Hypersensitivity of Skin Fibroblasts from Basal Cell Nevus Syndrome Patients to Killing by Ultraviolet B but not by Ultraviolet C Radiation

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ABSTRACT

Basal cell nevus syndrome (BCNS) is an autosomal dominant genetic disorder in which the afflicted individuals are extremely susceptible to sunlight-induced skin cancers, particularly basal cell carcinomas. However, the cellular and molecular basis for BCNS is unknown. To ascertain whether there is any relationship between genetic predisposition to skin cancer and increased sensitivity of somatic cells from BCNS patients to killing by UV radiation, we exposed skin fibroblasts established from unexposed skin biopsies of several BCNS and age- and sex-matched normal individuals to either UV-B (280–320 nm) or UV-C (254 nm) radiation and determined their survival. The results indicated that skin fibroblasts from BCNS patients were hypersensitive to killing by UV-B but not UV-C radiation as compared to skin fibroblasts from normal individuals. DNA repair studies indicated that the increased sensitivity of BCNS skin fibroblasts to killing by UV-B radiation was not due to a defect in the excision repair of pyrimidine dimers. These results indicate that there is an association between hypersensitivity of somatic cells to killing by UV-B radiation and the genetic predisposition to skin cancer in BCNS patients. In addition, these results suggest that DNA lesions (and repair processes) other than the pyrimidine dimer are also involved in the pathogenesis of sunlight-induced skin cancers in BCNS patients. More important, the UV-B sensitivity assay described here may be used as a diagnostic tool to identify presymptomatic individuals with BCNS.

INTRODUCTION

BCNS,3 also known as nevoid basal cell carcinoma syndrome or Gorlin’s syndrome, is an autosomal dominant disorder with high penetrance (>97%) (1–4). The principal manifestations of this syndrome are multiple BCC, palmoplantar pitting, and systemic (jaw cysts) and musculoskeletal abnormalities (scoliosis, bifurcated rib, spina bifida) (5–9). This high incidence of developmental anomalies suggests that the normal allele of the BCNS gene may play a role in growth and development. While BCC in normal individuals are generally solitary, occur on sun-exposed body sites, and do not usually appear until later in life, the nevoid BCC occur as multiple tumors (average, 50–100), primarily on sun-exposed skin, and usually appear at puberty and during the second and third decade of life. These observations suggest that the process of carcinogenesis is accelerated in BCNS. However, the cellular and molecular basis for BCNS is unknown.

That individuals with BCNS also develop other neoplasms such as medulloblastoma and ovarian and uterine fibromas (5–11) has led to the hypothesis that BCNS fits Knudson’s two-mutation model for carcinogenesis (12, 13). However, the number of such mutations required to induce cancers in BCNS individuals is unknown and could be higher than two. Nevertheless, in BCNS, one of the mutations is inherited as an autosomal dominant gene in all somatic cells, whereas the remaining mutation(s) is induced by radiation. This hypothesis is supported by the observation that presymptomatic BCNS children treated with radiation for medulloblastoma developed multiple BCC in the area that received radiation 6 months to 3 years after treatment (14). Radiosensitivity studies using fibroblasts from BCNS individuals have, however, led to contradictory results. While most studies have failed to show any unusual sensitivity of BCNS fibroblasts to cell killing by X-rays, γ-rays, α-particles, or mitomycin C (15–18), Chan and Little (19) reported that BCNS fibroblasts were hypersensitive to killing by ionizing radiation. The ability of BCNS fibroblasts to repair X-ray-induced DNA damage was also normal (18, 19). In contrast, Arlett and Priestley (21) have reported that cells from BCNS patients were defective in the repair of γ-ray-induced potentially lethal damage. Thus more studies are needed to resolve this controversy.

