BRIEF COMMUNICATION

AN INVESTIGATION OF THE EFFECT OF RADIOACTIVE LABELING OF DNA ON EXCISION REPAIR IN UV-IRRADIATED HUMAN FIBROBLASTS

URSULA K. EHMANN AND ERROL C. FRIEDBERG, *Laboratory of Experimental Oncology, Department of Pathology, Stanford University, Stanford, California 94305 U.S.A.*

ABSTRACT Previous studies on the kinetics of thymine dimer excision and unscheduled DNA synthesis in UV-irradiated human fibroblasts showed a significant discrepancy in these two parameters (Ehmann et al., 1978. *Biophys. J.* **22:** 249). In the present study we have investigated the effect of the level of the radioactive isotope used for labeling cells on the kinetics of a parameter that indirectly measures thymine dimer excision. We find no significant differences in the kinetics of this parameter in cells lightly or heavily labeled with radioactive thymidine.

INTRODUCTION

UV-irradiation of living cells at ~254 nm results in the formation of dimers between adjacent intrastrand pyrimidines in DNA (1). We have previously investigated the kinetics of the loss of thymine-containing pyrimidine dimers from the acid-insoluble fraction of several cultured human cell lines and compared these results to the kinetics of repair synthesis measured by autoradiography (2, 3). In those studies we observed that the time of half-maximal loss of dimers ranged from 12 to 22 h after irradiation. In contrast, the time of half-maximal repair synthesis of DNA was ~4.5 h.

We advanced several hypotheses to account for this kinetic discrepancy (2, 3), one of which addressed the possible effects of radioactive labeling of the DNA on cells in culture. Specifically, the measurement of dimer excision by the techniques we employed requires the prelabeling of the DNA of cells with radioactive thymidine for ~24 h before irradiation. On the other hand, the measurement of repair synthesis by autoradiography only utilizes labeling of the DNA after the irradiation, just before sacrifice of the cells. Radioactive labeling of mammalian cells in culture is known to cause DNA strand breaks, mutations, inhibition of cell division, chromosomal aberrations, and cell death (see discussion by Ehmann et al. [2, 4]). We have thus considered the possibility that the use of relatively high levels of radioactive isotope may have a toxic effect on cells in culture that manifests as a reduced efficiency of excision repair. The most direct method for testing this hypothesis would be to compare the kinetics of the loss of thymine-containing pyrimidine dimers in cells exposed to low and high

levels of radioactive thymidine. However, in our experience the accurate quantitation of the thymine dimer content of the DNA in cells undergoing active excision (and hence progressively losing dimers), requires a high specific radioactivity of the DNA that can only be achieved by including at least 2 μCi/ml and preferably 5–10 μCi/ml of labeled thymidine in the cultures. Such levels of isotope have been shown by others to cause significant perturbations of cellular metabolism in mammalian cells (See reference 2 and references cited therein).

The excision repair of UV-irradiated cells involves both the enzyme-catalyzed incision of DNA at pyrimidine dimer sites and their subsequent excision (5). The efficiency of the former step can be directly measured by quantitating the sensitivity of the cellular DNA to a preparation of enzyme(s) isolated from *Micrococcus luteus* or from phage T4-infected *Escherichia coli* known to catalyze the incision of DNA at pyrimidine dimer sites (6). Sensitivity of the DNA to such enzyme probes indicates that incision of the DNA failed to occur at these sites in the intact cell. The use of this technique (generally referred to as the enzyme-sensitive site [ESS] assay [6]) also requires prelabeling of cellular DNA with a radioisotope; however, a significantly lower specific activity of the DNA can be tolerated than for the direct measurement of the excision of thymine-containing pyrimidine dimers. If prelabeling of the DNA of human cells in culture with radioactive isotope impairs their capacity for excision repair in vivo, one might expect to observe differences in the kinetics of the loss of ESS from DNA of cells exposed to high or low levels of radioactive isotope. The results of such a comparison using normal human fibroblasts are reported here.

