Differential Repair of the Two Major UV-Induced Photolesions in Trichothiodystrophy Fibroblasts

Lydia Riou,1 Eric Eveno,1 Anneke van Hoffen,2 Albert A. van Zeeland,2 Alain Sarasin,1 and Leon H.F. Mullenders2

1Laboratory of Instability Genetic and Cancer, Centre National de la Recherche Scientifique, Institut Gustave Roussy, Villejuif, France and 2Medical Genetics Center, Department of Toxicogenetics, Leiden University Medical Center, Al. Leiden, The Netherlands

ABSTRACT

Defects in nucleotide excision repair have been shown to be associated with the photosensitive form of the disorder trichothiodystrophy (TTD). Most repair-deficient TTD patients are mutated in the XPD gene, a subunit of the transcription factor TFIIH. Knowledge of the kinetics and efficiency of repair of the two major UV-induced photolesions in TTD is critical to understand the role of unrepaird lesions in the process of carcinogenesis and explain the absence of enhanced skin cancer incidence in TTD patients contrarily to the xeroderma pigmentosum D patients. In this study, we used different approaches to quantify repair of UV-induced cyclobutane pyrimidine dimers (CPD) and pyrimidine (6–4) pyrimidone photoproducts (6–4PP) at the gene and the genome overall level. In cells of two TTD patients, repair of CPD and 6–4PP was reduced compared with normal human cells, but the reduction was more severe in confluent cells than in exponentially growing cells. Moreover, the impairment of repair was more drastic for CPD than 6–4PP. Most notably, exponentially growing TTD cells displayed complete repair 6–4PP over a broad dose range, albeit at a reduced rate compared with normal cells. Strand-specific analysis of CPD repair in a transcriptional active gene revealed that TTD cells were capable to perform transcription-coupled repair. Taken together, the data suggest that efficient repair of 6–4PP in dividing TTD cells in concert with transcription-coupled repair might account for the absence of increased skin carcinogenesis in TTD patients.

INTRODUCTION

Nucleotide excision repair (NER) is a versatile DNA repair pathway capable of removing a variety of structurally unrelated DNA lesions. In addition, two NER subpathways have been identified, i.e., transcription-coupled repair (TCR) and global genome repair (GGR). TCR is dependent on RNA polymerase I and II driven transcription, generally resulting in more rapid removal of lesions from the transcribed strand of active genes when compared with the nontranscribed strand or inactive DNA (1–3). GGR acts with variable efficiency on damage across the genome, including the transcribed strand of active genes.

Defects in NER affecting either TCR or GGR, or both, have been shown to be associated with three rare hereditary human disorders: (a) xeroderma pigmentosum (XP); (b) Cockayne syndrome; and (c) the photosensitive form of trichothiodystrophy (TTD). TTD is an autosomal recessive disorder characterized by sulfur-deficient brittle hair. The disease is also associated with physical and mental retardation but not with an increased incidence of skin cancer (4, 5). The clinical photosensitivity of TTD patients is usually associated at the cellular level with enhanced UV cytotoxicity and impaired NER (6–8). Photosensitive TTD patients exhibit genetic heterogeneity and fall in three groups: (a) TTD-A; (b) TTD/XP-B; and (c) TTD/XP-D. Although there is general agreement concerning the association of photosensitive TTD with defective NER, no consensus exists with respect to which types of the major UV-induced photolesions, i.e., cyclobutane pyrimidine dimers (CPD) and pyrimidine (6–4) pyrimidone photoproducts (6–4PP), are affected in their repair and which NER subpathways are involved. Previous studies (7) demonstrated that two TTD strains (TTD1RO and TTD1BI) with reduced levels of repair synthesis showed a marked reduction of 6–4PP repair but normal rates of repair of CPD. However, more recent studies by Eveno et al. (8), including the abovementioned TTD strains, revealed completely opposite results. In their study, all of the tested TTD strains within the three genetic groups thus far identified exhibited a main defect in repair of CPD, whereas repair of 6–4PP was relatively unaffected. Only in TTD cells with severe UV sensitivity was a clear reduction of 6–4PP (~70% of normal human cells) observed.

