Repair of Psoralen-induced Cross-Links and Monoadducts in Normal and Repair-deficient Human Fibroblasts

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ABSTRACT

SV40-transformed normal, xeroderma pigmentosum (XP) and Fanconi's anemia (FA) fibroblasts have distinct repair capacities for monoadducts and DNA interstrand cross-links produced by exposure to near-UV (320-400 nm) light in the presence of 8-methoxypsoralen or angelicin. Excision repair of monoadducts occurred rapidly in normal and FA cells after exposure but not in XP cells. Cross-links were repaired in normal cells with a t½ of about 10 h but not in XP or FA cells. When the total number of adducts induced by 8-methoxypsoralen in normal cells was kept constant, the amount of repair replication decreased as the ratio of cross-links to monoadducts increased. This suggests either that cross-link repair is significantly different from monoadduct repair, involving smaller patches and a much slower rate of patching or that cross-links can inhibit monoadduct repair. Our results show that XP group A and FAH12 cell lines are deficient in cross-link repair. The data also suggest that the mechanism of cross-link repair in human cells involves several enzymes and that different ones may be deficient in XP and FA cells.

INTRODUCTION

Some human diseases are characterized by reduced survival after various types of DNA damage (1, 2). Cells from two such diseases, a hereditary skin disorder, XP, and a hereditary hematopoietic and skeletal disorder, FA, display a reduced capacity to survive UV-induced DNA damage (1, 2) and DNA interstrand cross-link damage, respectively (3-6).

XP group A cells show reduced repair after exposure to UV and various chemical agents (1, 2) but measurements of cross-link repair have been as conflicting as those with FA cells. Fujiwara et al. (9) have shown very rapid removal of cross-links in SV40-transformed XP cells, whereas two other groups have shown no repair of cross-links in diploid XP group A cells (14, 15). Studies using different FA cell lines treated with either mitomycin C or 8-MOP showed both the presence of cross-link repair (15, 16) and lack of cross-link repair (9) by FA cells. Hypersensitivity of FA cells to DNA cross-link damage has been attributed to a deficiency in DNA interstrand cross-link repair (7, 9), and may involve several complementation groups (17, 18).

There are several possible reasons for the extensive disagreement about cross-link repair deficiencies in FA and XP cells. (a) Obtaining accurate measurements of the number of cross-links is difficult. Many methods require knowledge of both a cross-linked fraction of DNA and the molecular weight of DNA, or as with alkaline elution, the titration of breaks against cross-links, which increases the potential sources for error. (b) Every exposure condition results in a mixture of cross-links and monoadducts and repair mechanisms of these two kinds of lesions may interact with one another (19). (c) Variation in repair of cross-links according to the stages of the cell cycle (8) and the passage number for primary fibroblasts (20) is possible.

We investigated some of these possibilities with a method to measure cross-links using isopyknic gradients. Our method for measurement of cross-link repair is a density-shift method that involved unidirectional substitution of DNA by BrdUrd (21-23). This method was recently compared with several others for the ability to measure cross-link repair after the treatment of hamster cells with cisplatin (23) and we found it to be the most suitable for our studies.

As DNA damaging agents, we used 8-MOP and angelicin. 8-MOP and angelicin plus near-UV radiation form covalent mono- and diadducts with pyrimidine residues in the DNA (22, 24-29). Because 8-MOP forms more cross-links in human DNA than does angelicin at the same concentration and near-UV dose, it is possible to measure repair synthesis caused by similar agents at high and low ratios of cross-links to monoadducts and observe interactions between monoadduct and cross-link repair.

MATERIALS AND METHODS

Cells. The cells used in this study were normal human (GM637) excision-deficient xeroderma pigmentosum group A (XP12RO) and Fanconi's anemia (FAH12), which is hypersensitive to DNA cross-linking agents (17). All cells were SV40 transformed. We avoided variations due to contact inhibition (8) or to different passage numbers (20) by using cell lines transformed with SV40, although changes due to transformation cannot be totally excluded. Cells were maintained at 37°C in Eagle's minimal essential medium supplemented with 15% fetal calf serum and antibiotics under an atmosphere of 5% CO2 in air.

Psoralen Derivatives. 8-Methoxypsoralen and angelicin were the gift of Dr. M. J. Ashwood-Smith. Stock solutions were prepared in 100% ethanol at a concentration of 1 mg/ml. For treatment, aliquots of the stock solution were diluted to the appropriate concentrations with phosphate-buffered saline. Stock and treatment solutions were stored in the dark at -20°C and 4°C, respectively.

