Abnormal Sensitivity of Human Fibroblasts from Xeroderma Pigmentosum Variants to Transformation to Anchorage Independence by Ultraviolet Radiation

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ABSTRACT

One class of xeroderma pigmentosum (XP) patients, known as XP variants, inherit the characteristic predisposition to sunlight-induced skin cancer, but unlike the majority of XP patients, their cells do not exhibit a deficiency in rate of excision repair of ultraviolet (UV) radiation-induced DNA damage. XP variant cells are only slightly more sensitive than normal to killing by 254 nm UV radiation or simulated sunlight. But they are much more sensitive than normal to the induction of mutations by these agents. We investigated their sensitivity to UV-induced transformation to anchorage independence compared to that of normal cells. Low doses of UV (2 to 4.5 J/m²), doses which resulted in little or no measurable transformation in normal cells, caused a dose-dependent increase in the frequency of anchorage independent XP variant cells. Doses of 6 to 8 J/m² were required to elicit a comparable response in the normal fibroblasts. Even when the two kinds of cells were compared at doses adjusted to give equal cytotoxicity, the frequency of transformation in the XP variant cells was higher than normal. Thus, their sensitivity to induction of anchorage independence by UV paralleled their sensitivity to UV-induced mutations.

INTRODUCTION

Xeroderma pigmentosum patients, are characterized by a genetic predisposition to sunlight-induced skin cancer (1). Skin fibroblasts from the majority of such patients are very deficient in nucleotide excision repair of DNA damage induced by 254 nm UV radiation (1-3) and are abnormally sensitive to the killing and mutagenic effects of UV (4-7) or simulated sunlight (7). But cells from one set of XP patients, designated XP variants, carry out clinical characteristics of the disease. We and others showed excision repair of UV-induced DNA damage at the normal rate (4-7) or simulated sunlight (7). But cells sensitive than normal to the killing action of UV or simulated sunlight, but much more sensitive to their mutagenic effect (7, 9, 10). Because of the sensitivity of XP variant patients to sunlight-induced skin cancer, we compared fibroblasts from such patients with normal cells for their sensitivity to the transforming effect of UV radiation. The results showed that these XP variant cells are, indeed, transformed to anchorage independence by much lower doses of UV than are normal cells.

MATERIALS AND METHODS

Cells. XP variant cells XP4BE were obtained from the American Type Culture Collection; the normal cells were derived from foreskin material of normal newborn as described (11). Cells had attained passages 5 to 19 by the time they were used as the target populations for UV. Their cloning efficiency on plastic without a feeder layer ranged from 16 to 45% for the XP4BE cells and from 25 to 89% for the normal cells.

Media. Cells were routinely cultured in tissue culture plasticware in Ham’s F10 medium lacking hypoxanthine and supplemented with additional sodium bicarbonate to 2.2 g/liter for additional buffering capacity and with fetal bovine serum (10% for normal, 15% for XP variant cells). They were incubated at 37°C in an atmosphere of 5% CO2-95% air, with humidity as near to 99% as possible. Eagle’s minimum essential medium supplemented with 0.2 mM serine, 0.2 mM aspartate, and 1.0 mM pyruvate was sometimes substituted for Ham’s F10 medium for routine culturing of cells and during the expression period postirradiation. For selection of TG resistant cells, the modified Ham’s F10 medium was supplemented with 40 μM TG. For selection of Al cells, Ham’s F10 medium supplemented with the designated amount of serum was used most commonly. In a few experiments (designated in the chart legend), a more complex medium was used.

UV Irradiation. Cells were irradiated attached to the surface of 100-mm diameter dishes. The medium was removed and the cells were rinsed with phosphate buffered saline and irradiated with a Mineralight UVSL-54 germicidal lamp as described previously (7). The incident dose was 0.1 or 0.2 J/m² as determined with an International Light Radiometer IL570 (Newburyport, MA). After irradiation, the cells were re-fed with fresh medium and allowed to undergo cell replication in the original dishes.

Cytotoxicity Assay. Cells from exponentially growing cultures were trypsinized briefly, suspended in culture medium, and plated at cloning densities (i.e., 50 to 1000 cells/100-mm diameter dish, 6 to 12 dishes/dose). After 12 to 16 h, the cells were irradiated and allowed to form colonies in the original dishes. The percentage of survival was determined from the cloning efficiency of treated and untreated cells (11).