The differences in repair phenotype between the various studies are difficult to reconcile, although in the case of 6–4PP, different immunoassays and antibodies might account for the differences in results. Knowledge of the kinetics and efficiency of repair of the two major UV-induced photolesions in TTD is critical to understand the role of unrepaired lesions in the process of carcinogenesis and explain the absence of enhanced skin cancer incidence in TTD patients. Indeed, XP patients belonging to the group D carry mutations often very close to those found in TTD patients who, however, never exhibit increased levels of skin cancers.

In this study, we used biochemical approaches to quantify the frequencies of UV-induced photolesions (CPD and 6–4PP) in primary human fibroblasts, i.e., TTD (belonging to XPD category) and XP-D fibroblasts at the genome overall level or at the level of specific genes to detect differences in TCR. These studies were complemented with immunochemical analysis of photolesions in the primary diploid cells under identical conditions. Our results show that repair of both CPD and 6–4PP is impaired but that the effect is more severe in confluent cells than in exponentially growing cells and more drastic for CPD than for 6–4PP. Particularly, exponentially growing TTD cells exhibited complete repair of 6–4PP, albeit at reduced rate compared with normal cells. The efficient repair of 6–4PP in concert with TCR might account for the absence of increased skin carcinogenesis in TTD patients.

MATERIALS AND METHODS

Cell Culture, Irradiation Conditions, and Isolation of DNA. The cell strains used in this study were primary human fibroblasts: (a) VH25 and 198VI (wild-type human); (b) TTD1BI and TTD1RO (TTD; Refs. 9 and 10, respectively); (c) XP25RO (XP group A); and (d) XP1DU (XP group D).

In addition we used a SV40 transformed TTD cell strain (TTD9VI-SV) that contained either the expression plasmid p2E-ER2 with the ERCC2 c-DNA or the plasmid pcD2E lacking the 2.4-kb ERCC2 c-DNA (11). Cell strains were cultured in Ham’s F10 medium (without hyposoxanthine and thymidine) or in modified Eagle’s medium supplemented with 15% FCS and antibiotics, in a 5% CO2 atmosphere. Before irradiation, exponentially growing or confluent primary cells were rinsed with PBS, irradiated with UV (Philips T UV lamp, 254 nm) at a dose rate of 0.2 W/m2, and incubated in conditioned medium for 24 h. DNA was isolated and purified as described previously (2).
To obtain confluent cells, 3.10^6 fibroblasts were split 1:3 in 90-mm Petri dishes and grown for an additional 2 weeks with regular changes of the medium. For experiments with exponentially growing cells, confluent fibroblasts were split 1:3, and cells were UV-irradiated 36 h later when cells entered into S phase (2).

**Determination of CPD Frequencies in Specific DNA Sequences.** Confluent human fibroblasts were UV-irradiated with 10 J/m^2^ and post-UV incubated for various time periods. High molecular weight DNA was digested with appropriate restriction enzymes and purified by phenol and chloroform extractions as described previously (2). Equal amounts of DNA were either treated with appropriate restriction enzymes and purified by phenol and chloroform extraction or mock treated with the CPD-specific T4 endonuclease V and subjected to electrophoresis in 0.6% alkaline agarose gels. The DNA was transferred to Hybond N^+ membranes (Amersham) by vacuum Southern blotting (Pharmacia-LKB Vacugene 2016) and hybridized with 32P-labeled gene-specific probes. Radioactivity in full size fragments was quantified using an Instant Imager Electronic Autoradiography System (Packard). The number of CPD was calculated from the relative radioactivities of the bands in the lanes containing DNA that was either treated or mock treated with T4 endonuclease V, using the Poisson expression.

**Determination of 6–4PP Frequencies in Specific DNA Sequences.** For the determination of 6–4PP repair kinetics, human fibroblasts were UV-irradiated with 30 J/m^2^ and post-UV incubated for various times. High molecular weight DNA was restricted with EcoRI or BclI and processed for 6–4PP measurements as described previously (12). Briefly, CPDs were removed from the DNA using photolyase derived from Anacystis nidulans (kindly provided by Dr. A. Eker, Erasmus University, Rotterdam, The Netherlands) and visible light (13). After purification, the photoreactivation of CPD was checked for completeness by treatment of the DNA with T4 endonuclease V and subsequent Southern analysis. Equal amounts of photoreactivated DNA were treated with or mock treated with UvrABC endonuclease (2 pmol of each subunit per μg of DNA), purified, subjected to electrophoresis in alkaline agarose gels, and treated as described for CPD. The incision frequency calculated for each sample was corrected for nonspecific cutting of the enzyme complex by including in each experiment a DNA sample from unirradiated cells that had been processed simultaneously with the UV-irradiated samples.

