Role of Postreplication Repair in Transformation of Human Fibroblasts to Anchorage Independence

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ABSTRACT

Cellular capacity for postreplication repair (PRR) and sensitivity to transformation to anchorage independence (AI) were quantified in normal foreskin and xeroderma pigmentosum (XP) variant fibroblasts after treatment with UV or benzo[a]pyrene-diol-epoxide I (BPDE-I). PRR is defined here as a collection of pathways that facilitate the replication of DNA damaged by genotoxic agents. It is recognized biochemically as the process by which nascent DNA grows longer than the average distance between two lesions in the DNA template. PRR refers more directly to the elimination of gaps in the daughter-strand DNA by mechanisms which remain to be determined for human cells, but which may include translesion replication and recombination. PRR was measured in diploid human fibroblasts by analysis of the dose kinetics for inhibition of DNA strand growth in carcinogen-treated cells. Logarithmically growing foreskin fibroblasts (NHF1) displayed $D_0$ values of 4.3 J/m² and 0.14 $\mu$m for the inhibition of DNA synthesis in active replicons by UV and BPDE-I, respectively. XP variant cells (CRL1162) exhibited corresponding $D_0$ values of 1.5 J/m² and 0.16 $\mu$m. The increased sensitivity to inhibition of DNA replication by UV in these XP variant fibroblasts (2.9-fold greater than normal) was mirrored by an enhanced frequency of transformation to AI. XP variant fibroblasts (CRL1162) were 3.2 times more sensitive to transformation to AI by UV than were the normal foreskin fibroblasts.

INTRODUCTION

Replication of carcinogen-damaged DNA is believed to result in mutations and chromosomal aberrations that may contribute to carcinogenesis (1, 2). Prereplicative repair of such damage reduces cellular risk of transformation (3). Cells from individuals who exhibit enhanced sensitivity to the development of cancers are valuable tools for the examination of the roles of DNA repair and replication in mutagenesis and carcinogenesis. Individuals with the XP variants of the xeroderma pigmentosum (XP) syndrome have a linearized deficiency in excision repair of UV-induced DNA damage (6, 7). Fibroblasts from these individuals appear to exhibit a defect or deficiency in the replication of UV-damaged DNA templates (8-10).

The process which permits the complete replication of DNA that contains noninstructive base damage has been referred to as PRR. We use PRR as a collective term to describe an undefined number of pathways operating in concert to complete the replication of the entire cellular genome in damaged cells. We expect that some of these pathways operate only on certain structural lesions in DNA and/or in response to specific agents or defined classes of mutagens/carcinogens. XP variant cells express a reduced capacity for PRR of UV-damaged DNA, but they display the same ability as do normal cells to replicate DNA containing BPDE-I adducts (10, 11). XP variant fibroblasts are only slightly more sensitive than are normal cells to the killing action of UV radiation (10, 12) but have been found to be hypermutable (12, 13) and hypertransformable by UV (14). On the other hand, mutation frequencies induced in XP variant cells by exposure to BPDE-I were similar to those observed in normal cells (6). These results suggest that XP variant fibroblasts lack an error-free pathway of PRR of UV-induced lesions that is present in normal cells. However, this pathway in normal cells appears to be ineffective in bypassing BPDE-I-induced DNA lesions (10). Thus, one would expect that normal fibroblasts and XP variant cells should be equally sensitive to transformation to AI by BPDE-I. In this paper, we present experimental data that confirm this prediction. Thus, cellular sensitivity to transformation to AI by the carcinogens UV and BPDE-I appears to be quantitatively associated with sensitivity to inhibition of DNA strand growth caused by carcinogen-induced damage to the DNA template.

MATERIALS AND METHODS

Cell Culture Conditions. Normal human fibroblasts were derived from neonatal foreskin (NHF1) and established in culture according to published methods (15), or they were obtained from the Human Genetic Mutant Repository, Camden, NJ (GM3348, skin biopsy of a clinically unaffected 10-yr-old male). XP variant fibroblasts were obtained from the American Type Culture Collection (CRL1162, XP strain designation XP4BE) or as a generous gift from Dr. Seiji Kondo (X4P2T0). Cell stocks were grown in plastic tissue culture dishes, at 37°C in a humidified atmosphere of 5% CO₂, in Ham's F10 medium supplemented with 15 to 20% fetal bovine serum and either penicillin (100 units/ml) plus streptomycin (100 µg/ml) or gentamicin (50 µg/ml). Cells were subcultured 2 to 3 times/wk to maintain logarithmic growth and used between passages 1 and 18. The colony-forming efficiency on plastic ranged from 10% to 15% for the XP variant cells and from 25% to 40% for the NHFI cells.

