Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 250-257

COMPARISON OF NUCLEOID AND ALKALINE SUCROSE GRADIENTS IN THE ANALYSIS OF INHIBITORS OF DNA REPAIR IN HUMAN FIBROBLASTS

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Received June 2, 1982

SUMMARY: In ultraviolet-irradiated human fibroblasts inhibitors of DNA polymerases alpha (cytosine arabinoside, aphidicolin) and beta (dideoxythymidine) blocked polymerization of repair patches and accumulated large numbers of single-strand breaks in alkaline sucrose (up to 4-5 in 10^8 daltons). In nucleoid gradients, however, the restoration of DNA supercoiling to control levels after relaxation during excision repair was prevented only by aphidicolin, not by cytosine arabinoside or dideoxythymidine. These chain-terminating inhibitors must therefore generate breaks in DNA that are masked by proteins (possibly repair enzymes) that cannot be removed by the high-salt treatment of nucleoid gradients. Alkaline sucrose and nucleoid gradients therefore reveal different facets of the DNA breakage and rejoining during repair. The inhibitor of poly(ADP-ribose) synthesis, 3-aminobenzamide, had no effect on nucleoid sedimentation, implying that synthesis of this polymer plays no significant role in completion of repair of ultraviolet damage in human fibroblasts.

The production of strand breaks in DNA either directly by radiations or carcinogens or through excision repair of damaged sites has been extensively studied using nucleoid gradients (1-5) or alkaline sucrose gradients (6) as well as by other techniques (6). Nucleoid gradients supposedly reveal the influence of single-strand breaks on supercoiling of DNA in nuclei stripped of much cellular lipid and protein by 2 M salt. The sedimentation rate of nucleoids is fast and is sensitive to small numbers of single-strand breaks. Alkaline sucrose gradients, on the other hand, generally provide direct measurements of the numbers of

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0006-291X/82/130250-08$01.00/0

single-strand breaks, but with a resolution no better than about 1 break in 10^8 daltons (6).

In a recent study involving inhibitors of DNA polymerases and of poly(ADP-ribose) polymerase, nucleoid sedimentation and alkaline sucrose sedimentation gave qualitatively different results that were not due merely to differences in resolution (5). When repair of ultraviolet damage was blocked by cytosine arabinoside, numerous single-strand breaks were detected in alkaline sucrose but not in nucleoid gradients (5). We therefore have extended this observation to compare the results obtained with both kinds of sedimentation in cells grown in various inhibitors after ultraviolet irradiation.

MATERIALS AND METHODS

Normal human fibroblasts established from skin biopsies (2498, BD, ANA-1) or from embryonic tissue (MRC-5) were grown in Eagle's minimal essential medium with 10% fetal calf serum and antibiotics in 5% CO_2 at 37°C, and were monitored to ensure they were mycoplasma-free throughout the period of use (up to passage 30). Cultures were grown in 0.01 μCi/ml [^{14}C]thymidine (specific activity, 55 mCi/mol) until confluent and the medium was changed to fresh unlabeled medium for 2-3 days before use. All experiments were performed on confluent cells to minimize competition from the elevated nucleotide pools during exponential growth that are antagonistic to many inhibitors (7-9).

Stock solutions of inhibitors (hydroxyurea, aphidicolin, cytosine arabinoside, dideoxythymidine, or 3-aminobenzamide) were prepared at 100-fold excess concentrations in phosphate-buffered saline, pH 7 (except for aphidicolin, which was prepared in dimethyl sulfoxide), stored at 4°C, and added 30 min or longer before irradiation. Cultures were irradiated with ultraviolet light (254 nm, 1.3 J/m^2/sec, calibrated by a YSI radiometer model 52) and grown for various times in the inhibitors.