**Determination of CPD Frequencies at the Genome Overall Level.** Fibroblasts prelabeled with [3H]thymidine or [14C]thymidine were irradiated with UV light (5 J/m^2^), and subsequently, cells were pooled and collected by centrifugation. Cell pellets (2.10^6 cells) were resuspended in 50 ml of RM buffer [10 mm Tris/HCl (pH 8.0), 10 mm EDTA, 1 mg/ml BSA, and 10 mm 2-mercaptop-ethanol], permeabilized by freeze/thawing, and incubated with T4 endonuclease V. Lysis of cells and alkaline sucrose gradient centrifugation was performed as described previously (14), and the frequencies of T4 endonuclease V-sensitive sites in DNA derived from irradiated and unirradiated cells were estimated by analysis of DNA profiles.

**Immuno-Slot-Blot Analysis of CPD and 6–4PP Photoproducts.** Exponentially growing or confluent fibroblasts were UV-irradiated with a dose ranging from 5 to 30 J/m^2^ at different times after irradiation, fibroblasts were stores at −20°C until processing. Briefly, genomic DNA was isolated and purified as described previously (8) and loaded onto polyvinylidene difluoride membrane. The membranes were then immersed in PBS containing 5% low-fat dried milk and 0.1% Tween 20 for 1 h and then incubated with anti-CBP (TDM-2) or anti–6–4PP (64M-2) monoclonal antibodies (diluted 1:2000 and 1:500, respectively) for 1 h at 37°C on an orbital shaker. Chemiluminescent detection was performed using the ECF Western blotting kit (Amersham). The secondary antibody was linked to FITC; the tertiary anti-FITC antibody was linked to the alkaline phosphatase enzyme. Quantification of signals was obtained after scanning the blots on a FluorImager (Molecular Dynamics) using the Image Quant software (Amersham). The percentage of repair was expressed as the residual intensity of bands at post-UV irradiation times over samples processed immediately after UV irradiation. Results are the average of two independent experiments.

**Preparation of 32P-Labeled Double or Single-Stranded Probes.** The PstI fragment Bo of the human ADA cDNA (exons 6–11) recognizing the 18.5-kb EcoRI fragment of the human ADA gene was labeled randomly as described (2). The PstI fragment Ba of the human ADA CDNA (exons 1–5) recognizing the 19.9-kb BclI fragment of the human ADA gene was used for the preparation of strand-specific probes by linear PCR (15).

**RESULTS**

**Gene-Specific Repair of CPD in TTD Cells.** The UV-induced CPD (UV dose 10 J/m^2^) repair kinetics were measured in confluent cells from TTD patients (TTD1BI, TTD1RO, and TTD9VI-SV) using techniques that have been described previously (2). Repair was measured in a 3′ located 18.5-kb EcoRI fragment of the active ADA gene in which both strands are transcribed or a 5′ located 19.9-kb BclI fragment (only the coding strand transcribed; Fig. 1A). Representative autoradiograms are shown in Fig. 1B. In all cell lines used, the initial
frequency of CPD formed in DNA by UV amounted to 0.65 CPD/10 kb/J/m², which is close to the frequency found previously in other human fibroblasts (2). In TTD strains, repair of CPD in a transcribed strand of the 5' BclI fragment amounted ~30–50% after 24 h (Fig. 1C). Comparatively, a complete lack of repair of CPD has been found in the XP-D strain XP1DU (16). In both TTD1BI and TTD1RO cells, a preferential repair on the transcribed strand was observed; the repair level of CPD in the nontranscribed strand of the ADA genes was ~20%. In the transformed TTD9VI-SV expressing the ERCC2 gene, a much more efficient repair of the ADA gene was observed resembling the repair kinetics of immortalized normal human cells (40% for TTD9VI cells versus 100% for TTD9VI-ERCC2 cells; Fig. 1D).

