Hereditary Dysplastic Nevus Syndrome: Lymphoid Cell Ultraviolet Hypermutability in Association with Increased Melanoma Susceptibility

Mohan I. R. Perera, Kyung Il Um, Mark H. Greene, Haywood L. Waters, Anders Bredberg, and Kenneth H. Kraemer

ABSTRACT

The hereditary dysplastic nevus syndrome (DNS) is a well-characterized disorder in which affected individuals have increased numbers of premalignant (dysplastic) nevi and a markedly increased risk of developing cutaneous melanoma. Seeking evidence of a systemic disorder in DNS, we examined the effect of ultraviolet radiation on cultured lymphoid cells. Epstein-Barr virus-transformed lymphoblastoid cell lines from patients with hereditary DNS had similar survival values following treatment with 2.3 to 9.0 J of 254-nm ultraviolet radiation per m² as did lines from control individuals. Mutagenesis at the hypoxanthine-guanine phosphoribosyltransferase locus was assessed by measuring the induction of resistance to thioguanine using a microtiter well assay. Three lymphoblastoid cell lines from patients with hereditary DNS and melanoma had a 2- to 3-fold greater frequency of induced mutants per clonable cell than three normal lines following exposure to 4.5 to 9.0 J of ultraviolet radiation per m². Expanded clones of mutated DNS lymphoblastoid cell lines had <6% of normal hypoxanthine-guanine phosphoribosyltransferase activity. Inhibition and recovery of DNA synthesis following ultraviolet exposure were similar in 2 DNS and 2 normal lines. Repair by DNS lines of ultraviolet-induced DNA damage was in the normal range as measured by alkaline elution. Thus, hereditary DNS exhibits in vitro hypermutability which may reflect increased susceptibility to ultraviolet-induced somatic mutations in vivo. This abnormality may be related to the increased melanoma susceptibility of patients with hereditary DNS.

INTRODUCTION

The hereditary DNS is an autosomal dominant disorder characterized clinically by the appearance of multiple distinctive moles (dysplastic nevi) on the skin and greater than 100-fold increased risk of development of malignant melanoma (1-6). We performed studies on cultured lymphoblastoid cell lines from patients with the hereditary DNS in an attempt to find laboratory abnormalities which might further define syndrome and clarify its pathogenesis. We used as a model another genetic disease, xeroderma pigmentosum (7,8). Cells derived from xeroderma pigmentosum patients show hypermutability following ultraviolet exposure in vitro (7). Since ultraviolet irradiation is a melanoma risk factor (5,9), we decided to investigate whether cellular ultraviolet hypermutability is associated with the development of dysplastic nevi and melanoma in these patients.

MATERIALS AND METHODS

Cell Lines. Lymphoblastoid cell lines were established by Epstein-Barr virus transformation of peripheral blood lymphocytes from patients with hereditary dysplastic nevus syndrome in 3 unrelated families (Table 1): normal controls; KR6057, KR6058, and GM606; and a patient with xeroderma pigmentosum XP12BE (GM 2250). Lines designated KR or GM were transformed at the Institute for Medical Research, Camden, NJ. The other lines were transformed by Dr. L. Bonner, Dr. D. Buck, or Dr. R. Bennet. Line HH4, a subclone of line WI-L2 derived from a patient with hereditary spherocytosis (10), was obtained from Dr. W. Thilly and contains Epstein-Barr viral antigen. Cell lines were maintained in suspension culture in RPMI 1640 medium (GIBCO) supplemented with 16% fetal calf serum (selected for ability to support colony formation in microtiter wells) without antibiotics. Stock cultures were maintained in a 40- to 50-ml volume in 75 cm² flasks at 37°C with 5% CO₂ and fed at 2- to 3-day intervals to maintain exponential growth.