Treatment. Cell cultures were treated in 100-mm Petri dishes after removal of medium and rinsing with Puck's Saline A. Irradiations with 254-nm UV were carried out as described previously (19) using a germicidal UV lamp (1.3 J/m2/s). Cells exposed to near-UV (320-380 nm) in the presence of 8-MOP or angelicin were irradiated on a rotating turntable by a filtered mercury arc lamp (25 J/m2/s, 356-nm peak; MagnaFlux Corp., Chicago, IL). All treatment manipulations were carried out at room temperature in the dark and under yellow light. Dosimetry was performed with a Yellow Springs Radiometer (Yellow Springs Instrument Co., Yellow Springs, OH).

Repair Replication. Repair synthesis was measured by isopyknic
DNA was purified from cells which were incubated in BrdUrd 1 h before exposure to psoralen and UV and then in 10^{-5} M BrdUrd, 10^{-6} M fluorodeoxyuridine, 2 mM hydroxyurea, and [³H]dThd, 10 μCi/ml (50 Ci/mmol; New England Nuclear) for 2-h intervals after treatment (19). Growing the cells in medium containing BrdUrd makes it possible to distinguish semiconservative DNA synthesis from repair synthesis. The extent of repair synthesis was determined from the amount of ³H cpm associated with a given amount of normal-density (1.700 g/cm³) DNA. The concentration of DNA was determined from A_{260} measurements.

Cross-Link Repair. For measurement of cross-linking at various times after treatment, cells prelabeled with [¹⁴C]dThd were incubated in 10^{-5} M BrdUrd (21, 22). The cells were then grown in medium containing [¹⁴C]BrdUrd, 0.005 μCi/ml (57 mCi/mmol), 10^{-5} M BrdUrd, and 10^{-8} M fluorodeoxyuridine for different times before harvesting. Cellular DNA was isolated (31) and banded on alkaline CsCl-Cs₂SO₄ gradients. Substitution of BrdUrd for dThd in one of the DNA strands makes that strand of DNA of higher density than the opposite strand. Separation of the DNA strands on alkaline isopyknic gradients makes it possible to visualize and quantify cross-linked strands of DNA that are of intermediate density (21, 34). The extent of cross-linking was determined from the radioactivity profiles generated by the banded DNA (22) by calculating the proportion of total DNA that disappeared from the BrdUrd-substituted DNA band when compared to uncross-linked control DNA. Neutral sucrose gradient velocity sedimentation was used to measure the number average molecular weight (M₅₀) of the DNA that was banded in the alkaline isopyknic gradients (22). This measurement of M₅₀ permitted the determination of cross-link frequency per dalton of DNA. Alkaline sucrose gradients were not used because of artifacts due to the joining of denatured strands of DNA by cross-links. Gradients were calibrated with ³H-labeled SV40 as an internal standard.

RESULTS

Repair Synthesis. DNA from cells exposed to UV or to psoralens plus near-UV and labeled with [³H]dThd plus BrdUrd gave two peaks in alkaline CsCl-Cs₂SO₄ isopyknic gradients representing semiconservative and repair replication (Chart 1). The normal-density peak fractions in these gradients which represent repair replication were pooled and rebanded (Chart 1). After exposure to UV (254 nm), 8-MOP plus near-UV, or angelicin plus near-UV, FAH12 cells showed approximately the same amount of repair synthesis as did normal cells (Chart 2). The similarity in repair synthesis between normal and FA cells has not been shown previously and indicates that the polymerization step of excision repair in FA cells is normal. In contrast to normal and FA cells, XP group A cells show no repair synthesis after treatment with either 8-MOP or angelicin (Chart 2). These observations indicate a similarity between the repair of pyrimidine dimers and that of photoinduced 8-MOP or angelicin damage (15, 19, 35). The psoralen-stimulated repair synthesis of normal and FA cells reached a plateau that was between 15 and 21% of that for a saturation dose of UV (26 J/m²). The lower saturation level of repair replication of psoralen damage relative to UV damage...
CROSS-LINK VERSUS MONOADDUCT REPAIR

Chart 2. Repair replication in human cells measured during a 2-h interval immediately after exposure to 8-MOP or angelicin. GM637, XP12ro, and FAH12 cells were exposed to 7500 J/m² near-UV and 8-MOP or angelicin (ANG). Repair replication is expressed relative to the amount of repair synthesis induced in GM637 cells by 26 J/m² UV (254 nm) alone. Points, average of two or three experiments; bars, SE.

was observed previously when normal human cells were treated with similar 8-MOP concentrations and near-UV light doses (15).