Repair of 6–4PP in TTD Cells. Removal of UV-induced 6–4PP from the active ADA gene was measured in confluent cells using UvrABC endonuclease treatment to introduce DNA strand breaks specifically at the sites of 6–4PP in the DNA. A high UV dose (30 J/m²) had to be applied to induce sufficient levels of 6–4PP for gene-specific analysis, i.e., approximately one 6–4PP per restriction fragment of ~20 kb. Representative autoradiograms are shown in Fig. 2A. Quantification of the intensity of full size restriction fragments of DNA samples obtained from cells that were lysed immediately after UV irradiation revealed comparable initial frequencies of 6–4PP in the ADA gene for all cell lines investigated, i.e., 0.015 6–4PP/10 kb/J/m². The two TTD cell strains (TTD1BI and TTD1RO) that were investigated displayed a rather inefficient removal of UV-induced 6–4PP from the BclI fragment of the active ADA gene without significant strand specificity (Fig. 2B). Repair levels after 24 h were ~35–50%. For comparison, XP-D cells exhibited slow removal of 6–4PP (~25%) from the EcoRI fragment of the ADA gene, in which both strands are transcribed (16). In exponentially growing TTD cells (TTD1BI or TTD1RO), the repair of 6–4PP was, however, more efficient, being ~80% after 24 h of repair (Fig. 2B and representative autoradiograms Fig. 2C).

Repair of CPD at the Genome Overall Level. To quantify repair of CPD at a dose <10 J/m², we applied alkaline sucrose gradient centrifugation in concert with T4 endonuclease V treatment to cut specifically at CPD in permeabilized cells. To optimize the estimation of repair, we used differentially labeled cells that were exposed to UV or mock treated before mixing, T4 endonuclease treatment, and centrifugation. Fig. 3 shows the sedimentation profiles of representative gradients. After 24 h, considerable repair was monitored in TTD cells exposed to 5 J/m². The level of repair in the TTD cells was 44 and 49% for TTD1BI and TTD1RO, respectively, and 68% in normal cells. In contrast, no significant removal of CPD was found in XP1DU cells exposed to 5 J/m².

Imuno-Slot-Blot Analysis of Photoproducts. We determined the kinetics of the removal of 6–4PP and CPD in exponentially growing cells from two TTD patients (TTD1RO and TTD1BI) and made a comparison with the rate of repair in the normal 198VI cells. To investigate the role of UV dosage, we used UV doses from 5 to 30 J/m². Representative slots blots are shown in Fig. 4A. In all of the cases, the 5 J/m² dose gave rise to a repair level lower than for higher doses (Fig. 4, B and C). Even in normal cells, the initial slopes for 6–4PP and CPD repair are slightly increasing with increasing UV dose. With TTD cells, we consistently found that the level of repair after 30 J/m² was always the highest. If one takes an average UV dose (~10–15 J/m²), the extent of repair for both 6–4PP and CPD lesions was found to be higher in TTD1BI than TTD1RO; however, both TTD strains displayed reduced repair when compared with normal cells. After 24 h post-UV incubation, the three cell lines exhibited complete repair of 6–4PP but only partial repair of CPD: (a) 80% (198VI); (b) 40% (TTD1RO); and (c) 60% (TTD1BI).

In contrast, confluent cells repaired more slowly than exponentially growing cells. After 24 h, considerable repair was monitored in TTD cells exposed to 5 J/m². The level of repair in the TTD cells was 44 and 49% for TTD1BI and TTD1RO, respectively, and 68% in normal cells. In contrast, no significant removal of CPD was found in XP1DU cells exposed to 5 J/m².

