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Short communication

XPA protein as a limiting factor for nucleotide excision repair and UV sensitivity in human cells

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ABSTRACT

Nucleotide excision repair (NER) acts on a variety of DNA lesions, including damage induced by many chemotherapeutic drugs. Cancer therapy with such drugs might be improved by reducing the NER capacity of tumors. It is not known, however to what extent any individual NER protein is rate-limiting for any step of the repair reaction. We studied sensitivity to UV radiation and repair of DNA damage with regard to XPA, one of the core factors in the NER incision complex. About 150,000–200,000 molecules of XPA protein are present in NER proficient human cell lines, and no XPA protein in the XP-A cell line XP12RO. Transfected XP12RO cell lines expressing 50,000 or more XPA molecules/cell showed UV resistance similar to normal cells. Suppression of XPA protein to ~10,000 molecules/cell in a Tet-regulatable system modestly but significantly increased sensitivity to UV irradiation. No removal of cyclobutane pyrimidine dimers was detected in the SV40 immortalized cell lines tested. Repair proficient WI38-VA fibroblasts and transfected XP-A cells expressing 150,000 molecules of XPA/cell removed (6–4) photoproducts from the genome with a half-life of 1 h. Cells in which XPA protein was reduced to about 10,000 molecules/cell removed (6–4) photoproducts more slowly, with a half-life of 3 h. A reduced rate of repair of (6–4) photoproducts thus results in increased cellular sensitivity towards UV irradiation. These data indicate that XPA levels must be reduced to <10% of that present in a normal cell to render XPA a limiting factor for NER and consequent cellular sensitivity. To inhibit NER, it may be more effective to interfere with XPA protein function, rather than reducing XPA protein levels.

1. Introduction

Many agents used for chemotherapy induce DNA damage that is repaired by nucleotide excision repair (NER), one of the major DNA repair pathways in cells. Reducing the efficiency of the NER pathway in tumors might, therefore, increase the efficacy of DNA damaging chemotherapeutic drugs. However, such an approach requires detailed knowledge of the rate-limiting events. NER requires the action of about 30 polypeptides, which function by the stepwise assembly of interacting proteins at a site of DNA damage [1]. It is not known to what extent any of these factors are rate-limiting for the NER reaction in cells.

The 31 kDa XPA protein is part of the core preincision complex of NER and interacts with DNA as well as with other NER protein factors including RPA, TFIIH and ERCC1

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[2]. In the absence of XPA, no stable preincision complex can form [3,4] and no NER occurs. Consequently, cells deficient in XPA protein have no capacity for NER and are hypersensitive to killing by damage caused by UV radiation and chemical mutagens [5]. As a potential target for improving chemotherapy, XPA has some attractions. Unlike most other NER proteins, XPA does not appear to have additional functions in other biochemical processes such as recombination, transcription or DNA replication. Consequently, disruption of XPA function is expected to specifically affect NER, without concomitant effects on other metabolic pathways.

Previous studies suggested that amounts of XPA protein lower than those found in normal cultured cells are sufficient to confer normal resistance to UV radiation and repair of UV-radiation-induced damage [6–9]. However, a level at which XPA becomes a rate-limiting factor for NER has not been determined. Here, we have used a regulatable system and quantitative immunoblotting to determine the amount of XPA protein that is limiting for repair and sensitivity to UV radiation in living cells.

2. Materials and methods

2.1. Cell lines and culture

WI38-VA13, an SV40 transformed normal fibroblast cell line was obtained from ATCC. SV40 transformed fibroblast cells derived from XP-A patient XP12RO (homozygous for a nonsense mutation at the codon for Arg207) and XP129 cells, a revertant cell line derived from XP12RO [10], were obtained from James Cleaver (UCSF). WI38-VA13 was maintained in DMEM medium (Mediatech) supplemented with 10% heat-inactivated fetal calf serum and 1% antibiotics (Penicillin, Streptomycin). XP12RO, XP129 and the sublines were maintained in RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine and 1% antibiotics. Cell lines were cultivated in a humidified atmosphere of 5% CO_2 in air. G418 (650 μg/ml) was added to the medium for cultivation of the transfected sublines. New cultures were initiated from frozen vials every 2 months.