MATERIALS AND METHODS

A normal human diploid fibroblast cell line, GM 316, obtained from the Mutant Cell Repository, Medical Research Institute, Camden, N.J., was cultured in Eagle's Modified Essential Medium as described previously (2). The cells were plated in 100-mm plastic tissue culture petri dishes at a density of 5.7×10^5 cells/dish in 10 ml of growth medium and incubated overnight. The medium was then removed and replaced either with medium containing [^{3}H]thymidine (5.0 μCi/ml, 50 Ci/mmol, New England Nuclear, Boston, Mass.), [^{14}C]thymidine (0.05 μCi/ml, 49 mCi/mmol, New England Nuclear), or medium without label. After 39 h of incubation at 37°C, some of the cells were irradiated with 5 J/m^2 of 254 nm light at a dose rate of 0.17 J/m^{2}s^{-1}. The cells were then incubated for 0, 3, 6, or 12 h in normal growth medium. At the end of the postirradiation incubation period the cell sheets were washed twice with phosphate buffered saline and incubated for a few minutes with 2 ml of a solution containing 0.05% trypsin plus 0.025% EDTA to remove the cells. To this mixture 7 ml of phosphate buffered saline and 1 ml of growth medium were added to inhibit further trypsin digestion. Clumps of cells were disaggregated by pipetting the cells in the solution 4–5 times with a 10-ml pipette. 9 ml of each sample were used for cell counting and sizing. Cells were counted with a Coulter Model ZBI cell counter, and size spectra for the cell populations were determined with a Coulter Channelizer (Coulter Electronics Inc., Hialeah, Fla.).

For the determination of ESS in cellular DNA, [^{14}C]thymidine- and [^{3}H]thymidine-labeled cells were mixed and the DNA co-extracted and sedimented on the same gradient. An aliquot of (1.0 ml) of the cell suspension labeled with [^{3}H]thymidine from a particular incubation time was added to 1.0 ml of the suspension labeled with [^{14}C]thymidine from the same incubation time and the mixture was centrifuged for 7 min at 1,000 rpm in a GLC-1 table-top centrifuge (Sorvall, Newtown, Conn.). 2 ml of a solution of 0.15 M NaCl and 10 mM EDTA was added to each cell pellet and the mixture was vortexed and recentrifuged. To each pellet 100 μl of an enzyme incubation mixture at pH 7.5 consisting of 0.1 M NaCl, 0.02 M Tris-HCl buffer, pH 7.5, 0.01 M EDTA, 0.01 M β-mercaptoethanol, and 1 mg/ml bovine serum albumin was added. After mixing, the cells were permeablized by two cycles of rapid freezing and

thawing (7). For this procedure the suspensions were frozen by immersing the bottom of the centrifuge tubes in a mixture of dry-ice/acetone for 30 s and then thawed by quickly transferring to a water bath at 37°C for 90 s. An aliquot (10 μl) of a crude extract of *M. luteus* containing saturating amounts of UV-DNA incising activity prepared according to the procedure of Carrier and Setlow (8) was added to each tube of permeabilized cells and the tubes were incubated at 37°C for 15 min (7). After incubation, 0.5 ml of 0.15 M NaCl containing 0.01 M EDTA was added and the suspension was lysed at room temperature for 45 min on the top of 5–20% alkaline sucrose gradients as described by Lett et al. (9). The DNA was centrifuged at 18,000 rpm in a Beckman SW27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 292 min at 20°C, after which the gradients were fractionated from the bottom of the polyallomer centrifuge tubes. The number average molecular weights and the number of breaks per DNA strand were calculated from the sedimentation profiles as previously described (10). The position of ^{14}C-labeled T2 phage DNA sedimented on a separate gradient was used as a DNA molecular weight calibration marker.

RESULTS

It has been previously shown by one of us and others (4, 11, 12) that the proliferation of mammalian cells in monolayer culture is retarded by growth in high levels of radioactive thymidine, and that this isotope also causes cell cycle arrest in G_2 phase. Both these effects were observed in the present experiments. GM 316 fibroblasts that were prelabeled for 39 h with either 0.05 μCi/ml of [^{14}C]thymidine (low level of isotope) or 5.0 μCi/ml of [^{3}H]thymidine (high level of isotope) were washed and incubated in fresh medium without the addition of isotope for an additional 12 h. At this time the cell number in the culture exposed to the high level of isotope was about half that observed in the control cultures or the cultures prelabeled with low levels of isotope (Table I). We also observed by direct measurement that the cells treated with a high level of isotope had a median volume more than twice that of controls (Fig. 1, Table I). This result is expected of cells blocked late in the cell cycle (13–15). The volume spectrum of the cells labeled with low level of isotope was slightly larger than that of untreated cells (Fig. 1, Table I), indicating that even 0.05 μCi/ml of radioactive thymidine may have caused some perturbation of the cycle. However, this effect was marginal and the growth rate of these cells apparently normal.