Ultraviolet Treatment. Lymphoblastoid cells were washed and suspended in a phenol red-free solution containing the inorganic salts and glucose of RPMI medium (11) at 3 x 10⁶ cells/ml. Sixteen ml of cell suspension shaking in an uncovered 150-mm tissue culture dish were exposed at room temperature to ultraviolet radiation from an unfiltered germicidal lamp (General Electric G15T8) (predominantly 254 nm) at a rate of 0.11 J/m²/s as measured by a calibrated International Light Company IL770 radiometer with PT171C detector. The treated cells were then diluted in growth medium for further testing.

Cell Survival. Cell survival following ultraviolet exposure was measured using microtiter wells or growth in suspension culture as previously described (1). Briefly, for the microtiter well survival assay, serial halflog dilutions of cells were inoculated into 8 to 24 wells of a 96-well tissue culture tray (Linbro) in 0.2 ml of culture medium. Trays were wrapped in aluminum foil to minimize evaporation and incubated at 37°C with 5% CO₂ and 90% relative humidity for 2 to 3 wk without refeeding. Growth of colonies within the wells was scored by microscopic observation. The microculture initiating efficiency (frequency of clonable cells) was calculated by assuming a Poisson distribution: dividing the negative logarithm of the fraction of wells without colonies at a cell dilution by the number of cells inoculated per well (12). The percentage of survival was obtained by dividing the mean microculture initiating efficiency of an ultraviolet-treated culture by that of the untreated control culture. The frequency of clonable cells was 8 to 100% in the hereditary DNS lines and 4 to 100% in the normal control lines.

For the suspension culture assay, 40 ml of cell suspension at 100,000 cells/ml were placed in a 75-cm² flask in the CO₂ incubator. The concentration of viable (trypan blue excluding) cells was determined by daily hemocytometer counting.
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Mutagenesis Testing. Mutation frequency was determined as described by Thilly (13) with minor modifications. In preparation for mutagenesis experiments, 200-ml cultures containing 4 x 10^6 cells were grown in 750-cm^2 roller bottles (Corning) with growth medium containing hypoxanthine (27 ¿g/ml)-aminopterin (0.088 ¿g/ml)-thymidine (4.2 ¿g/ml) for 2 days to reduce the frequency of spontaneous HGPRT-negative cells in the culture. Cells were then washed, cultured in growth medium for an additional 24 h, and then treated with ultraviolet radiation.