2.2. Expression system for XPA

XPA cDNA was obtained by PCR from plasmid pET15b/XPA [11] containing the complete XPA cDNA, using *Pfu* DNA polymerase and primers containing terminal *Cla*I sites. The DNA was sequenced to confirm the absence of errors. The PCR product was digested with *Cla*I and ligated into the *Cla*I sites of pTet-tTA [12]. To generate stable XPA-expressing sublines 5 μg of vector pTet-tTA (XPA) were co-transfected with 5 μg of vector pTet-Neo into XP12RO cells. Stably transfected clones were isolated in medium containing 750 μg/ml G418 (Table 1). Antibodies and immunoblotting for detection of XPA and XPF proteins were as described [13].

2.3. Colony forming assays for measuring sensitivity to UV irradiation

Between 1500 and 10,000 cells (depending on the UV radiation dose) were plated in 100 mm × 10 mm Petri dishes containing 10 ml medium. Following incubation overnight cells in a thin layer of clear phosphate buffered saline were exposed to UV-C irradiation from germicidal lamps (peak emission 254 nm) and incubated in fresh medium for 10–14 days. The dose rate was 0.4–0.5 J/m^2/s, measured for each experiment with a UVX radiometer and a UVX-25 sensor (UVP, Upland, CA). Colonies were fixed and stained with a modified Giemsa solution (Fluka) for 20 min. Colonies of 50 or more cells were counted and data were expressed as the percentage of colony formation in untreated controls.

2.4. Immunocytochemistry

100,000 cells were plated per well in six-well plates and after 24 h fixed with 2% formaldehyde. After several washing steps with 0.2% Triton in PBS, cells were blocked with 5% normal goat serum (Chemicon International) for 30 min, followed by washing with 0.2% Triton, PBS. The monoclonal antibody 12F5 was raised against full-length XPA. Cells were incubated with 12F5 (1/100 in PBS) for 90 min, washed with 0.2% Triton followed by PBS and incubation with secondary antibody Cy3 goat anti-mouse (1/5000 in PBS) for 60 min. After washing 10 μl DAPI was added to the slides. Fluorescence images were

Table 1 – Number of XPA molecules per cell in parental cells and sublines

Cell line	XPA	XPA molecules/cell
WI38-VA	Wild-type genomic	157,000
HeLa	Wild-type genomic	200,000[a]
XP12RO	R207 stop genomic	
XP129	R207G/R207 stop	About 80,000
XP12RO/clone 12	Wild-type cDNA	153,000
XP12RO/clone 14	Wild-type cDNA	60,000
XP12RO/clone 15	Wild-type cDNA	50,000
Clone 15 + dox (1 μg/ml), 24 h	Wild-type cDNA	10,000
Clone 15 + dox (10 ng/ml), 7 days	Wild-type cDNA	Not detectable

[a] Ref. [15].

visualized using an Olympus AX70 microscope and captured with Spot Document software.

2.5. *Measurement of repair of CPDs and (6–4) photoproducts with lesion specific antibodies*

