Overlapping Pathways for Repair of Damage from Ultraviolet Light and Chemical Carcinogens in Human Fibroblasts

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ABSTRACT

DNA excision repair was measured in cultured human fibroblasts after single or dual treatments with ultraviolet radiation, 4-nitroquinoline 1-oxide, or N-acetoxy-2-acetylaminofluorene. Three approaches were used to monitor repair: unscheduled DNA synthesis, measured by autoradiography; repair replication, measured by the incorporation of a density-labeled DNA precursor into repaired regions; and excision of ultraviolet endonuclease-sensitive sites. When a single repair-saturating dose of one of the three carcinogens was administered, little stimulation of unscheduled DNA synthesis or repair replication could be observed by additional treatment with one of the other carcinogens. In no instance was total additivity of repair observed. These observations were confirmed by showing that the excision of endonuclease-sensitive sites produced by ultraviolet damage (i.e., pyrimidine dimers) was inhibited by exposure to 4-nitroquinoline 1-oxide and N-acetoxy-2-acetylaminofluorene. The data indicate that the repair of lesions induced by these substances may have common rate-limiting steps, a conclusion previously indicated by the repair deficiency in xeroderma pigmentosum cells in which a single mutation eliminates the repair of damage caused by each of these agents.

INTRODUCTION

The treatment of human fibroblasts with mutagens or carcinogens and the subsequent monitoring of DNA damage and its repair have been the subject of considerable research (7, 18). These studies generally involve exposing cells to a single substance and examining the fate of any DNA lesions induced. The environment, however, often contains several carcinogens, and an organism may be exposed to more than one agent simultaneously. How these agents may interact to influence DNA repair is important, especially in attempts to estimate permissible environmental levels of carcinogens.

In human fibroblasts, 2 broad classifications of DNA damage and its excision repair have been made. One kind of damage is repaired relatively quickly by the insertion of a small number of bases into the repair patch (19); the other requires a longer time for repair, and the patch size involved is considerably larger (19). The first process operates in cells from patients

with XP (19), whereas the "long-patch repair" is defective in most of these cells (5, 6, 19). Attempts have been made to classify DNA-damaging carcinogens by their long or short repair processes (6, 19). For instance, the damage induced by UV light and AAAF is repaired in a long-patch manner, and its repair is defective in XP cells (22), whereas the damage induced by 4NQO is repaired in both long- and short-patch fashion (13, 19); only the long-patch repair is defective in XP cells (6, 19).

The regulatory factors that regulate the maximum amount of repair that normal cells can perform after damage from UV light and chemical carcinogens may be the same factors that are affected by the repair defect(s) in XP cells. Exposure of normal cells to several agents simultaneously should therefore result in less than additive amounts of repair because of overlapping of the repair pathways for these agents. This has been observed in human cells exposed to a combined dose of UV and aflatoxin B (21) and in V79 hamster cells exposed to UV light and AAAF (2). Other investigators, however, have reported apparent additivity of repair in human cells exposed to UV and AAAF (1). Because of the varying results obtained in these investigations, we repeated the experiments for 3 carcinogens (UV, AAAF, and 4NQO) that form irreparable damage in XP cells (19), using each of the 3 possible pairs to measure UDS and repair replication. We also measured the excision of UV endonuclease-sensitive sites in cells exposed to UV and 4NQO. We concluded that saturation of repair by a high dose of one agent restricts the induction of more repair by a dose of another agent.

MATERIALS AND METHODS

UDS. Normal, nonembryonic human skin fibroblasts (designated E-11) were plated at 10⁵/35-mm Linbro well containing a glass coverslip and incubated in Eagle’s minimal essential medium plus 15% fetal calf serum for 2 days. Cells were prelabeled for 15 min with 10 µCi [³H]dThd per ml (specific activity, 60 Ci/mmol) to label S-phase cells heavily and to ensure their identification in autoradiographs. The cells were then irradiated without medium at 254 nm UV at an incident dose rate of 1.3 J/sq m/sec. Then AAAF (dissolved in dimethyl sulfoxide; a gift of A. J. Miller) or 4NQO (dissolved in ethanol; a gift of J. Epler) was added after dilution into larger (at least 100×) volumes of Eagle’s minimal essential medium without serum. Dilution in medium with serum was considered inadvisable because of the possibility of reactions with serum proteins.