Ultraviolet-treated and control cells were placed in flasks at 5 x 10^6 cells/ml and maintained in exponential growth phase by daily dilutions. Microrotr well survival and mutation assays were performed at Day 0 and at 2 to 4 additional days during the interval from Day 6 to Day 14 to permit expression of the mutated phenotype and to assure detection of the maximal number of mutants. For the mutation assay, 40,000 cells suspended in 0.2 ml of growth medium containing 5 ¿g of 6-TG per ml were inoculated per well of eight 96-well microtiter trays (total, 3.1 x 10^7 cells) and incubated as in the cell survival assay. The frequency of mutants per clonal cell was determined by dividing the microculture initiating efficiency in 6-TG medium by the microculture initiating efficiency in growth medium for a given posttreatment day. Reconstruction experiments with varying numbers of 6-TG-resistant cells added to 40,000 6-TG-sensitive cells per well in 6-TG medium showed a linear relationship between the number of 6-TG-resistant cells added and the microculture initiating efficiency in normal and hereditary DNS lines. Measurement of Relative HGPRT Activity. Subclones of untreated control cells, spontaneous 6-TG resistant, or ultraviolet-treated 6-TG-resistant mutated cells were established from hereditary DNS and normal lines. Exponentially growing cultures were incubated in growth medium containing [3H]thymidine (10 ¿Ci/ml; 29 Ci/mmol; ICN) and [8-14C]guanine (1 ¿Ci/ml; 54.4 mCi/mmol; ICN) for 2 and 4 h. Cells were harvested on Millipore filters as described previously (11). HGPRT activity was determined from the ratio of incorporated [14C]guanine to [3H]thymidine per h. Relative HGPRT activity was obtained by dividing the value obtained for each cell line by the value for the control clone. Measurement of DNA Synthesis after Ultraviolet Treatment. Cells were prelabeled by growth in medium containing [14C]thymidine (0.01 ¿Ci/ml; 57 mCi/mmol; ICN) for 24 h. Following ultraviolet treatment, cells were suspended in prewarmed medium containing [3H]thymidine (10 ¿Ci/ml) and quintuplicate samples were incubated at 37°C. Incorporation of radioactivity was determined by a Millipore filter assay as described in Ref. 11. The relative rate of DNA synthesis was determined from the ratio of [3H]thymidine to [14C]thymidine in ultraviolet-treated cells divided by that in the untreated cells. Measurement of DNA Repair by Alkaline Elution. Cellular induction of DNA strand breaks during excision repair following ultraviolet treatment was measured by an alkaline elution assay (14). Briefly, 2 x 10^6 cells/ml prelabeled by 24-h growth in medium containing [14C]thymidine (0.1 ¿Ci/ml) (test cells) or [3H]thymidine (0.1 ¿Ci/ml) (internal standard L1210 mouse lymphoma cells, 3 x 10^5/ml) were chased in nonradioactive growth medium for 1 h and then treated with ultraviolet. After incubation at 37°C for various intervals, cells were lysed on Nucleopore filters (pore size, 2 ¿m) with sodium dodecyl sulfate-EDTA and treated with proteinase K, and the DNA was slowly eluted with an alkaline solution of tetrapropylammonium hydroxide over a 15-h interval. An internal standard of L1210 cells treated with 300 rads of X-ray was added to each sample prior to elution. Calculation of the elution rate was determined as described in Ref. 14 using a computer program written by Dr. A. Peacock. Relative strand break induction at different times following ultraviolet treatment was measured by determining the fraction of the test sample DNA retained at the point of retention of 50% of the DNA of the internal standard L1210 cells.

RESULTS

Survival of hereditary DNS, normal, and xeroderma pigmentosum lymphoblastoid cell lines following exposure to 2.3 to 9.0 J of ultraviolet radiation per m² is shown in Fig. 1. As assessed by both growth curve assay (Fig. 1A) and the microrotr well assay (Fig. 1B), survival of the hereditary DNS lines was not significantly different from that of the normal lines. In marked contrast, the cell line from the patient with xeroderma pigmentosum was extremely sensitive to the growth-inhibiting effect of ultraviolet radiation (Fig. 1A).

The frequency of a 6-TG-resistant cells per 10^6 clonable cells in cultures not treated with ultraviolet following the expression period was similar in the hereditary DNS and the normal lines (mean, 19 in hereditary DNS, 17 in normals). In cultures treated with ultraviolet radiation, there was an absolute increase in the number of mutants detected at each dose before correction for survival. Fig. 2 shows that the frequency of mutants per clonal cell in the hereditary DNS and normal cell lines increased with increasing ultraviolet dose. The 3 normal lines had similar mutation frequency per clonable cell. The 3 hereditary DNS lines had a similar frequency of mutations per clonable cell. However, the mutant frequency per clonable cell was greater in the hereditary DNS lines than in the normal lines for doses of ultraviolet of 4.5 to 9 J/m². At 9 J/m², the mean frequency of mutants per survivor was 2.8-fold higher in the hereditary DNS than in the normal lines.

At the doses tested, survival of the hereditary DNS and normal lines was greater than 10%. At similar levels of survival, the hereditary DNS lines had a greater frequency of 6-TG-resistant cells per clonable cell than the normal lines.

In the hereditary DNS and normal lines, there was more than a 90% reduction in HGPRT activity in clones of 6-TG-resistant cells obtained following ultraviolet exposure or in spontaneous mutants following expansion in selective medium (Table 2).