10^6 cells were seeded into 10 mm Petri dishes and incubated overnight. Cells were irradiated with 10 J/m^2 254 nm UV-C light and either collected immediately after irradiation or incubated in medium for various repair periods. DNA was isolated by incubation of cell pellets in 5 ml lysis buffer (10 mM Tris–HCl [pH 8.0], 0.1 M NaCl, 0.1 mM EDTA, 0.5% SDS, 200 ng/ml RNase A) for 5 h at 37 °C, followed by treatment with Proteinase K (100 ng/ml) at 50 °C overnight. After two extractions with phenol/chloroform and one with chloroform, DNA was precipitated with 1/10 volume of sodium acetate (3 M) and 2.5 volumes of ice-cold ethanol, dried and dissolved in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA. For each time point 400 ng DNA was used in triplicate for detection of UV-induced photolesions. DNA was denatured by incubation at 100 °C for 5 min and placed on ice immediately. SSC to a final concentration of 6× was added to the DNA, DNA was applied to a Protran membrane (Schleicher & Schuell) using a slot blot apparatus (Minifold, Schleicher & Schuell). Four hundred nanograms of genomic DNA from unirradiated cells were also loaded for each experiment to control for non-specific binding of the monoclonal antibodies. The DNA was fixed onto the membrane by baking for 1 h at 80 °C. After soaking in blocking buffer (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20 and 5% milk) the membrane was incubated for 1 h with monoclonal antibodies specific for either CPDs (TDM-2) or (6–4) photoproducts (64M-2) [14] at a dilution of 1/1000 for (6–4) photoproducts and 1/4000 for CPDs in blocking buffer. Peroxidase-conjugated secondary anti-mouse antibody was used at a dilution of 1/5000 in blocking buffer. Chemoluminescence (Pierce Super Signal) and chemoluminescence analysis (Bio-Rad Chemi Doc system) were used for quantification. The percentage of lesions remaining at each time point was calculated in comparison to the lesions present immediately after UV irradiation. To assess DNA synthesis during the repair period, cells were labelled with 50 nCi/ml [^{14}C]thymidine for 24 h prior to UV irradiation. Dilution factors (specific activity of DNA at time point/specific activity of DNA at 0 h) were then determined for the 8 h repair period.

3. Results

3.1. *Reducing the amount of XPA protein to 30% of normal does not affect cellular resistance to UV radiation*

To obtain cell lines with a regulatable level of XPA protein, the XPA-deficient cell line XP12RO was stably transfected with a tet-regulatable system expressing the XPA cDNA (Fig. 1a). For comparison, endogenous XPA protein was quantified in WI38-VA cells and the XP revertant cell line XP129 (Fig. 1b). The number of XPA molecules per cell in the different cell lines was determined by calibration with purified XPA protein (Fig. 1d, Table 1, and data not shown). Repair-proficient WI38-VA cells and the transfected subline XP12RO/clone 12 contained similar amounts of XPA protein (150,000–160,000 molecules/cell), comparable to the 200,000 molecules/cell previously estimated for the human HeLa cell line [15]. The cell line XP129 (heterozygous for XPA)