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that would reduce the effective concentration reaching DNA. This is particularly important with AAAF, which is extremely reactive and does not require activation (1) and may contribute to differences between the absolute amounts of repair observed in our experiments and those of others in which serum was present during AAAF exposure (1). The medium was then removed, and the cells were washed once with 0.9% NaCl solution (10) and reincubated for 1 hr in medium containing 10 μCi [3H]dThd per ml. The cells were then incubated for an additional hr with fresh medium containing 10−5 M unlabeled dThd. After this medium was removed, the cells were fixed to the coverslips with 25% acetic acid in ethanol. The coverslips were removed from the wells and glued to the microscope slides, which were then dipped in photographic emulsion (NTB-2) and allowed to stand for 4 to 6 days. After developing, the slides were stained with Giemsa, coverslips were placed over them, and grains from 40 randomly selected nuclei were counted. Hydroxyurea was not required in these experiments because the S-phase cells (20 to 40% of the population) were black with grains because of the 15-min prelabel, and the number of cells entering S during the 1-hr labeling period after high UV and chemical doses would be negligible. S-phase cells could therefore be recognized and ignored in the grain counting, which was confined to G1 and G2 cells undergoing UDS. The average number of grains in untreated cells was 0.6 to 1.7, and in cells exposed to 20 J/sq m or more of UV radiation, it was 30 to 75. In each experiment, the value of UDS at 20 J/sq m UV was normalized to 100% to facilitate comparisons between experiments done on different occasions.

Repair Replication via Density Labeling. Normal, nonembryonic human skin fibroblasts (designated HSBP) (11), pre-labeled with 0.5 Ci 32P, per ml, were plated in 100-mm dishes at 2 × 105/dish and incubated in Dulbecco’s modified Eagle’s minimal essential medium plus 10% fetal calf serum at 37° in a 5% CO2 atmosphere. Forty hr later, BrdUrd (3 μg/ml) and FdUrd (0.25 μg/ml) were added for 1 hr. Dishes were then rinsed, and mutagen treatment was carried out in the presence of BrdUrd and FdUrd. Cells were irradiated with UV light after being rinsed with warm phosphate-buffered saline (10). AAAF and 4NQO were administered in medium without serum and 4NQO was dissolved in dimethyl sulfoxide (final concentration of solvent was 1% or less in media containing serum) was added to medium on the dishes for 1 hr. The media were then replaced, and, at times up to 24 hr, samples were taken by rinsing the plates once with phosphate-buffered saline and storing them at −80° for at least 30 min. Cells were then lysed. DNA was extracted, incubated with UV endonuclease (4), and centrifuged in alkaline sucrose gradients at 40,000 rpm for 80 min (16). Molecular weight of unirradiated DNA was 65 to 75 × 106.

DNA samples were analyzed on alkaline sucrose gradients to determine the number of single-strand breaks, i.e., the number of UV endonuclease-sensitive sites introduced by irradiation, as described previously (16, 26). Investigators at 2 of the laboratories collaborating in this study (Oak Ridge National Laboratory and Medische Biologische Laboratorium), who used micrococcal UV endonuclease preparations made separately, estimated the number of UV endonuclease-sensitive sites introduced into DNA to be 2.5 sites/107 daltons at 10 J UV per sq m. Investigators at the Laboratory of Radiobiology obtained values of 1.4 sites/i 0 daltons at 10 J/sq m using T-4 UV endonuclease V (a gift of E. C. Friedberg) (28) and of 2.0 sites/107 daltons at 10 J/sq m using thin-layer chromatography (9). These values indicate that the dosimetry in this investigation was consistent from one laboratory to another, and the incident dose and dose rate at each were used without correction. DNA isolated from cells exposed to 4NQO or AAAF alone was insensitive to attack by UV endonuclease under the above conditions.