After irradiation with 5 J/m^2 of UV light, cells were incubated at 37°C for varying periods of time. The number of sites sensitive to hydrolysis of phosphodiester bonds by the *M. luteus* enzyme preparation was determined by incubating permeabilized cells with the enzyme preparation, sedimenting the DNA through alkaline sucrose gradients and calculating the number average molecular weight and hence the number of enzyme-induced breaks per

TABLE I
EFFECT OF RADIOISOTOPE ON CELL GROWTH AND CELL VOLUME

Conditions	Final cell count per dish ($\times 10^{-5}$)	Final median cell volume (relative units)
No isotope added	12.6	35.4
[^{14}C] (*0.05 μCi/ml*)	13.6	41.5
[^{3}H] (*5.0 μCi/ml*)	6.8	78.9

Cells were plated at an intitial density of 5.7 × 10^5/petri dish, incubated for 39 h in the presence or absence of radioactive thymidine and then re-incubated in fresh nonradioactive medium for an additional 12 h before final cell counts and cell volumes were determined. See text for further experimental details.

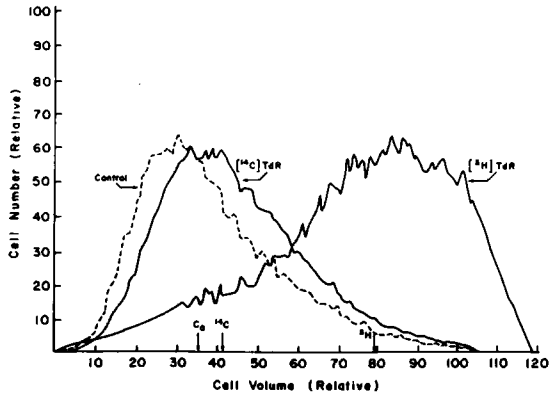

FIGURE 1 Volume distributions of GM 316 fibroblasts. Cells were grown for 39 h without the addition of radioactive thymidine or in the presence of 0.05 μCi/ml [^{14}C]thymidine or 5.0 μCi/ml [^{3}H]thymidine. The cells were then grown for a further 12 h in medium without isotope and cell volumes were measured in a Coulter Channelizer. The arrows indicate the median values of the cell volume profiles.

molecule. The number average molecular weight of the DNA from unirradiated cells labeled with ^{14}C or ^{3}H was 2.1×10^8 and 1.8×10^8 daltons, respectively. These values decreased to 1.8×10^7 and 1.9×10^7, respectively, in samples analyzed immediately after irradiation, indicating the presence of ~9 ESS in the DNA of both ^{14}C- and ^{3}H-labeled cells (Fig. 2). The number of ESS in both the ^{14}C- and ^{3}H-labeled DNA decreased progressively as a function of the time of postirradiation incubation. In repeated experiments the rate of loss of such sites from the ^{3}H-labeled DNA (high level radioactivity) was slightly faster than from the ^{14}C-labeled DNA (low level radioactivity); however, the reverse result was never observed, i.e., we have found no evidence that cells containing heavily labeled DNA are inhibited in the rate of loss of ESS relative to cells containing lightly labeled DNA. The results of a typical experiment are shown in Figs. 2 and 3.

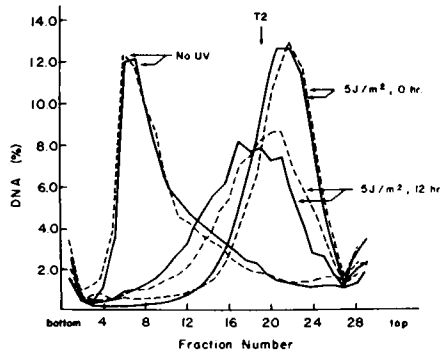

FIGURE 2 Sedimentation profiles of DNA from GM 316 cells centrifuged in alkaline sucrose velocity gradients. See text for experimental details. The arrow marks the position of sedimentation of bacteriophage T2 DNA. Profiles shown with continuous lines are from cells labeled with 5.0 μCi/ml [^{3}H]thymidine (high level radioactivity). Those shown with discontinuous lines represent DNA profiles from cells labeled with 0.05 μCi/ml [^{14}C]thymidine (low level radioactivity).