![Fig. 2. Removal of 6–4PP from the active gene ADA in wild-type and trichothiodystrophy (TTD) fibroblasts after UV irradiation (30 J/m²). A. autoradiograms showing the induction (0 h) and removal of 6–4PP at various times (6 and 24 h) after UV irradiation in confluent VH25 (wt) and TTD1RO (TTD) fibroblasts. B. repair kinetics of 6–4PP measured in the EcoRI restriction fragment of the ADA gene (TS, transcribed strand; NTS, nontranscribed strand) in confluent wild-type (VH25), confluent, or exponentially growing TTD (TTD1BI and TTD1RO) fibroblasts (average of three experiments). C. autoradiograms showing 6–4PP removal in confluent and exponentially growing TTD1BI after UV irradiation.](https://cancerres.aacrjournals.org)
growing cells (Fig. 5A); confluent normal cells (198 VI) repair only 50% of 6–4PP at 3 h, whereas they repair 100% of 6–4PP during that time period under exponentially growing conditions. It is the same for the two TTD cell lines because repair of 6–4PP is only 40–50% at 24 h, whereas it reaches 100% in growing cells. A similar difference in repair between the two growth conditions was observed for CPD (Fig. 5B).

We want to point out that because similar results are obtained over a broad range of J/m² and short time intervals after UV irradiation, dilution of photolesions by DNA replication cannot account for the accelerated repair of photolesions in growing TTD cells compared with confluent cells.

DISCUSSION

We have investigated repair of UV-induced DNA lesions in cells derived from a classical XP-D patient and from two TTD patients (TTD1BI and TTD1RO) belonging to the group of TTD cell strains characterized by almost normal levels of clonal survival after UV irradiation (≤10 J/m²) but significantly reduced repair replication and Unscheduled DNA Synthesis (6, 7). In the case of TTD1BI, this reduction in repair was seen in both dividing and nondividing cells. When UV survival was measured in nondondiving cells (6, 17), clear differences were observed between TTD and normal human cells; TTD1RO and TTD1BI cells appeared to be more UV sensitive than normal cells at doses >10 J/m². This result fits with the observation that in UV-irradiated nondondiving TTD1BI exposed to 15 J/m², RNA synthesis is severely repressed (6).

The gene-specific repair experiments with confluent TTD1BI and TTD1RO cells using a UV dose of 10 J/m² exhibited a clearly reduced level of CPD repair when measured over a period of 24 h; however, repair of CPD in these TTD strains is significantly better than in classical XP-D cells in which repair of CPD is virtually absent when measured at the gene level (16) or at the genome overall level by alkaline sucrose gradient centrifugation. Expression of ERCC2 in the SV40-transformed TTD9VI-SV conferred a repair level in transfectants comparable with normal human cells demonstrating that the repair defect can be complemented by ERCC2.

The residual repair in TTD appeared to be preferential in the transcribed strand of the ADA gene demonstrating that TTD cells are capable to perform TCR in contrast to XP-D cells. In mouse models, expression of TCR after UVB irradiation protects against erythema/edema in the skin (18). Although the UV-irradiated TTD fibroblasts displayed TCR, the efficiency of TCR is markedly reduced and obviously insufficient to restore UV-inhibited RNA synthesis (6), explaining the observed photosensitivity of TTD1BI and TTD1RO patients.

In addition, exponentially growing TTD cells displayed a reduced efficiency and slower rate of CPD repair compared with normal human cells as shown by alkaline sucrose gradient centrifugation and immunoblot analyses, although it is obvious that the results of both assays are quantitatively different, particularly for the TTD1BI strain. Compared with the confluent cells, repair of CPD in exponentially growing TTD cells appears to be more efficient. On the contrary, replication might account for the reduced photosensitive frequency per unit of DNA in growing cells. However, the cells used in the biochemical and immunological assays are diploid, nontransformed fibroblasts. After UV irradiation, DNA replication is severely inhibited (particularly at the high UV dose and short time intervals after UV) for ≤12 h in normal human and even more in the TTD cell lines. In the immunological approach, we used variable doses and short time intervals during which time period only a small fraction (if any) of the genome will be replicated and found consistent results: an enhanced repair

Fig. 4. Repair kinetics of 6–4PP and cyclobutane pyrimidine dimer (CPD) lesions in exponentially growing wild-type (198VI) and trichothiodystrophy (TTD; TTD1RO and TTD1BI) fibroblasts. A, examples of immuno-slot-blot experiments carried out with genomic DNA samples of irradiated (15 and 10 J/m², respectively, for 6–4PP and CPD) or CPD monoclonal antibodies. Repair kinetics of 6–4PP (B) and CPD (C) in 198VI, TTD1RO, and TTD1BI fibroblasts irradiated at 5, 10, 15, 20, or 30 J/m². Experimental points are from two independent experiments, each performed in triplicate.
over confluent cells. Most notably, repair at 30 J/m² in growing cells is much faster than in confluent cells. In addition, examination of the fraction of BudR containing replicated DNA in growing TTD cells exposed to 10 J/m² using cesium chloride density gradients revealed <10% replicated DNA consistent with previous experiments (2). Hence, DNA replication cannot account for the accelerated removal of photolesions in growing TTD cells.