Ultraviolet radiation has been previously reported to cause a prolonged inhibition of DNA synthesis in lymphoblastoid cell lines from some patients with a form of familial melanoma (15). Fig. 3A shows the effect of increasing doses of ultraviolet radiation on DNA synthesis during the first 2 h after treatment. The inhibition of DNA synthesis was similar in the hereditary DNS and the normal line. Fig. 3B demonstrates that recovery of DNA synthesis following exposure to 5 J of ultraviolet per m² was also similar in 2 hereditary DNS and 2 normal lines.

In order to demonstrate a possible DNA repair abnormality in the hereditary DNS cells, induction and sealing of DNA strand breaks following exposure to ultraviolet radiation were measured by alkaline elution (Fig. 4). This strand breakage reflects the first (incision) step of DNA excision repair. A xeroderma pigmentosum line served as a positive control, demonstrating the delayed onset of DNA strand breakage typical of repair-deficient cells.

Table 1

<table>
<thead>
<tr>
<th>Lymphoblastoid cell lines established from hereditary DNS patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>DNS3BE (JAK)</td>
</tr>
<tr>
<td>DNS5BE (LT/GMR21)</td>
</tr>
<tr>
<td>DNS6BE (MUKA)</td>
</tr>
<tr>
<td>DNS7BE (MUDA)</td>
</tr>
</tbody>
</table>

*At which lymphocytes were obtained to establish line.

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DISCUSSION

The 3 hypermutable hereditary DNS lymphoblastoid cell lines showed responses in the normal range with rapid onset of strand breakage during the first 30 min following 5 J of ultraviolet exposure per m² and substantial recovery by 4 h of repair. The pattern of strand breakage and recovery following exposure to 10 J of ultraviolet per m² was also similar in the hereditary DNS and normal lines (data not shown).

Table 2

<table>
<thead>
<tr>
<th>Cells</th>
<th>Clone</th>
<th>Thio guanine resistance</th>
<th>Relative HGPRT activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR8057</td>
<td>C3</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>S5</td>
<td>+</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>M3</td>
<td>+</td>
<td>1.5</td>
</tr>
<tr>
<td>DNS3BE</td>
<td>C6</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>M6</td>
<td>+</td>
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<td>+</td>
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<td>-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>M5</td>
<td>+</td>
<td>2.8</td>
</tr>
</tbody>
</table>

6 Clones designated "C" were established in growth medium without thioguanine. Thioguanine-resistant clones designated "S" were spontaneous mutants, and "M" clones were ultraviolet-induced mutants.

6 Resistant to 6-thioguanine (5 μg/ml) in growth medium.

6 Average relative incorporation per h of [3H]guanine to [3H]thymidine after 2- and 4-h incubation.

The hereditary DNS (DNS type D-2) (1, 5) is an inherited disorder which may be linked to the Rh locus on chromosome 1 (2, 6, 16). The present study demonstrates an abnormality in cultured cells derived from peripheral blood lymphocytes of hereditary DNS patients with cutaneous melanoma. This is evidence of a systemic abnormality not limited to the melanocytic cells from which dysplastic nevi and melanomas arise.

Howell et al. (17) previously reported that fibroblasts from 1 of 2 hereditary DNS patients tested were hypermutable following treatment with broad-spectrum (290 to 390 nm) ultraviolet radiation from an artificial sunlamp. These fibroblasts, 3072T and 3012T, were obtained from hereditary DNS patients we did not study. In the present study, we found lymphoblastoid cell lines from 3 of 3 hereditary DNS patients to be hypermutable to 254-nm ultraviolet radiation. The hereditary DNS is thus the second human genetic disease (along with xeroderma pigmentosum) to be associated with in vitro ultraviolet hypermutability. Both dis-
have an error-prone (hypermutable) DNA repair pathway. The relationship of such a defect to melanoma induction remains speculative. However, the finding of a mutated ras oncogene in some human melanoma cell lines (19, 20) suggests a possible mechanism. In addition, the reports by Krontiris et al. (21) describing the presence of rare restriction fragment polymorphism alleles to a Ha-ras DNA probe in persons with or at high risk of cancer (including patients with hereditary melanoma and dysplastic nevi) and that of Dracopoli et al. (22) showing loss of polymorphic restriction fragments in malignant melanoma are also consistent with the hypothesis that genetic instability may contribute to the development of melanoma in families. The recent observation that peripheral blood karyotypes from members of hereditary DNS families with dysplastic nevi with or without melanoma contain excessive numbers of apparently random chromosomal structural and numerical abnormalities may represent the morphological expression of this genomic instability (23).