RESULTS

Low doses of UV radiation are known to induce pyrimidine dimers linearly with dose (8). However, some chemical carcinogens, such as 4NQO, require activation before causing DNA damage (14, 25) (AAAF is already in activated form) and undergo many competing and branching reactions with media and cellular components; a linear relationship between exposure dose and damage to DNA cannot be assumed. We therefore determined the amount of [3H]4NQO that bound to DNA at various drug doses, using the repair-deficient cell line XP12RO (Group A, with less than 5% of normal amounts of UDS and repair replication of UV and 4NQO damage) (6) to minimize the effect of excision of 4NQO-DNA adducts during the exposure time. In subsequent experiments, the amount of 3H bound was a linear function of dose, which also implies linearity for the amount of 4NQO-induced DNA damage (Chart 1).
In experiments combining UV and 4NQO, the amount of UDS (Chart 2) and of repair replication (Chart 3) approached saturation at 20 J UV per sq m (24). However, a higher dose of UV (30 J/sq m) was used to ensure saturation to determine whether additional doses of 4NQO could increase repair further. Some additivity of UDS (Chart 2) was apparent during a 1-hr labeling period after saturating UV doses plus low (1 μM) 4NQO doses, but the amount of UDS did not approach the sum of 2 separate treatments. After higher 4NQO doses (2 μM and above), some additivity in UDS and repair replication was observed, but only when the UV dose was not saturating (i.e., 5 or 10 J/sq m).

In experiments combining UV and AAAF, 5 or 20 μM AAAF alone resulted in amounts of UDS that were the same, indicating that these doses of AAAF saturate the amount of UDS resulting from the drug. Exposure to UV plus AAAF increased the UV saturation level by only 10 to 20% (Chart 4). Repair replication measured during the 3 hr after treatment with UV and 2 or 10 μM AAAF also resulted in small increases, but much less than that required for complete additivity (Chart 5).

In experiments combining 4NQO and AAAF, the amount of repair replication from 4NQO alone saturated at 4 μM. Doses of AAAF that resulted in relatively large amounts of repair replication when administered alone did not result in an increase when combined with saturating doses of 4NQO (Chart 6).

These observations indicate that, when high doses of 2 agents are used, the amount of repair observed is less than the saturation level of each agent alone. We therefore determined whether similar interference in repair could be detected in measurements of an earlier step in excision repair, the removal of damaged sites. After exposure to a low UV dose (3 J/sq m), about one-half of the UV endonuclease-sensitive sites were removed in 8 to 10 hr. Exposure to 4NQO inhibited this removal (Chart 7). A dose of 3 μM 4NQO, which is below saturation, had a small effect detectable 15 to 24 hr after irradiation. A dose of 10 μM, which saturates repair for 4NQO alone, completely prevented excision of UV endonuclease-sensitive sites. Similarly, when nonsaturating doses of UV plus AAAF were used, no inhibition of the excision of UV endonuclease-sensitive sites could be detected but, at higher AAAF doses (10 μM), excision was inhibited distinctly (Table 1). It
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should be noted that, whereas UDS and repair replication measure the incorporation of new bases into DNA and have greatest resolution of small differences at early times after exposure, excision measures the loss of a fraction of damaged sites, and its resolution is greater at long times after irradiation. For this reason, a small inhibition of excision after 3 J/sq m and 3 μM 4NQO was detectable at long times after exposure by UV endonuclease techniques but not by UDS or repair replication.

DISCUSSION

In this study, we measured excision repair in DNA of normal human fibroblasts treated singly or with combinations of carcinogens, using UDS as measured by autoradiography, repair replication as measured by isopyknic gradient centrifugation, and excision of UV endonuclease-sensitive sites. Every combination that we investigated with UV, 4NQO, and AAAF gave similar results. The amount of repair observed after combined high (saturating) doses of 2 agents was much less than the sum of the repairs that resulted from each agent separately. These results indicate that there is considerable overlap in the mechanisms that regulate the total amount of repair performed for each of these agents; this was expected because the repair deficiency in XP cells reduces their capacity to repair damage from each of these agents coordinately (6, 19).