Attempts to demonstrate an increased sensitivity of somatic BCNS cells to killing by UV radiation have been unsuccessful. Studies by Little et al. (20) and Lehmann et al. (22) have shown that fibroblasts from BCNS patients exhibit normal survival following UV irradiation. However, Nagasawa et al. (23) have reported that BCNS fibroblasts were slightly more sensitive to UV radiation than normal fibroblasts. In any case, these studies were conducted using UV-C radiation (254 nm), a wavelength that is filtered out by the stratospheric ozone. Since epidemiological and clinical studies indicate that UV-B radiation (280–320 nm), which reaches the surface of the earth in sunlight, is responsible for the induction of most skin cancers in humans (24, 25), it is important to use these wavelengths in sensitivity studies on human cells, particularly from individuals genetically predisposed to sunlight-induced skin cancers. When we determined the sensitivity of skin fibroblasts from BCNS and age- and sex-matched normal individuals to killing by UV-B radiation, we found that skin fibroblasts from BCNS individuals were hypersensitive compared with the skin fibroblasts from age- and sex-matched normal individuals.

MATERIALS AND METHODS

Cell Cultures. None of the BCNS patients had undergone radiotherapy or chemotherapy for BCC at the time of skin biopsy. Three normal male (ages 32, 33, and 43 years) and two female (ages 33 and 50 years) volunteers, all Caucasians, were chosen as controls. Skin tissues (~6 mm in diameter) from non-sun-exposed areas from 5 BCNS patients and 5 normal individuals were surgically excised, cut into small fragments (<0.5 mm3), transferred to T-25 tissue culture flasks (Falcon), and grown in DMEM (GIBCO) containing 20% calf serum (Hyclone), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 95% air/5% CO2. The culture medium was renewed twice weekly. After 2–3 weeks, primary fibroblasts were treated

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3 The abbreviations used are: BCNS, basal cell nevus syndrome; BCC, basal cell carcinoma; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; ESS, endonuclease-sensitive sites; XP, xeroderma pigmentosum; (6-4) photoproducts, pyrimidine (6-4) pyrimidone photoproducts.
endonucleases were partially purified from M. luteus by the method of
out with the laminar flow hood and overhead room fluorescent lights
they were frozen in liquid N2 in 1-ml aliquots containing ~5 × 10⁵
cells/ml. As needed, frozen cells were thawed quickly and grown in T-
150 flasks in DMEM for 2–3 days before using in experiments.

Light Sources. A short-wave, 4-W germicidal lamp (Ultra-violet
Products, Inc., San Gabriel, CA) was used as a source of UV-C
radiation. This light source emits primarily 254 nm radiation. An
unfiltered FS40T12 sunlamp (National Biological Corporation, Twins-
burg, OH) was used as a source of UV-B radiation. The unfiltered FS40
sunlamp emits wavelengths predominantly between 280 and 400 nm
(99.4% of the total UV radiation) with a peak emission at 314 nm. The
fluence rates of the FS40 sunlamp and the germicidal lamp at a source
to target distance of 20 cm (from the light bulb to the bottom of the
tissue culture dish) were 4.4 and 1 W/m², respectively. The fluence was
measured by a calibrated Optronic model 742 spectroradiometer (Op-
tronics Laboratories, Orlando, FL). The scanning radiometer measures
the spectral emissions at 1-nm intervals and integrates the emitted
energies.

UV Survival Assays. Cells (750/dish) were plated in 60-mm tissue
culture dishes in complete DMEM. About 6 h after plating, the medium
was removed and the cells were washed once with 2 ml of PBS and
then irradiated at room temperature in 1 ml of PBS with the dish cover
off using an unfiltered FS40T12 sunlamp or a germicidal lamp. A set
of unirradiated cultures was treated similarly, except that they were
kept in the dark for the duration of the irradiation period. After
irradiation, PBS was removed, medium was added, and the plates were
incubated at 37°C. After 7–10 days of growth, the dishes were stained
with methylene blue and the colonies were counted using a dissecting
microscope. The colony forming efficiency of untreated BCNS and
normal human skin fibroblasts was 20–25%. All operations were carried
out with the laminar flow hood and overhead room fluorescent lights
off.