In the present study, survival of 254-nm ultraviolet-treated lymphoblastoid cell lines from the hereditary DNS patients was similar to that of the normal controls by both measurement of cytotoxicity and measurement of microtiter well cloning ability. A previous study of ultraviolet survival of lymphoblastoid cell lines from 2 kindreds with familial melanoma in Australia reported the dose of UV radiation that reduces survival by 63% on the exponential portion of the survival curve to be 53 to 64% of those of controls in family members with melanoma (15). According to this report, "some family members had dysplastic nevi, but neither family conformed in all respects to the B-K mole (dysplastic nevus) syndrome" (15). Smith et al. (24) reported values for the fluence of the UV radiation resulting in 10% survival of 43 to 63% of controls in 6 fibroblast strains from hereditary DNS patients including fibroblast strains 3248T, 3191T, and 3190T, which were derived from the patients that provided lymphoblastoid cell lines DNS5BE, DNS6BE, and DNS7BE, respectively. Howell et al. (17) studied 2 of the hereditary DNS strains (3012T and 3072T) examined by Smith et al. (24) and found only one (3072T) to be hypersensitive to killing by artificial
sunlamp treatment. The explanation for these contradictory laboratory findings is not readily apparent. Significant differences exist between the various laboratories regarding assay techniques, type of cell studied, and carcinogens used. Alternatively, hereditary melanoma might be etiologically heterogeneous, with some kindreds displaying in vitro ultraviolet hypersensitivity which others do not.

The hypermutability of xeroderma pigmentosum cells is associated with defective repair of ultraviolet damage to DNA (7). This prompted similar studies with hereditary DNS cells. Xeroderma pigmentosum cells have delayed recovery of DNA synthesis following ultraviolet radiation. Ramsay et al. (15) reported prolonged inhibition of replication following exposure of 2 Australian familial melanoma lymphoblastoid cell lines to 5 J but not to 12.5 J of 254-nm radiation per m². In the present study, DNA synthesis was inhibited equally in hereditary DNS and normal lymphoblastoid cell lines whether measured as a function of varying ultraviolet dose or of time after exposure to 5 J of 254-nm radiation per m². Smith et al. (25) similarly found normal recovery of DNA synthesis in hereditary DNS fibroblasts. Ramsay et al. (15) reported normal ultraviolet-induced unscheduled DNA synthesis and frequency of sister chromatid exchanges in familial melanoma lymphoblastoid cell lines. Smith et al. (25) found repair replication following ultraviolet or γ-ray exposure to be normal with hereditary DNS fibroblasts. In the present study, we demonstrated normal kinetics of ultraviolet-induced DNA repair strand breakage and recovery as measured by alkaline elution. Collectively, these normal posttreatment DNA synthesis and repair responses serve to differentiate the hereditary DNS from both the excision repair-deficient and the variant forms of xeroderma pigmentosum.

Clearly, these studies must be extended to affected and unaffected members of complete, well-characterized pedigrees to determine if cellular hypermutability is consistently associated with increased melanoma susceptibility in the hereditary form of the dysplastic nevus syndrome.

ACKNOWLEDGMENTS

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REFERENCES

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