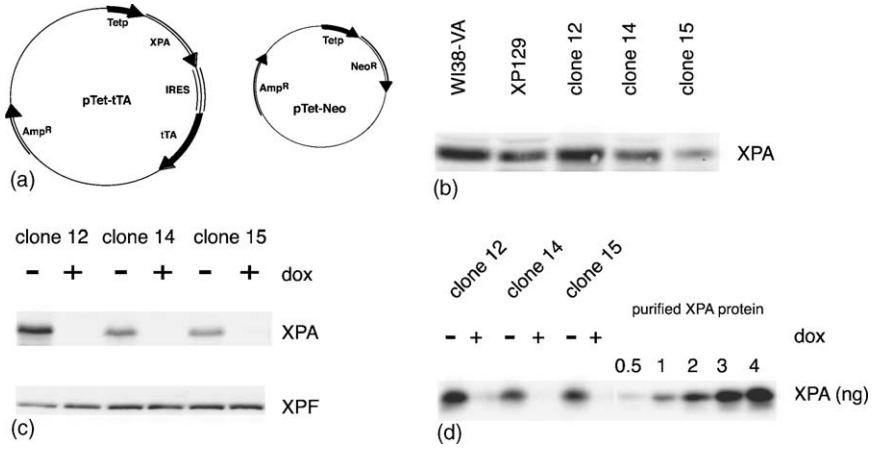

Fig. 1 – (a) The pTet-tTA expression system for expression of XPA protein. The system consists of vector pTet-tTa containing the XPA cDNA and the tetracycline transactivator (tTA), vector pTet-Neo contains the neomycin resistance gene for selection of stably transfected clones. The Tet promoter directs the expression of the XPA gene and the tetracycline transactivator. Transcription can be repressed by addition of the tetracycline analog doxycycline. Doxycycline prevents binding of the tTA to the transactivator-responsive Tet promoter and therefore abolishes transcription. (b) Immunoblot analysis of XPA protein in extracts from WI38-VA, XP129 and XP12RO derived sublines XP12RO/clones 12, 14 and 15. Fifty microgram protein extract were separated by SDS-PAGE using a 10% resolving gel. (c) XP12RO/clones 12, 14 and 15 were incubated in medium without or with doxycycline (dox) (10 ng/ml) for 9 days. Fifty microgram protein extract were separated by SDS-PAGE and immunoblotted for XPA and XPF. (d) Numbers of XPA molecules obtained by comparing band intensities of 4 × 10^5 cells with those given by dilutions of known amounts of purified XPA protein [11]. Examples are shown for XP12RO/clones 12, 14 and 15.

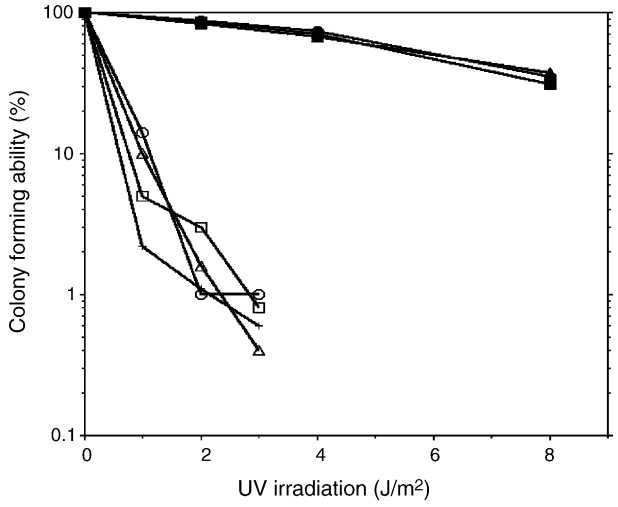

Fig. 2 – Clonogenic cell survival curves of XP12RO parental cell line and XPA-expressing sublines after exposure to UV radiation. Cells were cultivated in the absence or presence of doxycycline (dox) (10 ng/ml). Values are for XP12RO (cross), XP12RO clone 12 (squares), XP12RO/clone 14 (circles), XP12RO/clone 15 (triangles). Filled symbols represent cells grown in the absence of doxycycline, open symbols represent cells grown in doxycycline for 9 days prior to UV irradiation. The repression of XPA expression upon doxycycline treatment is irreversible, as transcription cannot take place without tTA and the tet promoter shows no intrinsic activity in a normal chromatin context.

contained about 80,000 XPA molecules/cell (Table 1). Two sublines designated XP12RO/clone 14 and XP12RO/clone15 stably expressed less XPA, 60,000 and 50,000 molecules/cell, respectively.

As measured by colony forming ability, all three sublines were similarly resistant to UV irradiation regardless of the difference in amounts of XPA molecules/cell (Fig. 2). After cultivation of the sublines for 9 days in medium containing doxycycline (10 ng/ml), no XPA protein was detectable (Fig. 1c). (The repression of XPA expression upon doxycycline treatment is irreversible as transcription cannot take place without tTA and the tet promoter shows no intrinsic activity in a normal chromatin context [16].) Doxycycline repression was specific for the XPA cDNA, as shown for example by the unchanged levels of the NER protein XPF in all of the cell lines (Fig. 1c). Under these conditions all three sublines were as sensitive to UV irradiation as the parental XP12RO cell line (Fig. 2).

3.2. *Further reduction of XPA protein level affects sensitivity to UV radiation*

As no difference in sensitivity to UV radiation was found by reducing XPA levels to ~1/3 of normal, XPA levels were further manipulated in the sublines by regulation of the tet promoter. Addition of doxycycline (1 μg/ml) decreased XPA protein levels in a time-dependent manner (Fig. 3a), with reduction to less than 50% after 12 h and to less than 10% after 24 h. Such reduction was seen for all three XPA-expressing XP12RO clones 12, 14 and 15 (Fig. 3a and b). In XP12RO/clone 15, incubation with doxycycline reduced the average level of XPA protein to about 10,000 molecules/cell (Table 1). Immunostaining of cells showed that XPA levels were reduced throughout the doxycyline-treated cell population of XP12RO/clone 15 (Fig. 3c). No specific staining was detected in parental XP12RO cells. Eighty-seven percent of XP12RO/clone 15 cells stained positively for XPA protein with an antibody specific for XPA, and after 24 h doxycycline treatment, 83% of cells stained positively, but with reduced intensity.