After combined treatments of UV and 4NQO, a small increase of repair by UDS was observed during 1 hr after treatment at high UV and low 4NQO doses, but this increase diminished at higher doses of 4NQO. Similar treatments analyzed by repair replication for 3 hr after exposure indicated that some additivity of UV and 4NQO repair occurred at nonsaturating UV doses, but this additivity diminished after higher, saturating UV doses. Regardless of the techniques used, total additivity of the repair seen after single, saturating UV and 4NQO doses was not seen. For example, the UV dose of 20 J/sq m resulted in 100% repair by UDS and 1 μM 4NQO resulted in 80% of this, but only 12% repair was observed after a combined treatment. This was the highest amount of additivity observed. When higher 4NQO doses were used after 20 J/sq m, 120% repair was obtained as measured by both UDS and repair replication. After 20 J UV per sq m and a low 4NQO dose, there was a discrepancy of about 25% between UDS at 1 hr and repair replication at 3 hr. This may have been due to the different lengths of assay times and the types of lesions involved. Two classes of 4NQO damage have been identified: (a) 4NQO-purine adducts that are stable during acid hydrolysis but are not repaired in excision-deficient bacteria or XP fibroblasts (12, 13); (b) a 4NQO-guanine adduct that is unstable during acid hydrolysis and is repaired in excision-defective bacteria (12) and XP fibroblasts (13). These classes are induced in a ratio of about 4/1 (acid stable/acid unstable) (12). The acid-unstable lesion is repaired in XP and normal fibroblasts by a short-patch, quick-repair mechanism (13, 19), whereas the acid-stable product is repaired by a long-patch process, which takes a longer time to remove the lesions (13, 19). Hence, at 1 hr, the contribution of the short-patch, quick-repair mechanism to the total repair synthesis would be greater than at 3 hr. This may account for the small difference between the UDS (1 hr) and repair replication (3 hr) estimates.
After combined treatments of UV and AAAF, the amount of repair was again less than the sum of that resulting from the action of the 2 agents independently. Single treatments with AAAF gave about 40 to 50% of the repair seen by UDS and repair replication after 20 J/sq m. Treatments with both UV and AAAF gave only 115% of the amount of repair seen by UV alone.

In experiments using UV endonuclease assays to detect the excision of UV-induced damage, the results were consistent with those obtained with UDS and repair replication. High doses of 4NQO (Chart 7) or AAAF (Table 1) interfered with excision of endonuclease-sensitive sites.

After combined treatments of 4NQO and AAAF, the clearest example of nonadditivity was observed (Chart 6). At saturating doses of 4NQO, addition of various doses of AAAF resulted in no increase in repair replication, indicating that the repair of damage from these agents completely overlapped and that saturation for one resulted in saturation for the other. This is not due merely to saturation of available sites for DNA damage, because the maximum repair after 4NQO alone occurred at 4 μM and above (Chart 6); but chemical binding to DNA was a linear function of dose to at least 200 μM (Chart 1).

Our results indicate that, at saturating doses of UV, additional exposure to 4NQO or AAAF results in limited increases in the amount of repair. This indicates that most of the rate-limiting elements in the regulation of excision repair of damage from these disparate agents are shared, even though UV predominantly damages pyrimidines, and these chemical carcinogens damage purines. When combined doses of 4NQO and AAAF were used, the rate-limiting elements appeared to be completely shared, because no additivity was observed. Our results are consistent with predictions made from the common classification of these agents with respect to the irreparability of their damage in XP cells (6, 19). Our results are also consistent with observations of human cells exposed to UV and aflatoxin (21) and of V79 cells exposed to UV and AAAF (2), in which repair after combined exposure was less than additive and in some cases mutually inhibitory. Our data from the UV endonuclease assay are also consistent with those obtained by the same method in human cells exposed to UV and AAAF (1), in which a dose of 20 μM AAAF inhibited the excision of UV endonuclease-sensitive sites to a small extent over a 24-hr period, although the effect was not measurable at 6 hr. We observed an inhibitory effect at 10 μM AAAF, which was lower than that in Ahmed and Setlow’s experiments (1), because (see “Materials and Methods”) our chemical carcinogen exposures were done in the absence of serum proteins which can interact with these reactive chemicals and reduce the effective dose to cellular DNA. Mutually inhibitory effects between combined doses of carcinogens may, in fact, be a general phenomenon, because inhibition of excision of methyl nitrosourea-induced O6-methylguanine by exposure to dimethylnitrosamine has been observed in vivo (17).