Transformation Protocol. Cells in exponential growth were trypsinized and plated into 100-mm diameter dishes (10 to 17 dishes/dose of UV, 10⁴-10⁵ cells/dish) and were irradiated after 12 to 16 h. The densities used were selected so that the number of surviving cells would be approximately the same in each set of dishes, and so that there would be sufficient room for the cells to replicate exponentially during the expression period. On Day 4 following irradiation, the cells in approximately one-half of the dishes for each dose and for the unirradiated controls were pooled and 1.5 to 2 x 10⁶ cells were subcultured at a lower density to be assayed for transformation and/or mutations on Day 9, 10.
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8 or 9. On Day 5 following irradiation the cells in the rest of the dishes were pooled and assayed for transformation to anchorage independence directly without being subcultured.

Anchorage Independence Assay. For each dose of UV and for the unirradiated controls, a total of 1.5 to 2 × 10⁶ cells were assayed at two densities: 5 × 10⁴ and 10⁵ cells/60-mm diameter dish. The cells (5 × 10⁴ or 10⁵), suspended in 1.5 or 3 ml of Ham’s F10 medium containing 0.33% Noble agar (Difco, Detroit, MI) and supplemented with the designated amount of serum (top agar), were plated into dishes that contained 5 ml of freshly solidified bottom agar (made with the same culture medium, but containing 2% Noble agar). (The few instances when medium other than Ham’s F10 was used are designated in Fig. 1 legend.

One to 2 ml of agar-free culture medium was added to the top agar layer the following day. This was repeated every 7 to 10 days thereafter, as needed to make up for evaporation, until the colonies were fully developed in size. An aliquot of the cell suspension that was to be used as the top agar layer was also appropriately diluted with agar-free culture medium and plated into a series of 100-mm diameter plastic culture dishes at cloning density to determine the cloning efficiency of the cells that were being assayed in soft agar (“replating efficiency”). This value was used to determine the viability of the cells used in the assay. The agar dishes, along with the set of dishes for determining replating efficiency, were incubated at 37°C in an atmosphere of 3% CO₂-97% air. Precautions were taken to maintain a humidity as close to 99% as possible. After 3 to 4 weeks of incubation, the frequency of transformation was determined from the number of colonies with a diameter equal to or greater than 60 μm, as determined with an inverted microscope equipped with an ocular micrometer.

Mutagenesis Assay. At the end of an 8- to 9-day expression period, 1 to 2 × 10⁶ cells were plated in selective TG medium into 48 to 96 dishes (100-mm) at a density of 400 cells/cm². The cloning efficiency of the cells at the time of selection was also determined by plating cells at cloning density in the absence of selection. The mutant frequency was determined as described (11) from the number of resistant colonies corrected for the number of clonable cells plated, as well as by determining the chance of a mutant cell per dish from the number of dishes containing no colonies (P₀ method).

RESULTS

Before attempting to compare XP variant cells with normal human fibroblasts for the frequency of anchorage independent cells induced in the population by UV, we adjusted the assay conditions until they gave comparable background frequencies of colonies with a diameter of 60 μm or greater. Unless one sees a low, but measurable background frequency, it is not possible to know if culture conditions are comparable (6, 12, 13). Using Ham’s F10 medium, this condition was met for normal cells by using 6% fetal calf serum, but for the XP variant cells 16% serum was required. As shown in Table 1, these respective percentages gave a background frequency of 5 to 8 Al cells/dish of 50,000 cells. The more complex medium used for initial experiments (see legend to Fig. 1) gave comparable background frequencies (data not shown).

Fig. 1 compares the sensitivity of the XP4BE variant cells (squares) with that of several normal cell lines (circles) to the cytotoxic, mutagenic and transforming effects of UV. The data from the four individual experiments with the XP4BE cells are distinguished by different kinds of square symbols. Similarly, the data from six individual survival and transformation and/or mutation experiments, carried out with normal fibroblasts, are designated by specific kinds of circles. There was no significant difference in the frequency of transformants obtained with a particular cell type using more than one type of media (see legend). Because we had already published data on the frequency of TG resistance induced by UV in normal cells (4, 7), we did not routinely assay the normal cells for this marker. Thus, the survival and mutation data for normal cells indicated by solid circles were obtained previously (4, 7) and have been included in Fig. 1 for purposes of comparison.