Treatment with UV and BPDE-I. Cells to be irradiated with UV were first rinsed with warm (37°C) HBSS. A short-wave UV lamp emitting mostly 254-nm radiation was used to irradiate cells. The incident fluence rate was 0.23 to 1.35 J/m²/s as determined with a UV radiometer (UV Products, Inc., San Gabriel, CA). For treatment with BPDE-I, culture medium was removed, and cells were rinsed twice with...
warm HBSS and then covered with 5 ml of the same solution. An aliquot of 25 μl of anhydrous dimethyl sulfoxide containing varying concentrations of BPDE-I was added, and cultures were incubated at 37°C for 10 min. The BPDE-I-containing solution was then removed, and the cells were rinsed once with warm HBSS and refed with fresh medium containing serum. The concentration of the stock solution of BPDE-I was determined from absorbance readings at 345 nm (extinction coefficient of 48,600 cm⁻¹/mol), and the activity was determined using the method of MacLeod and Lew (16).

Inhibition of DNA Replication. Effects of UV and BPDE-I on DNA strand growth were measured using the method of Kaufmann and Cleaver (9). Briefly, cells from logarithmically growing cultures were seeded into 100-mm dishes and treated 20 to 24 h later with 5 nCi/ml of [³H]thymidine (ICN Pharmaceuticals, Inc., 50 Ci/mmol) for 2 days to label DNA uniformly. One to 2 h before carcinogen treatment, the medium was replaced with fresh unlabeled medium. Fibroblasts were then treated with UV or BPDE-I as described above. After treatment, cells were incubated at 37°C in reserved medium for 30 min and then pulse labeled with 50 μCi/ml of [³H]thymidine (ICN Pharmaceuticals, Inc., 40 to 50 Ci/mmol) for 15 min. Cells were harvested, and the size distribution of nascent DNA was analyzed in alkaline sucrose gradients (9, 17). The sum of the incorporation of ³H cpm into large intermediates of DNA replication (5 × 10⁴ to 2 × 10⁷ daltons), after normalization to the number of cells added to each gradient (³H cpm), was expressed as the percentage of the same sum measured in control cells (10).

Anchorage Independence Assay. Assays were conducted according to the methods of McCormick et al. (18, 19). Briefly, 2 to 3 × 10⁵ cells were seeded into 100-mm dishes and treated 20 to 24 h later with carcinogen as described above. Treated cultures were refed with fresh medium and maintained in logarithmic growth by subculturing at a 1:3 (XP variant cells) or 1:10 dilution (NHF1 cells). After the optimal expression period of 5 population doublings (Refs. 14 and 18; Footnote 5), 5 × 10⁵ cells per 60-mm dish were seeded into 3 ml of 0.33% Noble agar (Difco) over a 3-ml base layer of 0.5% agar in Ham’s F10 supplemented with 7% (NHF1) or 20% (XP variant) fetal bovine serum (Hyclone). The serum concentration was adjusted to produce about 10% of larger (4 to 6 wk after seeding), cells were stained by adding 1 ml of a solution of nitroblue tetrazolium (1 mg/ml) and fi-NADH (0.5 mM). The blue-stained colonies were counted microscopically. The colony counts were normalized to the relative plating efficiency on plastic of the aliquot of cells taken from the top agar cell suspension. This normalization corrected for artefacts which may have resulted from either heat-induced cell death (i.e., during the suspension of cells into molten agar) or from the seeding of dead or nonproliferative cells that had persisted during passage of the original carcinogen-treated cultures. For example, in one experiment in which cells were treated with a dose of UV that killed 70% of the population, 41 colonies were produced per 5 × 10⁵ cells seeded in soft agar. These same cells exhibited an 88% relative colony-forming efficiency on plastic at the time of the AI assay. This gives a normalized frequency of 41/0.88 = 46 colonies per 5 × 10⁵ cells. Control cells were sham irradiated or treated with dimethyl sulfoxide, cultured in parallel, and plated in soft agar for the determination of the frequency of background colonies. In this example, the background frequency of 13 colonies per 5 × 10⁵ cells was subtracted from the treated cell frequencies. Thus, 46 − 13 = 33 colonies per 5 × 10⁵ normalized cells were induced by UV. Because S-phase cells are believed to be most at risk during carcinogen treatment (21) and because the number of S-phase cells observed in logarithmically growing cultures of XP variant fibroblasts (15 to 45%) was routinely lower than that observed in cultures of foreskin fibroblasts (35 to 60%), the frequency of soft agar colonies was also normalized to the percentage of cells in S phase at the time of carcinogen treatment. The frequency of S-phase cells in culture was determined by autoradiography (22) in parallel cultures pulse labeled with [³H]thymidine and cyt centrifuged onto microscope slides.

