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ULTRAVIOLET-INDUCED DNA REPAIR SYNTHESIS IN LYMPHOCYTES FROM PATIENTS WITH ACTINIC KERATOSIS

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Actinic keratosis is an epidermal cancer in situ. Extensive exposure to sunlight is considered as a contributing factor to the etiology of this tumor. Ultraviolet (UV) light of solar radiation induces structural damage in DNA, which may give rise to mutations and transformed cells if the damage is not repaired. Repair of UV-induced DNA lesions is an essential property of human cells. The conditions so far reported to have defective DNA repair are all associated with an increased incidence of malignancy. Do patients with actinic keratosis also exhibit a reduced capacity to repair UV-induced DNA lesions?

DNA repair synthesis in peripheral leukocytes was studied in 10 patients with actinic keratosis and 10 healthy subjects of corresponding age. After irradiation with various doses of UV light the leukocytes were incubated for 2 hr with [^{3}H]thymidine in the presence of hydroxyurea. A dose-response relationship for the UV-induced DNA repair synthesis was established for each individual. The average repair capacity in the patients with actinic keratosis was about 30% below that of the controls. The difference is statistically significant ($p < 0.02$). Reduced DNA repair synthesis may therefore be an important factor in the etiology of actinic keratosis.

Actinic keratosis is a premalignant lesion of the epidermis. Extensive exposure of the skin to sunlight is generally considered as an important factor [1] in the etiology of this condition. Subjects with fair complexions and people in outdoor occupations develop these changes more frequently than others, which indicates that individual pigmentation as well as the degree of exposure are contributing factors. This type of cancer in situ may slowly progress to squamous cell carcinoma in 12 to 13% of the patients left untreated [2].

The mechanism by which solar radiation causes transformation of epidermal cells is not known. However, the carcinogenic effect of ultraviolet (UV) radiation is well documented in animal experiments and epidemiologic evidence suggests a causal relationship of sunlight to skin cancer in man [3]. UV light causes structural damage of DNA [4] which may give rise to mutations and transformed cells if not properly repaired. Normal human cells possess enzymatic mechanisms for the repair of UV-induced DNA lesions [5]. Lymphocytes and fibroblasts from patients with the autosomal recessive disorder xeroderma pigmentosum show defects in the repair of UV-induced DNA changes [6–10]. These patients suffer from an extreme sensitivity to sunlight and characteristically develop skin cancer [11]. Thus, defective repair mechanisms may decrease the resistance of epidermal cells to the harmful effects of solar radiation, and predispose to cutaneous malignancies. It then appears plausible that a decreased ability to repair UV-induced DNA lesions could predispose to the development of actinic keratosis as well. We have studied the UV-induced DNA repair synthesis in peripheral leukocytes from 10 patients with actinic keratosis and from 10 healthy control subjects.

MATERIALS AND METHODS

Patients

Ten patients, 5 men and 5 women 53 to 81 years old, with actinic keratosis were analyzed. Only cases with multiple epidermal lesions were included and the diagnosis was confirmed by histologic examination in each case. Besides the actinic keratosis the patients were considered healthy. Control material was collected from 10 apparently healthy individuals, 5 men and 5 women, in the age range of 53 to 80 years. The white cell count and percentage of peripheral lymphocytes were within the normal range for all subjects. Blood smears were made and analyzed for the differential distribution of leukocytes. No differences were found between the two groups of patients and controls.

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Abbreviations:
HU: hydroxyurea
PBS: phosphate-buffered saline
TCA: trichloroacetic acid
UV: ultraviolet

DNA Repair Assay

The UV-induced DNA repair was measured essentially according to Evans and Norman [12]. Peripheral leukocytes were obtained from 10–20 ml of freshly collected, heparinized venous blood. White cells from each donor were washed and resuspended in phosphate-buffered saline (PBS) and distributed into 7 plastic Petri dishes at a cell density of about $5–10 \times 10^6$ cells/5 ml of PBS. Five dishes were exposed to UV light (254 nm) at four doses between 3.2–19.2 J/m^2 while slowly rocking to allow optimal dispersion of the cells. The other dishes were left unexposed on the bench during the irradiation and served as controls for background incorporation. The UV light was delivered by two parallel, low-pressure mercury vapor lamps (Philips, TUV 6 w) which produced 0.64 J/m^2/sec. The cells were transfered to centrifuge tubes immediately after irradiation, pelleted, and resuspended in 1 ml of Parker-199 medium (Flow Laboratories) supplemented with 25% of fetal calf serum, 125/μg of streptomycin, and 125 IU of benzyl penicillin. Samples from each tube were taken for cell counting. Hydroxyurea (HU) at a final concentration of 10^{-2} M was added to 5 of the tubes, including 1 of the unexposed controls. Two tubes, including 1 unexposed control and 1 sample which was given a UV dose of 9.6 J/m^2, did not receive HU. All tubes were preincubated for 30 min at 37°C before addition of tritiated thymidine ([^{3}H]thymidine, 5 Ci/mM, 1 mCi/ml, The Radiochemical Centre, Amersham, England) to a final concentration of 10 μCi/ml. Incubation continued for 2 hr, and was stopped by the addition of 1 ml of cold 10% trichloroacetic acid (TCA) to each tube. Extraction of free nucleotides took place for 30 min at +4°C. Two more washes in cold 5% TCA were carried out. The pellet was then resuspended in 70% ethanol and collected on a glass-fiber filter. After drying at 60°C for 2 hr or overnight, the filters were placed in scintillation vials and treated with 0.6 ml of solubilizer (60 ml of Soluene-350, Packard, 20 ml of methoxyethanol, 8 ml of water) to release the radioactivity. Ten milliliters of scintillation fluid (5.5 gm of Permablend, Packard, in 1000 ml of toluene) was added and the vials analyzed for radioactivity in a Packard liquid scintillation spectrometer, at an efficiency of about 30% and a background of 20 cpm.