XP12RO/clone 15 cells expressing only 10,000 XPA molecules/cell were modestly but significantly more sensitive to UV radiation than cells containing higher amounts of XPA, as measured by colony-forming ability (Fig. 3d).

3.3. *Repair of UV-induced DNA damage with regard to amount of XPA protein*

To investigate whether repair of UV-radiation-induced DNA damage was affected by reducing XPA protein levels to 10,000 molecules/cell, experiments were performed to measure the removal of the major lesions induced by UV radiation, CPDs and (6–4) photoproducts, from genomic DNA. Cells were irradiated with 10 J/m^2 and DNA isolated directly after treatment or after 1, 3, 6 and 8 h. None of the cell lines containing XPA protein could repair CPD within 8 h (Fig. 4a and b), consistent with the fact that compromising p53 function by SV40 virus immortalization interferes with CPD repair [17]. XP12RO cells contain no XPA protein and have no capacity for NER. The apparent reduction of ~20% in total genomic content of both CPDs and (6–4) photoproducts in XP12RO after 8 h can be accounted for by equivalent DNA replication taking place during this time period as measured by [^{14}C]thymidine incorporation (data not shown). Both WI38-VA13 fibroblasts and XP12RO/clone 15 cells efficiently removed (6–4) photoproducts, with a half-life of ~1 h (Fig. 4c and d). In XP12RO/clone 15 cells expressing 10,000 molecules/cell after 24 h incubation with doxycycline, the rate of (6–4) photoproduct repair was reduced about 3-fold, with a half-life of removal of ~3–4 h (Fig. 4c and d). Cultivation of XP12RO/clone 15 for 9 days to completely eliminate XPA expression (Fig. 1c) also eliminated repair of (6–4) photoproducts (Fig. 4c and d).

4. Discussion

4.1. *XPA as a rate-limiting factor for NER and UV sensitivity*

Under what circumstances is endogenously expressed XPA protein a rate-limiting factor for NER? In human beings, 50% of the normal gene dosage appears adequate as the parents of XP-A individuals are heterozygous for XP gene expression, and do not display acute sensitivity to sunlight or have cutaneous or neurological symptoms [18]. Mice heterozygous for disruption of *Xpa* do not differ from wild-type littermates in their propensity to develop UV-radiation-induced skin cancer [19,20].

The issue of how much XPA protein is needed for effective NER in cells has occupied investigators for some time. Cleaver et al. constructed a *lac*-controlled inducible system to