Pyrimidine Dimer and Excision Repair Assays. Exponentially grow-
ing cells (5 × 10⁴/dish) were grown in complete DMEM containing 0.5
μCi/ml of [³H]thymidine (specific activity, 20 Ci/mmol). After over-
night incubation at 37°C, the medium was replaced with fresh nonra-
dioactive DMEM and incubated for 1 h. The cells were washed with
PBS and exposed to 100 J/m² of UV-B radiation from a FS40T12
sunlamp, as described above. The numbers of pyrimidine dimers present
in the DNA of UV-B-irradiated BCNS and normal human skin fibro-
blasts were measured either immediately after UV irradiation or after
6 or 12 h of post-UV recovery using the damage-specific endonucleo-
ases from Micrococcus luteus, as described before (26). Damage-specific
endonucleases were partially purified from M. luteus by the method of
Carrier and Setlow (27). Briefly, crude extracts of spray-dried cells
(Miles Chemical Co., Elkhart, IN) were precipitated with ammonium
sulfate (final concentrations, 65%) and then fractionated on a DE52
cellulose column. The resulting eluate, referred to as fraction III (27),
represented about 6-fold purification and contained most of the endo-
nuclease activity. This fraction was used in ESS assays.

ESS (pyrimidine dimers) were determined by incubating 200 μl
(≈2000 cpm) of DNA with 20 μl of " assay mix" (50 mM EDTA, 100
μg crude yeast RNA/ml, and 200 μg sonicated calf thymus DNA/ml
in 50 mM phosphate buffer, pH 7.0) and 20 μl of M. luteus extract for
30 min at 37°C. The reaction was stopped by the addition of 100 μl of
1 N NaOH. The incubation was continued for another 30 min to assure
complete denaturation of the DNA; layered onto a 4.6-ml alkaline 5–
20% sucrose gradient containing 0.7 M NaCl, 0.3 M NaOH, and 0.01
M EDTA; and centrifuged in a Beckman SW50.1 rotor at 30,000 rpm
for 180 min. The distribution of radioactivity in the gradients was
determined by the paper strip method of Carrier and Setlow (28).

Number-average molecular weights (Mn) were calculated from the
weight-average molecular weights since direct calculation of Mw is very
sensitive to small fluctuations in the low molecular weight region of
the gradients. The number of ESS per 10⁸ daltons of DNA was
calculated as

\[
\text{No. of ESS/10}^8 \text{ daltons DNA} = 10^8 \left( \frac{1}{M_n} - \frac{1}{M_w} \right)
\]

where \(M_n\) and \(M_w\) are the \(M_n\)s of the DNA observed with and without
 treatment with \(M. luteus\) enzyme, respectively.

RESULTS

We have assembled a group of pedigrees in which the BCNS
is inherited as an autosomal dominant trait. On the basis of
clinical observations and personal interviews with the patients
and their family members, we were able to follow the occurrence
of BCNS in 3 families from Houston and 1 from Galveston,
representing 3–4 generations. As can be seen in Fig. 1, the
mode of inheritance of the BCNS gene in these families appears
to be dominant with multiple and variable expression, so that
affected children have an affected parent, and there is no sex
predilection. All the patients participating in this study had
multiple BCC (100+). The case histories of the 5 adult BCNS
patients participating in this study are shown in Table 1. In
addition to multiple BCC, all the patients also exhibited one or
more of the other abnormalities (such as palmar pits, jaw cysts,
scoliosis, etc.,) that are commonly associated with BCNS. Pa-
tients 1, 2, and 3 had palmar pits (since age ~15 years), jaw
cysts, mild scoliosis, pectus excavatus, and milia. Although
patients 4 and 5 had palmar pits, it is not certain at what age
this symptom manifested. In addition, no clear information is
available to indicate whether patients 4 and 5 have jaw cysts or
musculoskeletal abnormalities.

Fibroblast cell strains established from biopsy samples of the
non-sun-exposed skin of 5 BCNS patients and 5 normal age-

![Fig. 1. Partial pedigree of four skin cancer-prone families. All four families
are Caucasians. The families studied here have BCNS characterized by multiple
BCC, palmar and planter pitting, and eye or jaw cysts. The 5 BCNS patients
participating in this study are indicated by the arrows pointing to the numbers /
to 5. Patients 1 and 2 are identical twins. The kindreds with question marks in
families 1 and 4 have not yet shown any symptoms of BCNS. All of them are
young children ages 1.5 to 7 years. The father of the 3 male children in family 4
is 33 years old and is either unaffected or presymptomatic. D, unaffected male;
O, unaffected female; Ü, affected male; ©, affected female; Ü, deceased.]