DISCUSSION

These experiments demonstrate that the labeling of GM 316 fibroblasts in culture with the high levels of radioactive thymidine used to directly measure excision of thymine-containing pyrimidine dimers has profound effects on cellular metabolism as evidenced by retarded growth rate and by cell cycle arrest. Despite these effects the kinetics of the loss of ESS from the DNA of cells labeled with high levels of radioactivity are not reduced relative to the kinetics observed with lightly labeled cells. Indeed, the rate of loss of ESS from the former DNA appears to be slightly faster.

There is substantial evidence in the literature (16, 17) indicating that sites in UV-irradiated DNA sensitive to *M. luteus* enzyme(s) are pyrimidine dimers and that their loss reflects either their incision or incision plus excision during post-UV incubation of cells (18). It is thus evident that the labeling of cells with high levels of radioactive isotope does not inhibit enzymatic events required for incision of DNA in vivo. The use of the ESS assay does not discriminate between sites lost by incision only and those lost by incision followed by excision. Thus we cannot eliminate the possibility that high levels of radioisotope selectively depress the excision of dimers at preincised sites. However, we consider such a selective cellular toxicity unlikely. This consideration is supported by the results of Williams and Cleaver (19) who tested the effect of radiotoxicity on the kinetics of repair synthesis in mammalian cells by pretreating cells with ionizing radiation to mimic chromosomal damage caused by isotope decay. They observed no significant impairment in the capacity of those cells to carry out subsequent repair synthesis in response to UV radiation damage. Recently, a study on the kinetics of thymine dimer excision and of repair synthesis in human fibroblasts was reported by Konze-Thomas et al. (20). These authors reported no differences in the kinetics of these two parameters. It should be noted however that the experimental protocol (including the level of isotope) and the cell strains used by those authors are different from ours and thus their results are not directly comparable.

In a previous study we suggested four possible explanations for the discrepancy in the kinetics of thymine dimer excision and unscheduled DNA synthesis observed in our hands (2).

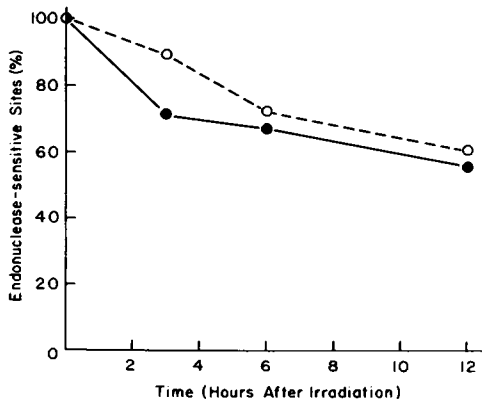

FIGURE 3 Kinetics of loss of sites in GM 316 DNA sensitive to UV-DNA incising activity from *M. luteus*. See text for experimental details. ●—●, cells labeled with 5.0 μCi/ml [^{3}H]thymidine (high level radioactivity); O—O, cells labeled with 0.05 μCi/ml [^{14}C]thymidine (low level radioactivity).

One hypothesis was that pyrimidine dimers are excised initially as acid-precipitable fragments that are then slowly degraded to acid-soluble products intracellularly. This hypothesis was experimentally tested and negative results obtained (2). A second model proposed was that addressed in the present report. The third hypothesis was that, in human fibroblasts exposed to UV irradiation, incision of DNA adjacent to pyrimidine dimers and repair synthesis precede the actual excision of the dimers, i.e., a "patch and cut" rather than a "cut and patch" mechanism operates. The results of the present experiments directly support this model, since the kinetics of loss of ESS from both ^{3}H- and ^{14}C-labeled DNA (Fig. 3) are faster than the kinetics of the loss of thymine-containing pyrimidine dimers we previously reported (2). A final possibility is that a significant fraction of measured unscheduled DNA synthesis in UV-irradiated cells in culture does not reflect repair synthesis at sites of pyrimidine dimer excision but at other sites in DNA which may or may not be sites of damage.

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