RESULTS

Effect of Hydroxyurea on DNA Replication and DNA Repair Synthesis

Hydroxyurea has an apparently differential effect on DNA replication and DNA repair synthesis [13]. The drug does not affect UV- or alkylation-induced DNA repair synthesis in mammalian cells, but effectively inhibits replicative DNA synthesis. HU has therefore been used to uncover the relatively small thymidine incorporation due to repair synthesis from the incorporation due to replicative DNA synthesis of cells in S-phase [12,14].

The results in Table I demonstrate the effect of HU on DNA synthesis in nonirradiated cells. The replicative DNA synthesis in the absence of the drug is about 25% lower in leukocytes from patients with actinic keratosis, but not statistically different from that of controls. HU inhibits the uptake of [^{3}H]thymidine by over 90%, and the remaining background incorporation in the two groups (66 vs 77 cpm/10^6 cells) is not statistically different.

TABLE I. *The effect of hydroxyurea on DNA synthesis in nonirradiated cells*

Concentration of hydroxyurea	Incorporation of [^{3}H]thymidine (cpm/10^6 cells ± SD)	
	Controls (n = 10)	Actinic keratosis (n = 10)
0	1,238 ± 417	955 ± 343
10^{-2} M	77 ± 15	66 ± 21

Cells irradiated with a UV dose of 9.6 J/m^2 demonstrated about 40% reduction in the incorporation of [^{3}H]thymidine when incubated in the presence of HU (Tab. II). The results in Tables I and II show that HU affects the incorporation of [^{3}H]thymidine into nonirradiated cells as well as into irradiated cells to about the same extent in the control group as in the group of patients with actinic keratosis.

It is also clear (Tab. III) that the UV-induced increase in [^{3}H]thymidine incorporation is greater in the presence than in the absence of HU. This difference may be explained by the inhibitory effect of UV irradiation on DNA replication, which tends to reduce the replicative DNA synthesis onto which incorporation due to repair synthesis is added. Thus, the UV-induced DNA repair synthesis is uncovered by HU (Tab. III).

In the absence of HU the UV-induced incorporation of [^{3}H]thymidine is more than 2-fold lower in the group of patients with actinic keratosis compared to the group of control subjects, while in the presence of the drug, the incorporation in the patient group is only 30% lower than the controls (Tab. III). Therefore, for valid comparisons between DNA repair activity in groups of controls and diseased subjects, it is essential that the background incorporation due to DNA replication is maximally inhibited, and that measurements are carried out in the presence of HU.

Dose Dependence of UV-Induced DNA Repair Synthesis

Leukocytes from patients with actinic keratosis and from healthy controls of the same age range were irradiated by four different UV doses, and incubated in the presence of 10^{-2} M HU to accomplish an optimal reduction of DNA replication. The incorporated activities at each dose level for all subjects are shown in the Figure, together with the group mean values. In both groups the incorporation plateaus at UV doses above 9.6 J/m^2. The average amount of incorporated activity was approximately 25 to 30% less in the patients with actinic keratosis compared to the control subjects at all UV doses given. The individual variation was great, however, and there was a considerable overlap in repair activities between subjects of the two groups.

TABLE II. *The effect of hydroxyurea on DNA synthesis irradiated cells*[a]

Concentration of hydroxyurea	Incorporation of [^{3}H]thymidine (cpm/10^6 cells ± SD)	
	Controls (n = 10)	Actinic keratosis (n = 10)
0	1,613 ± 348	1,106 ± 377
10^{-2} M	1,012 ± 259	702 ± 246

[a] The cells received a UV dose of 9.6 J/m^2.

TABLE III. *The effect of hydroxyurea on the UV-induced DNA repair synthesis*

The UV-induced DNA repair synthesis was calculated by subtracting the incorporation of [^{3}H]thymidine into nonirradiated cells (Tab. I) from the incorporation into irradiated cells which received a UV dose of 9.6 J/m^2 (Tab. II).

Concentration of hydroxyurea	UV-induced DNA repair synthesis (cpm/10^6 cells)	
	Controls	Actinic keratosis
0	375	151
10^{-2} M	935	636

Statistical Comparison Between DNA Repair Synthesis in Leukocytes from Patients with Actinic Keratosis and Controls

For the statistical analysis, the UV-induced DNA repair synthesis was calculated for each individual. This value was obtained by subtracting the HU-inhibited background incorporation of nonirradiated cells from the mean of the incorporated activities at the two highest UV doses.