Taken together, all data (generated by either the biochemical or antibody approach) suggest that CPD repair is impaired in TTD cells and that the impairment is more pronounced in confluent cells than in exponentially growing cells. Analogous to CPD repair, the repair efficiency of 6-4PP in TTD cells appeared to be dependent of the stage of the cell cycle. In confluent cells exposed to 30 J/m², repair of 6-4PP at the gene and genome overall levels (immuno-slot-blot analysis) was significantly impaired: ~40–50% removal after 24 h in both TTD1RO and TTD1BI cells, whereas normal human cells almost completely repair 6-4PP in this time period. When repair of 6-4PP was assayed in growing cells, both gene-specific repair and immuno-slot-blot analysis showed efficient repair of 6-4PP, albeit at a slightly slower rate than normal fibroblasts.

How do these results fit in with the published data on TTD1BI and TTD1RO? First, it is important to note that we used the same cell line (TTD1BI) and technique (alkaline sucrose gradient centrifugation) as in the study by Broughton et al. (7). The impaired repair of CPD in TTD1BI observed in our study contrasts with the normal repair levels of CPD in TTD1BI measured by Broughton et al. We checked that a dose of 10 J/m² induced CPD at an approximately similar frequency than in the early study by Lehmann et al. (6). Moreover, our results show that the difference in repair between normal human and TTD cells is conserved over a broad dose range. Sequence analysis of the XPD mutation showed that the cell line used in our study was indeed TTD1BI, excluding a possible intermixing of cell strains. Taken together, these findings exclude possible differences caused by cell strains or dosimetry. Our data on 6-4PP repair in TTD are, however, in agreement with the impaired removal of 6-4PP (in cells exposed to 10 J/m²) as shown previously by a radioimmunoassay (6, 7).

In conclusion, our results demonstrate that the mutations in the XPD gene in TTD1BI and TTD1RO cells affect repair of both CPD and 6-4PP but, more dramatically, the repair of CPD, most notably in TTD1RO. Moreover, the repair defect is less manifest in exponentially growing cells compared with confluent cells. The differential repair of CPD and 6-4PP in TTD may rely on the helicase activity of XPD in the NER reaction. The XPD gene product is a component of the transcription factor TFIIH essential for basal transcription initiation and NER (19). From the known sequence of steps to recruit the transcription factor TFIIH essential for basal transcription initiation,4 and, hence, that the lesion recognition (DDB) (22) and p53 (23). Preliminary experiments using local UV action is strongly stimulated by Damaged DNA-Binding protein

Second, NER activity is dependent on XPD and XPD-mediated helicase activity to open up the DNA around the damage site to allow damage-specific endonucleases to cut. Particularly, TFIIH is the most attractive candidate to position the XPD endonuclease in the incision complex (26). In TTD cells, the XPD 5’→3’ helicase activity might be impaired because of altered binding of the p44-kDa subunit of the TFIIH complex (27). It is possible that in TTD cells, helix unwinding is more dependent on lesion structure than in normal cells, i.e., the unwinding of DNA and hence repair might be facilitated by the larger helix distortion induced by the 6-4PP compared with CPD.

The efficient repair of 6-4PP in dividing TTD cells in concert with TCR might contribute to the absence of increased skin carcinogenesis in TTD patients. Epithelial cells are at the origin of most tumors, and this type of cells in TTD patients might carry out sufficiently efficient repair of 6-4PP. Moreover, it is likely that the reduced level of TCR accounts for the enhanced apoptotic response in TTD cells on UV exposure (28), a phenomena also observed in several mouse models (29). Although the enhanced apoptotic response contributes to the photosensitivity, it might contribute to reduced mutagenesis as shown in the mouse (30) and particularly to the destruction of the damaged cells in exposed skin (sunburn cells) exhibiting, therefore, an antitumoral effect.

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