<table>
<thead>
<tr>
<th>Patient</th>
<th>Family</th>
<th>Age (yr) at 1st BCC (approximate)</th>
<th>Total BCC no. to date (approximate)</th>
<th>Age (yr) at skin biopsy for this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>15</td>
<td>200+</td>
<td>32</td>
</tr>
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<td>15</td>
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<tr>
<td>5</td>
<td>F</td>
<td>23</td>
<td>100+</td>
<td>49</td>
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* As shown in Fig. 1.
and sex-matched individuals were exposed to UV-B radiation from an FS40 sunlamp. The UV-B survival curves for BCNS and normal human skin fibroblasts are shown in Fig. 2. Skin fibroblasts from all 5 BCNS patients were hypersensitive to killing by UV-B radiation compared to the fibroblasts from age- and sex-matched normal individuals. In addition, the BCNS skin fibroblasts did not exhibit a shoulder, whereas the normal human skin fibroblasts exhibited a broad shoulder, which are suggestive of single-hit and multiple-hit kinetics, respectively. The dose required to reduce survival by 50% for BCNS fibroblasts was ~25 J/m², whereas for normal human skin fibroblasts, it was ~150 J/m², about 6-fold increase in sensitivity. However, at doses of >50 J/m², BCNS skin fibroblasts were about 3-fold more sensitive to killing by UV-B than normal human skin fibroblasts. This difference stemmed largely from the biphasic nature of the curve for BCNS patients which exhibited a steep decline in survival initially followed by a relatively more resistant component. The latter component appeared to be running more or less parallel to that of the normal human skin fibroblast survival curve.

We next performed survival experiments using a germicidal lamp to determine whether skin fibroblasts from BCNS patients were also hypersensitive to UV-C radiation. The results shown in Fig. 3 indicate that the sensitivity of BCNS skin fibroblasts to killing by UV-C radiation was similar to that of normal human skin fibroblasts.

UV radiation induces primarily cyclobutane-type pyrimidine dimers in the DNA (29). In order to ascertain whether the increased sensitivity of BCNS skin fibroblasts to killing by UV-B radiation was due to a defect in the excision repair of UV-B-induced pyrimidine dimers, we measured the number of pyrimidine dimers remaining in cellular DNA at 6 or 24 h post-UV-B. These time points were chosen because it has been reported that human cells exhibit a biphasic excision repair kinetics where ~50% of the pyrimidine dimers are repaired in the first few hours and the remaining are repaired by 24 h (30, 31). The results shown in Fig. 4 reveal that the percent of induced pyrimidine dimers remaining at 6 or 24 h post-UV-B recovery was similar in both BCNS and normal human skin fibroblasts. The rate of excision repair of pyrimidine dimers in BCNS and normal human skin fibroblasts was 40–50% at 6 h and 70–80% at 24 h. This indicates that the excision repair of UV-B-induced pyrimidine dimers is very efficient in both BCNS and normal human skin fibroblasts.

DISCUSSION

The results presented here demonstrate that early passage fibroblasts established from unexposed skin biopsies of BCNS patients were hypersensitive to killing by UV-B radiation as compared with the skin fibroblasts from age- and sex-matched normal individuals. The skin fibroblasts from BCNS patients were 3- to 6-fold more sensitive, depending upon the survival point at which the sensitivity factor was calculated, to the cytotoxic effects of UV-B than the skin fibroblasts from normal individuals. Furthermore, the BCNS skin fibroblasts exhibited a biphasic survival curve in which there was a sharp decline in survival in the early portion of the survival curve. These results suggest that an unusually sensitive subpopulation of cells may exist in cultures derived from BCNS patients. In contrast to the increased sensitivity of BCNS skin fibroblasts to killing by UV-B radiation, their sensitivity to killing by UV-C radiation was similar to that of normal human skin fibroblasts. These results are in agreement with those of Little et al. (20) and Lehmann et al. (22) who also found that skin fibroblasts from BCNS patients exhibited normal survival following UV-C irradiation. However, Nagasawa et al. (23) have reported that BCNS fibroblasts were slightly more sensitive to UV-C radiation than normal fibroblasts.