As shown in the Fig. 1 (bottom), the XP4BE cells were significantly more sensitive than normal to UV-induced transformation

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Table 1

<table>
<thead>
<tr>
<th>% of serum used</th>
<th>Normal</th>
<th>XP4BE</th>
</tr>
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<tbody>
<tr>
<td>4</td>
<td>1, 3</td>
<td>ND</td>
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<tr>
<td>6</td>
<td>5, 8, 14</td>
<td>0.1</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>2.3</td>
</tr>
<tr>
<td>12</td>
<td>ND</td>
<td>6.6</td>
</tr>
<tr>
<td>16</td>
<td>ND</td>
<td>82.2</td>
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Colonies of diameter 60 μm or greater. The cells were seeded at 5 × 10⁴/dish. ND, not determined.
UV-INDUCED TRANSFORMATION OF XP VARIANT CELLS

to anchorage independence. Low doses (2 to 4 J/m²), which in
the normal cells resulted in little or no increase in frequency of
Al cells, gave a dose-dependent, linear increase in frequency of
Al cells in the XP4BE population. As shown in Fig. 1 (middle),
these low doses also induced a linear, dose-dependent increase
in the frequency of TG resistant cells in the XP4BE population,
but little or no increase in the normal cells. Data from repre-
sentative transformation experiments are shown in Table 2.

We (7, 9) and others (10) showed previously that XP variant
cells are more mutable than normal fibroblasts, even if one
compares the two types of cells for the frequency of mutants
induced by doses of UV adjusted to result in equal cytotoxicity.
The data in Fig. 1 show that this was also the case for XP4BE
transformation frequencies. For example, at a 37% survival
dose for the XP4BE cells (~3.2 J/m²), the frequency of
Al cells was approximately 500/10⁶; whereas at a 37% survival
dose for the normal cells (~5.2 J/m²), the induced frequency of
Al cells was approximately 185/10⁶. Similarly for mutation induc-
tion, the frequency of TG resistant cells induced in the XP variant
cells by a 37% survival dose was approximately 200/10⁶, but
only about 80/10⁶ for the normal cells.

Table 2 summarizes the relationships between the curves
shown in Fig. 1. The frequencies of transformants and mutants
induced by UV doses causing 0.5, 1.0, and 1.5 mean lethal
events (60, 37, and 22.5% survival) in the two kinds of cells have
been extrapolated from the curves shown in Fig. 1. The ratios in
Columns 6 and 7 show that for either cell type, the frequencies

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Dose (J/m²)</th>
<th>Original surviving fraction</th>
<th>Cloning efficiency at Al selection</th>
<th>Mean no. of Al colonies/ dish</th>
<th>Al cells/10⁶ cells</th>
<th>Induced Al cells/10⁶ cells</th>
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<tr>
<td>NFLG1</td>
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<td>0.48</td>
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<td>0.48</td>
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<td>280</td>
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<tr>
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<td>8</td>
<td>0.12</td>
<td>0.32</td>
<td>31</td>
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<td>3</td>
<td>60</td>
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<td>440</td>
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<td>8</td>
<td>0.21</td>
<td>0.55</td>
<td>45</td>
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<td>840</td>
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<td>XP4BE</td>
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<td>0.27</td>
<td>6</td>
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<td>0</td>
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<td></td>
<td>2.5</td>
<td>0.50</td>
<td>0.20</td>
<td>26</td>
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<td>400</td>
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<tr>
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<td>3.5</td>
<td>0.31</td>
<td>0.23</td>
<td>36</td>
<td>720</td>
<td>600</td>
</tr>
</tbody>
</table>

* These values were not used in calculating the frequency of Al cells, but only
to check the viability of the cells.

* A total of 10 dishes (60-mm) containing 5 x 10⁶ cells were used for each
determination. The medium used was Ham’s F10, 6% serum was used for NFLG1
cells; 16% for XP4BE cells.