Molecular weights of single-stranded DNA were determined from alkaline sucrose gradients and the numbers of single-strand breaks were calculated as described previously (6). Sedimentation rates of nucleoids from cultures labeled with [^{14}C]thymidine were determined using the procedures of Cook and Brazell (1-5). Whereas DNA in alkaline sucrose gradients consists of independently sedimenting single strands (6,9), nucleoids produced by gentle lysis of cells in nonionic detergent consist of extended, supercoiled, and entangled structures containing DNA, nuclear RNA, and a small percentage of nuclear protein (1-5). Preliminary mixing experiments indicated that

there was extensive cell-to-cell interaction in these gradients, whereas this is not true of alkaline sucrose gradients (6). For example, when an irradiated and a control culture that separately sedimented at very different rates were mixed, lysed, and sedimented in one tube, nucleoids from each culture sedimented together at an intermediate rate (Cleaver, J.E., unpublished data). Therefore, the nucleoid sedimentation rates observed after various treatments are an average over the culture and are subject to a more complex and less well-understood set of parameters than are the sedimentation rates in alkaline sucrose gradients (1-4).

RESULTS

Alkaline sucrose gradients. Cultures irradiated with 2.6 to 13 J/m^2 ultraviolet light and incubated without inhibitors showed no detectable strand breakage in alkaline sucrose (Table I) because the excision breaks are too few and transient to be resolved (6). Cultures irradiated with ultraviolet light and grown for 4 hr in aphidicolin, cytosine arabinoside, or dideoxythymidine, with or without hydroxyurea, showed an accumulation of single-strand breaks through inhibition of repair polymerization (Table I). 3-Aminobenzamide showed no detectable effects, although under these conditions it clearly inhibits poly(ADP-ribose) synthesis (10) and delays completion of repair patches in lymphoid cells (11).

Nucleoid gradients. The DNA in nucleoid gradients sedimented in peaks occupying only one or two fractions of the gradient, and the sedimentation rate was very sensitive to low doses of ultraviolet light. After irradiation with 2.6 J/m^2 the sedimentation rate decreased rapidly, and then slowly increased to control rates (Fig. 1). Incidental to our main interest, we noted that the rate of increase was slower in the embryonic fibroblast cell line (MRC-5) than in the skin fibroblasts (BD, ANA-1, 2498), and results were therefore compared carefully to the appropriate control.

Of all the inhibitors, aphidicolin was the only one that maintained sedimentation at a very slow rate (Fig. 1). Cytosine

Table I

Numbers of single-strand breaks in DNA of human fibroblasts grown for 4 hr in various inhibitors after irradiation with ultraviolet light*

Inhibitor	Hydroxyurea (mM)	Skin fibroblasts		Embryonic fibroblasts
		2.6 J/m^2	13 J/m^2	13 J/m^2
None	0	0	0	0
Cytosine arabinoside (10 μM)	0	1.1	4.5	
Cytosine arabinoside (10 μM)	2	2.0 ± 0.8	4.4 ± 0.2	4.1 ± 0.5
Aphidicolin (4 μM)	0	1.0	4.2 ± 1.3	4.7
Dideoxythymidine (100 μM)	2	0.75	2.7 ± 0.4	2.0 ± 0.2
3-Aminobenzamide (2 mM)	0	0.13 ± 0.19	0	0

*Calculated as number of breaks per control DNA molecule (2 x 10^8 daltons). Standard errors are given for 3 or more determinations; some of the values for cytosine arabinoside at 13 J/m^2 were included in a previous report (9).

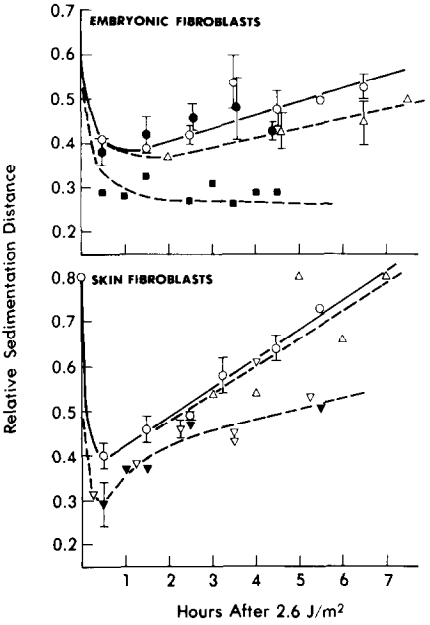

Fig. 1. Relative sedimentation distance, as fractions of tube length, of nucleoids as a function of time after irradiation with 2.6 J/m^2 ultraviolet light. Top: Embryonic skin fibroblasts grown in (○) fresh medium; (△) dideoxythymidine (100 μM) plus hydroxyurea (2 mM); (●) 3-aminobenzamide (2 mM); (■) aphidicolin (4 μM). Bottom: Skin fibroblasts grown in (○) fresh medium; (△) dideoxythymidine (100 μM) plus hydroxyurea (2 mM); (▽) cytosine arabinoside (10 μM); (▼) cytosine arabinoside (10 μM) plus hydroxyurea (2 mM). Standard errors are shown for 3 or more determinations.