Kinetics of Repair Synthesis. Repair replication which was determined for 2-h intervals after 8-MOP or angelicin treatment declined within 4 h (Chart 3). There was a greater decrease in the rate of repair synthesis after 8-MOP than after angelicin treatment in both normal and FA cells and a greater decrease after each treatment in FA cells than in normal cells. In parallel studies using 3H-labeled psoralens, we likewise observed that the majority (75%) of excision occurred within 4 to 6 h after exposure (36).

Repair as a Function of Near-UV Dose. Analysis of repair replication in normal cells after exposure to near-UV at an 8-MOP or angelicin concentration of 25 µg/ml showed maximum repair at approximately 15,000 J/m² (Chart 4). At higher near-UV doses, there was a sudden decrease in the amount of repair replication in 8-MOP-treated cells. In a split-dose protocol, cells were first irradiated in the presence of 8-MOP and then phosphate-buffered saline without 8-MOP to enhance cross-linking without changing the absolute number of lesions in DNA (22, 37). A second irradiation with 30,000 J/m² showed a decline in repair replication to 10% of that with exposure to 7500 J/m² alone (Chart 4). These observations suggest that conversion of monoadducts to cross-links causes a reduction in repair replication.

Cross-Link Removal. XP and FA cells showed very little cross-link removal when compared to normal cells (Table 1). In normal cells, cross-link removal had a t₁/₂ of about 10 h and was slower than that for repair synthesis (Chart 3) or excision of angelicin adducts (36). A slower rate of cross-link than monoadduct repair has been observed previously in normal cells (15, 38).

DISCUSSION

In this study we have sought to elucidate some aspects of cross-link and monoadduct repair in transformed normal, XP group A, and FA cells. XP group A cells were clearly deficient in both 8-MOP monoadduct and cross-link repair (15, 35). FA cells were proficient in the repair of monoadducts caused by DNA cross-linking agents, as has been shown previously for mitomycin C and 8-MOP (9, 15) but were defective in the repair of cross-links (9).

Repair replication measurements after UV damage showed similar levels in FA and normal cells. FA and normal cells were also similar in repair replication of DNA lesions caused by angelicin or 8-MOP indicating no major abnormality in FA cells by this measurement of DNA repair. The plateau in repair replication as a function of drug concentration indicates a saturation of the enzymatic mechanism involved in excision repair of angelicin and 8-MOP lesions. Similar saturation in DNA repair has been observed for pyrimidine dimer damage but at higher levels of saturation (19).

Repair replication measurements reflect the sum of both monoadducts and cross-link repair (15, 35, 38). Our findings show that an increase in the proportion of total adducts which are cross-links by reirradiating cells which contain monoadducts in the absence of any unbound psoralen results in decreased repair.
CROSS-LINK VERSUS MONOADDUCT REPAIR

Cross-link repair in human fibroblasts

<table>
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<th>Cell type</th>
<th>0</th>
<th>1-5</th>
<th>6-10</th>
<th>11-15</th>
<th>21-25</th>
</tr>
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<td>GM637</td>
<td>9.2 ± 0.84*</td>
<td>8.8 ± 0.69</td>
<td>7.3 ± 1.03</td>
<td>3.0 ± 0.60</td>
<td>0.9 ± 0.74</td>
</tr>
<tr>
<td>XP12RO</td>
<td>8.5 ± 0.92</td>
<td>7.8 ± 0.58</td>
<td>9.2 ± 0.77</td>
<td>7.1 ± 0.85</td>
<td>7.3 ± 1.28</td>
</tr>
<tr>
<td>FAH12</td>
<td>10.1 ± 1.09</td>
<td>8.4 ± 0.72</td>
<td>8.3 ± 0.93</td>
<td>10.6 ± 1.12</td>
<td>8.0 ± 0.71</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Concomitant exposure of cells to UV and 8-MOP plus near-UV has indicated that repair of monoadducts is inhibited by cross-links (19). In this previous study, we also showed that inhibition of UV-induced repair replication by 8-MOP and near-UV was reduced from that observed from near-UV alone. The decrease in repair synthesis might be due simply to the conversion of lesions that require large amounts of repair replication to ones that require less. However, the data indicate that the repair system is saturated, so conversion of monoadducts to cross-links would have to eliminate the excess monoadducts before a decrease in repair replication is detected. Maximum conversion of monoadducts to cross-links would result in ≤44% cross-links in the total population of photolatd. At less than 50% cross-links in the total photolatd present at 8-MOP, 25 µg/ml, repair synthesis is still detectable. Therefore the reduction of repair replication by enhanced cross-linking is more complex than elimination of monoadducts from the total population of lesions.