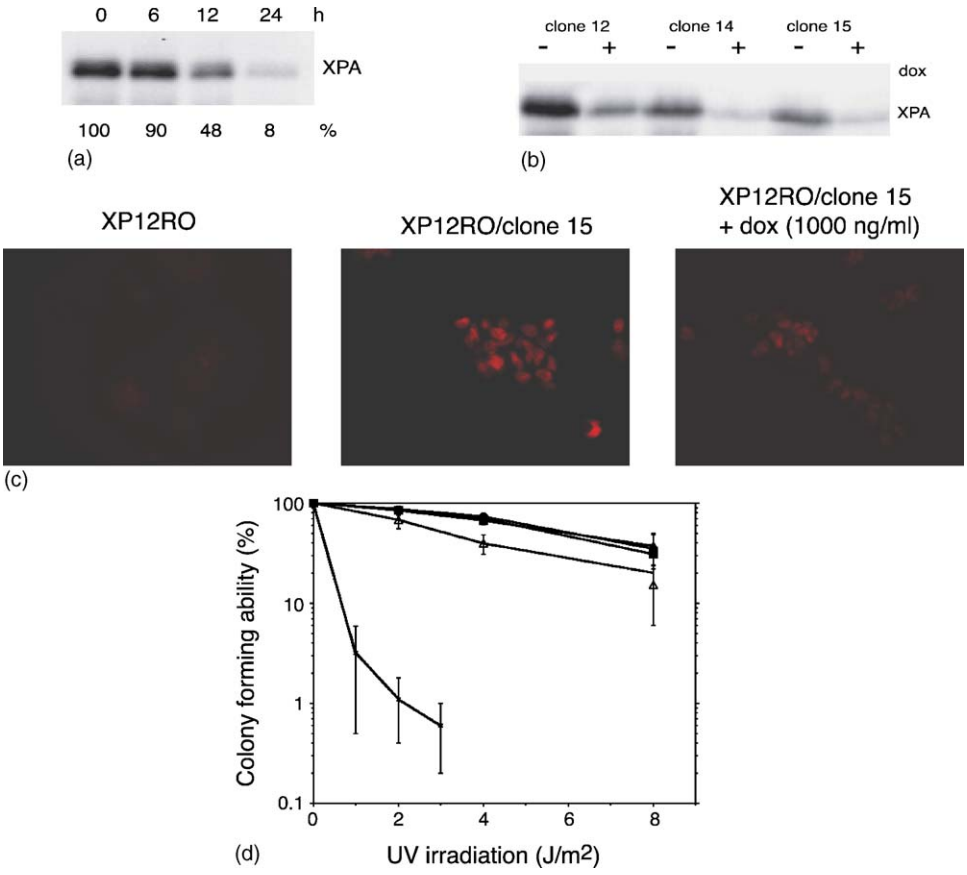

Fig. 3 – (a) Time course showing decrease in XPA protein levels in XP12RO/clone 12 following addition of doxycycline (1 μg/ml) to the medium. Cells were harvested at the indicated time points after addition of doxycycline. (b) Immunoblot analysis of XPA protein in extracts from XP12RO/clones 12, 14 and 15 cultivated in medium without or with doxycycline (dox) (1 μg/ml) for 24 h. (c) Immunostaining for XPA protein in parental XP12RO cells, XP12RO/clone 15, XP12RO/clone 15 cultivated in medium with doxycycline (dox) (1 μg/ml) for 24 h. Nuclei positive for XPA protein are stained bright red. (d) Clonogenic cell survival curves of XP12RO (cross), XP12RO/clone 12 (squares), XP12RO/clone 14 (circles), XP12RO/clone 15 (triangles), XP12RO/clone 15 cultivated in medium with doxycycline (1 μg/ml) for 24 h prior to UV irradiation (open triangles).

modulate XPA protein levels in an XPA-deficient background [6]. However, even in cells without addition of IPTG to induce XPA expression, resistance to UV radiation and repair of UV-irradiated plasmids (as measured by a host-cell reactivation assay) was comparable to normal wild-type cells. No measurements of XPA protein were made. Another inducible system for modulation of XPA expression was used by Kobayashi et al., but some expression of XPA protein was detected in uninduced cells which resulted in UV resistance similar to WI38-VA13 fibroblasts and repair of CPDs in the transcribed strand of an active gene [7]. The amount of XPA protein in uninduced and induced sublines was not quantified. Muotri et al. used a Muristerone A-inducible system, in which uninduced cells expressed about 20% of the XPA protein seen in the human cell lines HeLa and MRC5-V1 [8]. This much XPA protein was sufficient to confer normal resistance to UV irradiation and UDS at almost normal levels. In an ecdysone-inducible system, XPA protein was also present in the absence of an inducing agent, conferring substantial UV resistance and efficient repair of UV-induced damage [9].