arabinoside and dideoxythymidine (plus hydroxyurea) retarded the recovery of sedimentation rate only slightly, and 3-aminobenzamide was without effect. Therefore, even though cytosine arabinoside and dideoxythymidine accumulated many single-strand breaks in 4 hr after irradiation (Table I), these were not detected by nucleoid sedimentation and after 4 hr the nucleoids had sedimentation rates close to control.

DISCUSSION

These results indicate that there are differences in the experimental observations made with alkaline sucrose gradients and nucleoid sedimentation of the same biological state. Some differences seem merely a matter of resolution, because the

breaks involved in excision repair of ultraviolet damage are few and transient and are undetectable in alkaline sucrose but clearly detectable in nucleoid sedimentation (Table I, Fig. 1). Other differences seem to be caused by more complex qualitative features of the respective gradients.

The single-strand breaks produced by inhibition of DNA repair by aphidicolin maintained complete relaxation of DNA during nucleoid sedimentation, but those produced by cytosine arabinoside or dideoxythymidine did not. Similar results have been recently observed in other studies of nucleoid sedimentation (5,14). The reason for the failure of cytosine arabinoside and dideoxythymidine to affect nucleoid sedimentation is not merely that they generate alkaline labile bonds. When the DNA is isolated under neutral conditions many excess 3'OH termini are detected with exonuclease III (9), demonstrating the existence of true single-strand breaks.

An important difference between the inhibitors used is that aphidicolin binds directly to polymerase alpha (15,16), whereas the others are incorporated into DNA and act as chain terminators (15,17). The differences in the nucleoid and alkaline sucrose sedimentation results can be resolved if repair enzymes become trapped at aborted repair sites that contain incorporated chain terminators. The single-strand breaks could then be masked by proteins whose binding to DNA is resistant to high salt and Triton X-100. Aphidicolin, bound specifically to polymerase alpha, could prevent polymerases from binding to the repair site, and the excision breaks would then be clearly visible by their effects on nucleoid sedimentation.

Skin and embryonic fibroblasts differed in the rate at which their nucleoids recovered rapid sedimentation rates after

irradiation, despite their similarities in repair. Both fibroblasts have the same capacity for pyrimidine dimer excision (Cleaver, J.E., and Clarkson, J., unpublished data) and repair replication (12,13). One possibility to explain the differences in nucleoid sedimentation is that, because all the DNA from a culture sediments in a narrow zone within the gradient, the sedimentation rate may vary with differences in the cell population distributions. We noted that the embryonic cells had a more rapid growth rate and a larger fraction of cells in DNA synthesis than the skin fibroblasts. These differences may modify the changes in sedimentation rate that are due to excision repair itself.

The absence of any effect of 3-aminobenzamide on nucleoid sedimentation rates indicates that poly(ADP-ribose) synthesis is not involved in the repair of ultraviolet damage in human fibroblasts (10). This contrasts strongly with the effects that 3-aminobenzamide has on repair of ultraviolet and alkylation damage in human lymphoid cell lines (10,11), in which poly ADP-ribosylation modulates the efficiency of polynucleotide ligase (18). Our observations are consistent with those made recently in which 3-aminobenzamide had no effect on ultraviolet-induced sister chromatid exchanges but was synergistic with methyl methanesulfonate-induced sister chromatid exchanges (19).

This comparison of two forms of sucrose gradient sedimentation provides two very different views of the same stages of excision repair of ultraviolet damage. Clearly, the two kinds of gradients cannot be used interchangeably, but comparative studies can provide useful insights into the intermediate stages of DNA repair.

ACKNOWLEDGEMENTS

We are grateful to F. McEnroe for secretarial assistance, M. McKenney for editorial assistance, and the NCI Drug Development Branch for the gift of aphidicolin. This work was supported by the U.S. Department of Energy.

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