Table 3 summarizes the relationships between the curves
shown in Fig. 1. The frequencies of transformants and mutants
induced by UV doses causing 0.5, 1.0, and 1.5 mean lethal
events (60, 37, and 22.5% survival) in the two kinds of cells have
been extrapolated from the curves shown in Fig. 1. The ratios in
Columns 6 and 7 show that for either cell type, the frequencies

<table>
<thead>
<tr>
<th>Fraction of surviving cells</th>
<th>Frequency of induced transformants/10⁶ cells</th>
<th>Frequency of induced mutants/10⁶ cells</th>
<th>Transformant: mutant ratio</th>
<th>XP4BE:NF frequency ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP4BE</td>
<td>290</td>
<td>50</td>
<td>2.9</td>
<td>5.8</td>
</tr>
<tr>
<td>NF</td>
<td>200</td>
<td>50</td>
<td>2.5</td>
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</tr>
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<td>XP4BE</td>
<td>500</td>
<td>185</td>
<td>2.5</td>
<td>2.7</td>
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<tr>
<td>NF</td>
<td>200</td>
<td>80</td>
<td>2.2</td>
<td>2.5</td>
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</tbody>
</table>

* These frequencies are taken from the respective curves shown in Fig. 1 (middle and bottom) at the doses of UV required to lower the survival of the respective cells
to the fraction shown in Column one.

DISCUSSION

The frequency of Al cells induced in the normal population by
6 to 8 J of UV/m² agree with our earlier results (6) and those
obtained by Sutherland er al. (17) using early passage cells. The
transformation data for the XP4BE variant cells show that, like
excision repair-deficient XP cells (6), they can be transformed to
anchorage independence by much lower doses of UV than are
required for normal cells. Thus, their sensitivity to transformation
parallels their sensitivity to UV-induced mutations. This is in
keeping with the fact that both kinds of XP patients are abnor-
mally sensitive to sunlight-induced skin cancer (1).

The inability of excision repair-deficient XP cells to remove UV-
induced DNA damage at the normal rate could account for their
abnormal sensitivity to mutations and transformation of UV.
However, XP variant cells, unlike cells from the majority of XP
patients, have normal rates of excision repair (1, 3). The mech-
anism(s) responsible for the abnormal sensitivity of the XP variant
cells to induction of mutations and of anchorage independence
by UV is not known. We recently showed (18) that if synchronous
populations of XP variant cells or normal cells are irradiated in
early G₁ under conditions that allow them to carry out excision
repair during a 14-h period before DNA synthesis begins, the
frequency of UV-induced TG resistant cells is greatly reduced
compared to cells irradiated at the onset of S phase. No such
decrease is seen in XP cells that are virtually incapable of excision
repair (XP12BE) (5). These results suggest that excision repair
in normal and XP variant cells is equally “error free” and that the
abnormally high frequency of mutants induced by UV in the
XP4BE cells is not the result of mutations being put in by the
excision repair process.

Although the nature of the mechanism causing higher than
normal frequencies of mutants (TG resistant cells) in the XP
variant cells is not known, the same mechanism could account
for the higher frequency of transformed Al cells. We speculate
that during DNA replication on a damaged template, either the
XP variant cells use an abnormally error prone process to bypass

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such lesions, or normal cells possess a special error-free mechanism for bypassing some of the UV-induced lesions while using the same mechanism or process the XP4BE cells use for replicating past the remaining damage (18). This would result in more mutations and, by analogy, more transformation events, being “fixed” (made permanent) in the XP variant cell per dose of UV than in normal cells. Experimental evidence in support of either hypothesis is lacking. However, it is known that in XP variant cells, chain elongation is more readily blocked by pyrimidine dimers than in normal cells or in excision repair-deficient XP cells (19–21). This is the case in spite of the ability of the XP variant cells to carry out nucleotide excision repair at the normal rate (1, 3, 8).

In summary, our data indicate that XP variant cells, XP4BE, are abnormally sensitive to transformation to anchorage independence induced by UV, when compared on the basis of applied dose (equal number of DNA lesions), but also on the basis of doses adjusted to give equal cytotoxicity. This finding, taken together with data showing that this relationship is also true for mutation induction (7, 9, 10, 18) and that excision repair-deficient XP cells also are abnormally sensitive to transformation (6), supports the hypothesis that transformation to anchorage independence results from damage to DNA.

It is not surprising that a single exposure to UV radiation did not cause the cells to acquire all the characteristics of tumor-derived neoplastic cells. This is predicted by the multisteped nature of the process of in vitro neoplastic transformation as demonstrated, for example, in Syrian hamster embryo cells (22, 23) or in rat embryo fibroblasts (24).

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REFERENCES


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