Our quantitative studies show that 10,000 molecules of XPA are rate-limiting for repair of (6–4) photoproducts. Why is this the case? A dose of 10 J/m^2 introduces about 300,000 (6–4) photoproducts per diploid human genome, as calculated from measurements that UV-C light induces 0.6 (6–4) photoproduct per 10^8 Da per J/m^2 [21]. About half of the (6–4) photoproducts, 150,000, are removed during the first hour after radiation. Each NER event takes a total of about 4 min [22,23] and so each XPA molecule could optimally cycle through 15 events of repair in an hour, if all molecules were stable and turned over efficiently. Thus the minimal number of XPA molecules required would be 150,000/15 = 10,000. We find that this number of molecules of XPA are limiting for repair, which may suggest that the catalytic turnover of XPA is less than optimal. Further, Rademakers et al. observed that at doses of 10 J/m^2 or higher, only about 15% of the XPA in human cells appeared to be engaged in NER events [22]. The reason for this limitation is unknown, but not all XPA may be available for use in repair.

4.2. *p53 Status, NER and XP129 revertant cells*

XP129 cells were isolated as a UV-radiation-resistant revertant of XP12RO [24]. XP129 was found to efficiently repair (6–4)

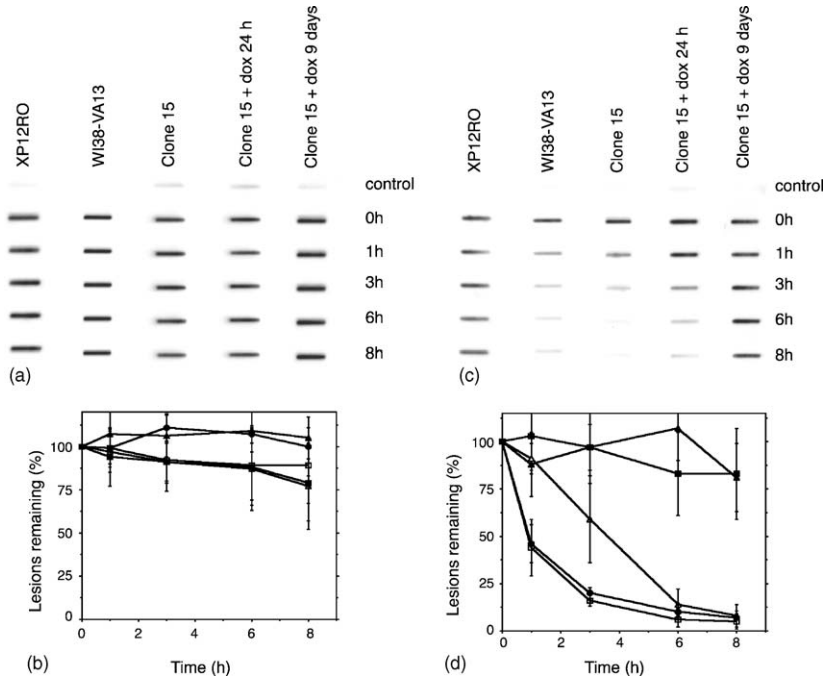

Fig. 4 – Time-course of removal of CPDs or (6–4) photoproducts from the overall genome of cell lines irradiated with UV (10 J/m^2). (a) Representative immunoslot blots of DNA from XP12RO, WI-38VA13, XP12RO/clone 15, clone 15 cultivated in medium with doxycycline (1 μg/ml) 24 h prior to UV irradiation, clone 15 cultivated in medium with doxycycline (10 ng/ml) 9 days prior to UV irradiation. Cells were UV irradiated, DNA was isolated at the time points indicated and examined for the presence of CPDs using a lesion specific antibody TDM2. (b) Percent of CPDs lesions remaining at the time points indicated. XP12RO closed squares), XP12RO/clone 15 (open squares), XP12RO/clone 15 cultivated in 1 μg/ml dox (open triangles), XP12RO/clone 15 cultivated in 10 ng/ml dox (closed triangles), WI38-VA (closed circles). Each point represents the value of at least four independent determinations. (c) Representative immunoslot blots of DNA from cell lines indicated. Cells were UV irradiated, DNA was isolated at the time points indicated and examined for the presence of (6–4) photoproducts using a lesion specific antibody 6–4M2. (d) Percent of (6–4) photoproducts remaining at the time points indicated. XP12RO (closed squares), XP12RO/clone 15 (open squares), XP12RO/clone 15 cultivated in 1 μg/ml dox (open triangles), XP12RO/clone 15 cultivated in 10 ng/ml dox (closed triangles), WI38-VA (closed circles). Each point represents the value of at least four independent determinations.