The one exceptional result among various studies with combined doses of UV and chemical carcinogens (Refs. 2 and 21 and the present report) is the study by Ahmed and Setlow (1) reporting complete additivity between apparently saturating doses of UV and AAAF. A companion paper by Amacher et al. (3), which also reported different rate-limiting steps for the excision of pyrimidine dimers and AAAF lesions, cannot be taken as supportive evidence because the excision kinetics for pyrimidine dimers in that report (3) is anomalous. These investigators found that dimer excision, as measured chromatographically, was completed within 1 hr of irradiation; all other reports, including that of Ahmed and Setlow (1), indicate that excision occurs more slowly and progressively over much longer time periods and that little excision is detectable during the first 4–5 hr after irradiation (11, 15, 16, 20, 23, 27). In view of this disparity, we feel we must suspend judgment on the significance of the observations of Amacher et al. (3) and should concentrate on possible explanations for the different conclusions reached by Ahmed and Setlow (1).

In our observations (Charts 2 to 5), saturation of UDS and repair replication was just attained at 20 J/sq m, but the convergence of the UV-plus-carcinogen exposure curves was difficult to interpret conclusively without proceeding to higher UV doses to be certain that saturation had been reached. When we used higher doses of 30 J/sq m, we were able to ascertain that the amounts of UDS and repair replication were less than additive when chemical and UV damages were combined. Consideration of the corresponding curves in the report of Ahmed and Setlow (1) shows that they recorded UDS at only 5, 10, and 20 J/sq m and that UDS was still increasing at the highest dose. If we had not extended our own observations to higher doses, we would have had difficulty in deciding whether there was complete or only partial additivity, because the repair system for UV alone was not saturated. We therefore believe that our results are consistent with theirs but that their experiments were not performed at a sufficiently high UV dose to validate their conclusions. Further evidence supporting our interpretation is that their dosimetry with UV endonuclease from Micrococcus luteus indicated a yield of 1.8 sites/105 daltons at 10 J/sq m in contrast to our 2.5 sites, so that their highest dose of 20 J/sq m corresponds to our dose of 14 J/sq m; however, subtle differences in endonuclease assays make this argument less substantial than the one discussed above. Other interpretations of the differences between Ahmed and Setlow (1) and ourselves, based on different cell lines or the use of hydroxyurea, are unlikely. Both they and we used primary human fibroblasts, and we obtained consistent results with cultures from 3 separate donors. We also obtained essentially similar results using UDS without hydroxyurea and repair replication with hydroxyurea. Results with other combinations of agents (21) and another cell type (2) are also consistent with our results.

In conclusion, we find that repair synthesis following combined doses of UV, AAAF, and 4NQO is not totally additive in the 2 normal cell lines used. In most instances, repair after a saturating dose of UV light was stimulated only about 20% by exposure to chemical carcinogens. These results may indicate that repair of DNA damage from these agents involves common rate-limiting steps, as was suggested by their classification into a common group of agents whose repair is defective in XP cells (6). Because a small amount of stimulation was observed when the 2 agents in combination damaged different bases in DNA (pyrimidines by UV and purines by AAAF or 4NQO), there may be slight differences in some of the steps of repair for these agents as well as a high degree of overlap. When 2 different agents damaged purines, there appeared to be no stimulation and hence a higher degree of common regulation for the repair.
of damage from these agents. These results should be borne in mind when calculating the environmental risks of individual carcinogens. Exposure to low concentrations of a large number of DNA-damaging compounds that are repaired by a common pathway may correspond to exposure to a saturating level of one compound and may result in the accumulation of unrepaired damage that increases the relative risk of exposure.

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