Our results also indicate a decline in the rate of repair synthesis which is slightly more rapid in 8-MOP-treated cells than in those treated with angelicin. In addition, repair synthesis persists for a longer time in normal cells than in FA cells. Both these observations can be explained by the presence of residual cross-links which interfere with excision repair of the monoadducts. Although normal cells repair cross-links, the greater rate of monoadduct repair would result in a greater cross-link to monoadduct ratio in 8-MOP-treated cells. These cross-links could inhibit the repair of monoadducts. A similar argument could be presented for FA cells which remove cross-links less efficiently. Experiments involving radioactive cross-linking agents would be useful in further elucidating the phenomenon.

It is possible to measure 1-2 cross-links/10^6 daltons by the density-shift technique we used (22, 34). This highly sensitive technique showed that cross-links were removed in GM637 cells with a t_1/2 of about 10 h, which is slower than the 2 h for the removal of mitomycin C cross-links reported by Fujiwara et al. (9). This may indicate a different mechanism for the repair of pyrimidine-pyrimidine cross-links than for purine-purine cross-links.

The deficiency in 8-MOP cross-link repair we observed in XP12RO cells is consistent with previous reports (14, 15) but does not support the findings obtained with mitomycin C (8, 9). This discrepancy may be due to the differences in cross-linking agents because mitomycin C forms cross-links involving purine residues (39). These cross-links appear to be unstable at high pH (40) and methods that depend on alkaline denaturation for detection of mitomycin C cross-links may be unreliable.

To avoid complications in assessing cross-link repair caused by cell cycle and contact inhibition (8, 20, 41), we used SV40-transformed cells. Our findings show efficient repair of cross-links within 24 h and observed no cross-link repair in exponen-

J. E. Hearst, personal communication.
tially growing XP12RO cells, again contrasting with the results of Fujiwara (8) and Fujiwara et al. (9) with both transformed and untransformed cells. The lack of detectable cross-link repair we noted in FAH12 cells, however, does confirm reports by these investigators (8, 9). The general trend of the data suggests much slower removal of cross-links by both XP12RO and FAH12 cells than by GM637 cells. This point needs to be clarified by longer posttreatment incubation times and requires further investigation on other transformed and untransformed FA cells before we can generalize our results to the disease as a whole.

Monoadducts and interstrand cross-links appear to be repaired by distinct noncooperative processes. In general, cross-link repair appears to have elements in common with the removal of monoadducts and pyrimidine dimers as well as elements unique to repair of cross-links. There are also possible differences between the repair of pyrimidine-pyrimidine and purine-purine cross-links (8, 9). It has been proposed that the repair deficiency in XP group A cells for UV damage involves the initial endonuclease cleavage near the pyrimidine dimer (1, 3, 4, 6, 42). An early step in cross-link removal could then involve a similar endonuclease cleavage.

Cole (43) has proposed a model for cross-link repair in Escherichia coli that involves two endonuclease cleavages on the same strand on both sides of the cross-link followed by homologous recombination between homologous duplexes and eventual excision of the resulting monoadduct. Recent studies in yeast (44, 45) and in human cells (8) gave a correlation between DNA synthesis and the completion of cross-link repair. The reason for the correlation is not known but a role for recombination and DNA replication in cross-link repair is conceivable.

The lack of cross-link repair in both FAH12 and XP12RO cells indicates that endonuclease cleavage is not the only requirement for cross-link removal. A mechanism which requires glycosylase cleavage in addition to endonuclease incision for cross-link removal is consistent with our findings (46).

Similarly it has been observed recently in E. coli that although UV repair genes are involved in cross-link repair the purified gene product for pyrimidine dimer excision alone cannot remove DNA-DNA cross-links. Cross-link repair therefore is probably one of the more complex repair processes and will require extensive investigation for the future.

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34. Roberts, J. J., and Friedlos, F. Quantitative aspects of the formation and loss of DNA interstrand crosslinks in Chinese hamster cells following treatment with cis-diamminechloroplatinum(ii) (cisp-)


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