photoproducts but shows no repair of CPDs. Two explanations were considered for this ability to repair one photoproduct and not the other. A lower level of XPA protein in XP129 was noted in comparison to the level in the cell line 1BR.3N, and it was proposed that this lower level of protein might account for the deficiency in CPD repair, as CPDs are less well-recognized by the NER system than are (6–4) photoproducts. Earlier estimates of the XPA levels in XP129 estimated that these cells had about 30% of the XPA found in the 1BR.3N cell line [25]. This challenging experiment required fractionation of cell extracts through two chromatographic steps before detection with an antipeptide antibody. Using an antibody that recognizes XPA in whole cell extracts, we found here that XP129 have about 80,000 molecules of XPA per cell (∼50% of that in the repair-proficient cell line WI38-VA and ∼40% of that in HeLa cells). An alternative explanation for the poor CPD repair in XP129 cells was that reversion of a nonsense mutation in XP12RO cells resulted in replacement of the highly conserved Arg207 with a Gly at this position [10].

Neither of these previously proposed explanations for the deficiency in CPD repair in XP129 appears to be the most relevant one. Instead, the poor repair of CPD is likely due to the fact that XP129 is an SV40 transformed cell line, as are all cells used in the present investigation. The original DNA repair study with XP129 used primary fibroblasts for comparison with XP129, rather than SV40-immortalized normal cells [26]. SV40 transformation causes expression of large T antigen, which binds to and inactivates p53 as a transcription factor. A number of studies indicate that efficient removal of CPDs, but not (6–4) photoproducts from the overall genome requires wild-type p53 function [17,27]. There may be several reasons that p53 dramatically affects the ability of the global NER system to repair CPDs. XPC protein participates in DNA distortion recognition early in NER [28]. XPC protein levels or recruitment to specific photoproducts are influenced by UV irradiation in a p53-dependent manner [29,30]. Further, efficient repair of CPDs in cells is aided by the DDB1–DDB2 protein complex [31], which binds preferentially to DNA containing a CPD [32]. Expression of DDB2 is dependent on p53 in some cell lines [33], but not others [34].

A 3-fold difference in rate of removal of (6–4)PP, as found between XP12RO/clone 15 with and without 24 h doxycyline treatment (Fig. 4) has only a modest effect on the ability of cells to survive UV irradiation (Fig. 3d). Cells that repair their DNA

more slowly may nevertheless be able to eventually initiate a colony during the 10–14-day course of the experiment.

4.3. *Implications for cancer therapy*

Many agents used for cancer therapy induce DNA lesions in tumor cells, which are dealt with by NER. Down-regulation or inhibition of NER activity would therefore be expected to increase the efficiency of chemotherapy. However, this requires detailed information about the level at which the individual NER factors are rate-limiting for the repair process. Here we found that the amount of XPA protein has to be reduced to ~5% of that present in a normal cell to render this factor rate-limiting for NER and sensitivity to DNA damage. RNAi has proven to be a powerful tool to suppress gene expression [35]. Though reduction of protein levels to 5% of normal is possible, it cannot always be achieved. Using antisense RNA we have reduced XPA levels to 30% of normal in a bladder cancer cell line (unpublished data), but this had no effect on the cisplatin sensitivity of the cells. Like XPA, the NER core factors RPA, TFIIH, XPG and ERCC1-XPF are also present at about 100,000–200,000 molecules per unirradiated HeLa cell, with XPC present at about 25,000 molecules per cell [15]. Efficient reduction of XPC protein levels by RNAi methods might prove most tractable.

These considerations suggest that it might be more successful to target XPA by interfering with its function. XPA has no known catalytic activity but instead is a core structural factor that interacts with DNA and with the RPA and ERCC1 proteins [28]. As these interactions are absolutely necessary for the NER process an attractive idea may be to inhibit NER by disrupting these protein–protein interactions.

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