle et. al., reference 19).

ACKNOWLEDGMENTS

We are grateful to Dr. Alan Katz and J. Daniel House for assistance with the statistical analyses. The research was supported by a grant (AG 0154B) from the National Institutes of Health.

REFERENCES

1. Szilard, L. (1959) Proc. Natl. Acad. Sci. USA 45, 30-45.
2. Hart, R.W., and Setlow, R.B. (1974) Proc. Natl. Acad. Sci. USA 71, 2169-2173.
3. Hart, R.W., Sacher, G.A., and Hoskins, T.L. (1979) J. Gerontol 34, 808-817.
4. Paffenholz, V. (1978) Mech. Age. Dev 7, 131-150.
5. Hart, R.W., and Setlow, R.B. (1976) Mech. Age. Dev 5, 67-77.
6. Lambert, B., Ringborg, U., and Skoog, L. (1979) Cancer Res 39, 2792-2795.
7. Engelman, G.L., Richardson, A., Katz, A., and Fierer, J.A. (1981) Mech. Age Dev 16, 385-395.
8. Hanks, J.H., and Wallace, R.E. (1949) Proc. Soc. Exp. Biol. Med 71, 196-200.
9. Althaus, F.R., Lawrence, S.D., Sattler, G.L., Longfellow, D.G., and Pitot, H.C. (1982) Cancer Res 42, 3010-3015.
10. Williams, G.M. (1976) Cancer Lett 1, 231-236.
11. Yager, J.D., and Miller, J.A. (1978) Cancer Res 38, 4385-4394.
12. Hart, R.W., D'Ambrosio, S.M., Ng, K.Y., and Modak, S.P. (1979) Mech. Age. Dev 9, 203-223.
13. Knook, D.L. (1978) In Genetic Effects of Aging, pp. 171-180, (ed. D. Bergsma and D.E. Harrison) Alan R. Liss, Inc., New York, 1978.
14. Ricca, G.A., Liu, D.S.H., Coniglio, J.J. and Richardson, A.J. (1978) J. Cell Physiol 97, 137-146.
15. Jayaraj, A., and Richardson, A. (1981) Mech. Age. Dev 17, 163-171.
16. Russell, G.R., and Partick, E.J. (1980) Cancer Res 40, 3719-3722.
17. Khilobock, I.Y., Mozzhukhina, T.G., Chabanny, V.N., and Kadura, S.N. (1983) Gerontology 29, 9-18.
18. Zongza, J., and Mathias, A.P. (1979) Biochem. J 179, 291-298.
19. Castle, T., Katz, A., and Richardson, A. (1979) Mech. Age. Dev 8, 383-395.