The increased sensitivity of BCNS skin fibroblasts to killing by UV-B radiation cannot be attributed to a defect in the repair
of cyclobutane-type pyrimidine dimers because the excision repair kinetics of UV-B-induced pyrimidine dimers in BCNS skin fibroblasts was similar to that of normal human skin fibroblasts. Once again, these results are in agreement with those of Lehmann et al. (22), who found that BCNS fibroblasts irradiated with UV-C radiation exhibited normal levels of dimer excision and postreplication repair. However, Nagasawa et al. (23) and Ringborg et al. (32) reported that BCNS cells may have a reduced capacity for the repair of some type of UV-induced DNA damage, as determined by unscheduled DNA synthesis. Our results suggest that BCNS cells may have a defect in the repair of some other, as yet unidentified, UV-B-induced photoproduct. Alternatively, the increased sensitivity of BCNS skin fibroblasts to killing by UV-B radiation could be due to reduced repair of specific gene lesions. Recent studies have shown that fibroblasts from Cockayne’s syndrome patients exhibit normal repair of genomic DNA lesions but are deficient in the repair of specific DNA lesions in actively transcribing regions of DNA (33). In addition, Bohr et al. (34) found that efficient removal of pyrimidine dimers in the dihydrofolate reductase gene in Chinese hamster ovary cells correlated with survival, indicating that repair in essential genes is perhaps the ultimate criterion for cell survival following UV irradiation.

Analogous to BCNS, individuals with the genetic disorder XP are also predisposed to sunlight-induced skin cancers. However, our studies indicate that skin fibroblasts from BCNS patients exhibit unique characteristics that are quite different from skin fibroblasts from XP patients. While XP skin fibroblasts are hypersensitive to killing by UV-C radiation (35, 36) and are defective in excision repair of UV-induced pyrimidine dimers (37, 38), BCNS skin fibroblasts exhibit similar UV-C survival and excision repair of pyrimidine dimers as normal human skin fibroblasts. This suggests that the molecular lesions responsible for cell killing and induction of skin cancers in XP and BCNS patients may be different. If this is true, then somatic cells from BCNS patients may be of value as a new model system for studying the molecular mechanisms of UV carcinogenesis.

Recent studies indicate that UV radiation also induces non-dimer photoproducts such as (6-4) photoproducts (39-41), cytosine photohydrates (42-44), purine photoproducts (45), and single-strand breaks in the DNA (46-48). Various data suggest that (6-4) photoproducts may play a role in UV-induced lethality and mutagenesis (49-51). Interestingly, Cleaver et al. (52) found that a revertant XP cell line obtained by ethyl methanesulfonate mutagenesis of XP12RO cell line exhibited differential repair of pyrimidine dimers and (6-4) photoproducts. While the parent XP cell line failed to repair both these photoproducts, the revertant XP cell line was able to repair (6-4) photoproducts but not pyrimidine dimers. These results suggest that in human cells (6-4) photoproducts may play a more important role in UV-induced lethality and mutagenesis than pyrimidine dimers. On the other hand, it is also possible that generation of Dewar bases from (6-4) photoproducts, a process that is preferentially catalyzed by UV-B radiation (53-57), may be involved in the UV-B hypersensitivity of BCNS skin fibroblasts. It is reported that cytosine photohydrates and purine photoproducts, while sparsely formed by UV-C irradiation, are maximally formed by UV-B irradiation (42-45). Therefore, these two photoproducts may also be involved in the hypersensitivity of BCNS skin fibroblasts to killing by UV-B radiation. It remains to be determined whether deficient repair of any of these non-dimer photoproducts is involved in the preferential killing of BCNS cells by UV-B radiation.

In summary, our studies indicate that there is an association between hypersensitivity of somatic cells to killing by UV-B radiation and the genetic predisposition to BCC in BCNS patients. In addition, our results suggest that DNA lesions (and repair processes) other than the pyrimidine dimer may also be involved in the pathogenesis of sunlight-induced BCC in BCNS patients. More important, we may be able to use the UV-B sensitivity assay as a diagnostic tool to identify presymptomatic children and adults of BCNS-affected families who are predisposed to cancer. Lastly, since the FS40 sunlamp used in these studies also emit substantial wavelengths in the UV-A (320-400 nm) region, it is possible that the skin fibroblasts from BCNS patients may also be hypersensitive to killing by UV-A radiation. This is currently under investigation.

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