Role of Postreplication Repair in Transformation of Human Fibroblasts to Anchorage Independence¹

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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 D₀ values of 4.3 J/m² and 0.14 μm for the inhibition of DNA synthesis in active replications by UV and BPDE-I, respectively. XP variant cells (CRL1162) exhibited corresponding D₀ values of 1.5 J/m² and 0.16 μm. These 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. As predicted by the PRR studies, both cell types exhibited similar frequencies of AI colonies induced by BPDE-I. Apparent thresholds were observed for induction of AI by UV (normal fibroblasts, 2.7 J/m²; XP variant fibroblasts, 0.3 J/m²) and BPDE-I (both, 0.05 μm). Doses of UV and BPDE-I above these thresholds produced proportional increases in the inhibition of DNA replication in operating replication and in the induced frequency of anchorage-independent colonies. At doses of UV and BPDE-I that produced the same degree of inhibition of DNA strand growth, BPDE-I induced a greater number of cells capable of anchorage-independent growth than did UV in both normal and XP variant 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 XP⁴ syndrome are affected by a genetic predisposition to sunlight-induced skin cancer (4). Cells of individuals expressing the classical form of XP display the same ability as do normal cells to replicate DNA containing BPDE-I adducts (10, 11). XP variant fibroblasts are 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. Therefore, 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 (XP42T0). 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% 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 NHF1 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/mmol), 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 at 4 × 10⁵ cells per 100-mm dish and treated 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 serum-free 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 × 10⁶ 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 × 10⁴ 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 × 10⁴ cells per 60-mm dish were seeded into 3 ml of 0.33% Noble agar (Hyclone). The serum concentration was adjusted to produce about 10 to 20 background colonies per 5 × 10⁴ cells seeded. In order to achieve this frequency of background colonies when using XP variant fibroblasts, the top layer of the agar was also supplemented with epidermal growth factor (10 ng/ml), insulin (2 μg/ml), and putrescine (50 μM). In some experiments with XP variant cells, hydrocortisone (10 μg/ml) was also used in the top agar layer (20). It was found to increase the size of the colonies, but had no effect on their frequency (data not shown). An aliquot of the agar suspension was diluted with Ham’s F10 containing 15% fetal bovine serum to determine the colony-forming efficiency of these cells on plastic (10, 18, 19). Agar dishes (8 per dose of carcinogen used) were fed 1 ml of medium containing the appropriate concentration of serum and supplements on the first day after seeding and 0.5 ml every 7 days thereafter. When colonies reached a size of 50 μm 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 β-NADH (0.5 mg/ml) per 60-mm dish, followed by incubation overnight at 37°C. The blue-stained colonies were counted microscopically. The colony counts were normalized to the relative plating efficiencies 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 passaging 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 × 10⁴ cells seeded in soft agar. These cells were selected 30 to 90% reduction of cell colony formation efficiency (plastic) (10). No differences in the appearance or size of XP variant versus normal fibroblasts 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 were determined 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

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
**POSTREPLICATION REPAIR AND CELL TRANSFORMATION**

![Diagram](image)

**Fig. 1.** Effect of UV and BPDE-1 on DNA strand growth. Cells were seeded at 4 × 10⁵ cells per 100-mm plate and incubated with [³²P]thymidine for 2 days to label DNA uniformly. One to 2 h before carcinogen treatment, the medium was replaced with fresh unlabeled medium. Fibroblasts were treated with UV (A) or BPDE-1 (B) in log phase as described in "Materials and Methods." Cells were incubated after treatment in reserved medium for 30 min at 37°C and then pulse labeled with 50 μCi/ml of [³²P]thymidine for 15 min. Cells were harvested, and the size distribution of nascent DNA was analyzed in alkaline sucrose gradients. The sum of the incorporation of ³²P cpm into large intermediates of DNA replication (5 × 10⁸ to 2 × 10⁹ daltons), after normalization to the number of cells added to each gradient (³²P cpm), was expressed as the percentage of the same sum measured in control cells. In A, each point represents the average of 1 to 5 determinations at the indicated UV fluences. Different symbols represent experiments performed with different cell types: •, normal fibroblasts (NHF1, 5 experiments, 4 or 5 fluences of UV in each); O, XP variant fibroblasts (CRL1162, 2 experiments, 4 fluences of UV in each). The data for CRL1162 have been published (10), but are included here for comparison. In B, each point represents the average of 1 to 3 determinations at the indicated BPDE-1 concentrations. Different symbols represent experiments performed with different cell types: •, normal fibroblasts (NHF1, 4 experiments, 5 concentrations of BPDE-1 in each); O, XP variant fibroblasts (CRL1162, 2 experiments, 5 concentrations of BPDE-1 in each). For simplicity, only one regression line was drawn through the data points. However, Dₜ values were determined for each cell strain independently. Bars, SD.

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-1-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-1-induced growth in soft agar.

It was also observed that there was a threshold for induction of AI by UV and BPDE-1 (Fig. 2). The threshold fluences for XP variant and normal cells treated with UV were 0.3 J/m² and 2.7 J/m², respectively. Both cell types exhibited a threshold dose of about 0.05 μM BPDE-1.

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-1 were normalized to their effects on DNA strand growth, BPDE-1-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-1 have been shown to inhibit DNA synthesis by two mechanisms: down-regulation of replicon 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-1, 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-1 (30). The frequencies of UV- and BPDE-1-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 Dₜ 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-1 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
POSTREPLICATION REPAIR AND CELL TRANSFORMATION

Fig. 2. Induction of anchorage independence by UV and BPDE-I. Fibroblasts were treated with UV (A) or BPDE-I (B) as described in "Materials and Methods." Cells were suspended in agar after being cultured for approximately 5 population doublings. Colonies were enumerated after 4 to 6 wk of growth in agar. Colonies per 50,000 cells that were induced per dose of carcinogen. Closed symbols denote fibroblasts derived from normal individuals, and open symbols represent strains of XP variant fibroblasts. A: NHF1 (n = 7); O, CRL1162 (n = 6); B: , NHF1; C, CRL1162; , GM3348; , XP42T0. Bars, SD.

Fig. 3. Relationship between inhibition of DNA strand growth and induction of anchorage independence. Normal and XP variant fibroblasts were treated with UV (- - - - , NHF1: - - - - , CRL1162) or BPDE-I (-----, NHF1: -- - - - , CRL1162). Doses of carcinogen that induced AI (Fig. 2) were expressed as the observed level of DNA strand growth (relative to untreated controls) at the respective doses (Fig. 1). The lines were drawn only within the range of carcinogen doses at which AI was measured.

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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