RESULTS

The objective of this study was to test the hypothesis that cellular sensitivity to inhibition of DNA replication by carcinogen-induced template damage was predictive of sensitivity to transformation to AI. Therefore, the effects of UV and BPDE-I on DNA replication and transformation were compared in normal and XP variant fibroblasts. Fig. 1 depicts the inhibition of DNA strand growth by UV and BPDE-I. These results were generated from sedimentation profiles in alkaline sucrose gradients as described in “Materials and Methods.” We determined D₀ values representing the increment of carcinogen dose that reduced to 37% of control the amount of [³H]thymidine incorporation into large intermediates of DNA replication. XP variant fibroblasts displayed an enhanced sensitivity to UV-induced inhibition of DNA strand growth when compared with normal cells (Fig. 1A). The calculated D₀ value for foreskin fibroblasts (NHF1) was 4.3 J/m². We have included in Fig. 1 our results in this assay previously published (10) for CRL1162 (D₀ = 1.5 J/m²). Other previously published D₀ values for UV-induced inhibition of DNA strand growth were: normal skin fibroblasts, GM3348 (D₀ = 6.3 J/m²); foreskin fibroblasts, HS1 (D₀ = 4.1 J/m²); XP variant fibroblasts, GM2359 (D₀ = 2.0 to 2.4 J/m²); and XP30R0 (D₀ = 1.9 J/m²). Another XP variant fibroblast strain, XP4270, exhibited a sensitivity to UV (D₀ = 2.6 J/m²) which was very similar to that observed with the XP variant fibroblasts listed above. As illustrated in Fig. 1B, DNA strand growth in both normal and XP variant cells was equally inhibited by BPDE-I (NHF1, D₀ = 0.14 μM; CRL1162, D₀ = 0.16 μM). The D₀ values that were previously published for BPDE-I-induced inhibition of DNA strand growth (10) were greater than those presented here. This is because the doses of BPDE-I used in the previous experiments were not corrected for the loss of activity due to hydrolysis of the diol-epoxide (16).

For quantitation of the induction of cell transformation, carcinogen-treated cell populations were assayed for colony formation in soft agar. The doses of carcinogen that were selected produced 30 to 90% reduction of cell colony formation efficiency on plastic (10). No differences in the appearance or size of XP variant versus normal fibroblast colonies were seen in the AI assay. Both cell types exhibited a linear dose-response relationship for transformation to anchorage independence by UV and BPDE-I (Fig. 2). The slopes of the lines representing UV-induced growth in soft agar, normalized to cells in S phase at the time of carcinogen treatment, were 33.6 and 10.4 colonies/50,000 cells per J/m² for UV-treated XP variant and normal fibroblasts, respectively (Fig. 2A). This 3.2-fold difference in slope was found to be statistically significant (P < 0.05) by analysis of variance with linear regression for multiple y observations at given x values (23). The same analysis of the lines representing BPDE-I-induced growth in soft agar by the

² Unpublished results.
two cell types (Fig. 2B) showed that the slopes were not significantly different ($P > 0.50$). In these experiments, the foreskin fibroblasts, NHF1, the normal dermal skin fibroblasts, GM3348, and two XP variant fibroblast strains, CRL1162 and XP42T0, exhibited similar BPDE-I-induced frequencies of soft agar growth. Thus, XP variant fibroblasts (CRL1162) exhibited an approximately 3.2-fold increased sensitivity to transformation to AI by UV as compared with normal cells, but normal sensitivity to BPDE-I-induced growth in soft agar.

It was also observed that there was a threshold for induction of AI by UV and BPDE-I (Fig. 2). The threshold fluences for XP variant and normal cells treated with UV were 0.3 J/m$^2$ and 2.7 J/m$^2$, respectively. Both cell types exhibited a threshold dose of about 0.05 μM BPDE-I.

As a final element in this analysis, the induction of AI colonies was expressed as a function of the level of inhibition of DNA replication by template lesions (Fig. 3). Doses of carcinogen that inhibited replication to the same degree in normal and XP variant cells produced similar frequencies of soft agar. When doses of UV and BPDE-I were normalized to their effects on DNA strand growth, BPDE-I-induced DNA damage appeared to produce more AI colonies than did UV-induced damage, in both normal and XP variant fibroblasts.

**DISCUSSION**

The carcinogens UV and BPDE-I have been shown to inhibit DNA synthesis by two mechanisms: down-regulation of replication initiation and inhibition of chain elongation (9, 17, 24, 25). Carcinogen lesions in the DNA template interfere with the polymerization step and may lead to the production of gaps in the lagging-strand DNA by interrupting the synthesis of Okazaki fragments (26). Several pathways of PRR have been proposed that may facilitate the replicative bypass of template lesions and/or the sealing of gaps in daughter-strand DNA (reviewed in Ref. 2). XP variant cells exhibit defective PRR after exposure to UV (8–11, 27) and were used to study the relationship between cellular capacity for PRR and the sensitivity of human fibroblasts to induction to anchorage independence.

Low doses of UV or BPDE-I, which are known to inhibit primarily replicon initiation (9, 17), failed to induce anchorage independence in human fibroblasts. However, higher doses of these carcinogens, which inhibited DNA synthesis in operating replicons by more than 45%, caused a dose-dependent induction of growth in soft agar. Down-regulation of replicon initiation is believed to be a protective response that provides time for the repair of DNA lesions prior to replication (2, 25, 28, 29). Thus, it is not surprising that anchorage-independent colonies were not induced by doses of carcinogen that only inhibit replicon initiation. Higher doses, however, interrupted strand growth and caused gaps in the daughter-strand DNA. We presume that it is in the process of overcoming the blockage of the replication machinery at the site of DNA damage and/or eliminating gaps in daughter DNA through pathways of PRR that mutations are produced and transformation is induced. Other researchers have successfully transformed human fibroblasts to AI with UV (14) and BPDE-I (30). The frequencies of UV- and BPDE-I-induced AI presented in this paper compare favorably with those published by these other authors, who also observed threshold doses of carcinogen below which AI was not observed.

Based on $D_0$ values for UV-induced inhibition of DNA replication, the XP variant fibroblasts (CRL1162) expressed approximately 34% of normal PRR activity, or a 2.9-fold higher sensitivity to UV than the foreskin fibroblasts (NHF1). After BPDE-I treatment, however, both of these cells displayed similar PRR activities. The enhanced sensitivity to inhibition of replication by UV was mirrored in XP variant fibroblasts by an enhanced frequency of transformation to AI. XP variant cells

![Graph A and B](image-url)
were 3.2 times more sensitive to the induction of AI by UV than were the normal fibroblasts. Both cell types exhibited similar frequencies of AI colonies induced by BPDE-I.

We have previously reported that, in normal fibroblasts, approximately 5 cyclobutane pyrimidine dimers per replicon are needed to inhibit replication to 37% of control levels, whereas only 1 or 2 dimers produced the same effect in XP variant cells (10). In the same analysis, it was found that, in both cell types, just 1 or 2 BPDE-I-DNA adducts per replicon inhibited replication to 37% of control levels. From the data presented here and the lesion frequencies needed to inhibit replication (10), it appears that normal cells may be endowed with a mechanism to bypass a majority of UV photoproducts without significant blockage of replication forks (Fig. 1A) and in a error-free manner (Fig. 2A). This would be in contrast to the error-prone PRR pathway(s) that may be used to bypass UV lesions in XP variant cells, a minority of UV photoproducts in normal fibroblasts, and BPDE-I-induced adducts in both cell types. The occurrence of error-free and error-prone pathways of PRR can be inferred from the diverse types of genetic alterations that result from replication of damaged templates. For example, sister chromatid exchanges may be derived from homologous recombination which is a putative error-free process and may play a role in the repair of gapped DNA (31, 32). Deletions and base substitution mutations are expected to arise from the operation of error-prone PRR pathways. Furthermore, our definition of PRR would also include the correction of replication errors that might be incurred by the eukaryotic DNA polymerases (33).

One model which may account for the data holds that XP variant cells lack a factor that prevents the interruption of polymerization at DNA sites containing pyrimidine dimers (i.e., permits error-free read-through by DNA polymerases). This factor, by binding DNA sequences containing dimers, may change the conformation of the DNA such that coding information can be regained, allowing fast and error-free bypass. A protein that binds UV-damaged DNA has been identified in human cells. It is present in XP complementation Groups A to H, but is absent in Group E (34). XP variant cells were not tested in this study. This binding factor may serve to identify damaged DNA for excision repair proteins, and its absence in XP Group E cells may explain the molecular defect in these individuals. Other proteins with binding activity for UV-damaged DNA may exist in human cells which aid in the replicative bypass of these lesions. The absence or lowered binding affinity of this putative factor may explain the XP variant phenotype.

When induced frequencies of AI were compared with the extent of inhibition of DNA replication by UV or BPDE-I (Fig. 3), similar slopes were observed for normal and XP variant fibroblasts. Thus, at equal levels of inhibition of DNA strand growth, similar frequencies of AI were observed in both cell types. This suggests that, in either normal or XP variant fibroblasts, once replication becomes blocked at a DNA lesion, there exists the same probability of producing a mutation that will lead to the AI phenotype. When compared to UV, the higher level of BPDE-I-induced AI per increment of inhibition of DNA strand growth in human fibroblasts may be related to a slower rate of repair or to a greater mutagenic potency of BPDE-I-induced DNA adducts than UV-induced photoproducts.

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REFERENCES


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