The total intra-individual variation of UV-induced DNA repair synthesis was estimated by repeated measurements on one separate control subject. Blood samples were taken at 9 different occasions during a 2-month period. The UV-induced [^{3}H]thymidine incorporation was calculated at each occasion. The mean ± SD was found to be 812 ± 133 (n = 9) (data not shown). The variation between individual subjects among those who had actinic keratosis and among those within the control group of the present study was considerably greater (Fig. and Tab. IV). According to F-analysis, the intra-individual variance is significantly smaller than the variance among the 10 control subjects ($F_{8;9} = 3.9$, $p < 0.05$, one-tailed test). It is likely, therefore, that there is a biologic difference between individuals with regard to DNA repair efficiency.

TABLE IV. *UV-induced DNA repair synthesis in leukocytes from patients with actinic keratosis and control subjects: statistical analysis*

Subjects		UV-induced DNA repair synthesis (cpm/10^6 cells)[a]		Rank No.
Sex	Age	Controls	Actinic keratosis	
f	58	1,560		1
m	71		1,145	2
f	61	1,028		3
m	80	1,025		4
m	62	991		5
m	59	971		6
f	62	872		7
m	60		862	8
f	81		852	9
m	77	820		10
m	62	774		11
f	53	761		12
f	62	692		13
f	78		672	14
f	68		668	15
f	53		605	16
m	70		568	17
m	67		516	18
m	74		409	19
f	53		389	20
Mean value ± SD		949 ± 245	669 ± 231	
t-test		$0.01 < p < 0.02$		
Sum of rank numbers		72	138	
Rank test		$0.01 < p < 0.02$		

[a] This value was calculated for each individual by taking the mean of the incorporated activity at the 2 highest UV doses (9.6 and 19.2 J/m^2) minus the HU-inhibited background activity (O J/m^2)

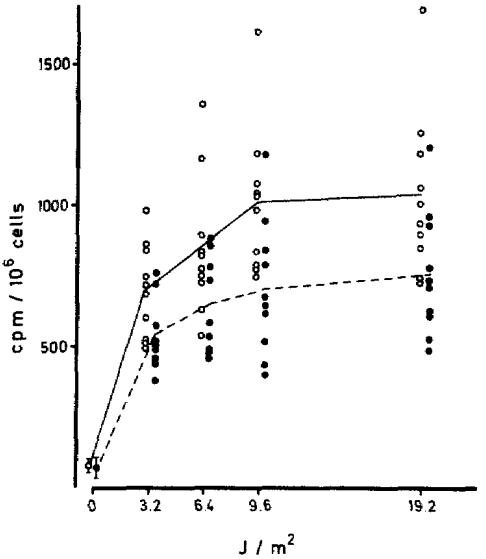

FIG. Dose dependence of UV-induced DNA repair synthesis. Peripheral leukocytes were irradiated with various UV doses, preincubated for 30 min in 10^{-2} M hydroxyurea, and subsequently incubated for 2 hr in the presence of 10 μCi/ml of [^{3}H]thymidine and 10^{-2} M HU. The *solid line* connects the group mean values of each UV dose for control subjects (○), and the *broken line* represents the group mean values of each UV dose for patients with actinic keratosis (●).

The statistical comparison between patients with actinic keratosis and controls with regard to UV-induced DNA repair synthesis is shown in Table IV. According to both the *t*-test and the Wilcoxon parameter-free rank test, the UV-induced DNA repair synthesis is significantly lower ($p < 0.02$) in leukocytes from patients with actinic keratosis than in normal subjects.

DISCUSSION

The most common lesions introduced into DNA by UV irradiation are thymidine dimers, i.e., the formation of cyclobutane structures between neighboring thymidine molecules in the same strand [4]. In the repair of these lesions the dimer is excised together with approximately 100 bases producing a single-strand gap, which is subsequently healed by a polymerase-dependent reaction using the complementary strand as a template [5]. Thus, the measurement of UV-induced DNA repair synthesis by incorporation of [^{3}H]thymidine, as adopted in this report, essentially reflects the gap-filling process, although the excision step should be regarded as a prerequisite for the polymerase action.

In a heterogeneous population of cells, such as peripheral leukocytes, UV-induced DNA repair synthesis goes on simultaneously with DNA replication in S-phase cells. Thus, it is essential to sort out which amount of incorporation of the labeled precursor is due to DNA repair synthesis and which is due to DNA replication. As shown by the present results this can be effectively brought about by the use of HU, which inhibits replication but does not affect repair synthesis to any greater extent [13,14].

Peripheral lymphocytes have an initial rate of DNA-repair synthesis which is greater than that of peripheral granulocytes by a factor of about 10 [15]. Therefore, the repair activity of the total, heterogeneous mixture of peripheral leukocytes is likely to be dominated by the repair synthesis in the lymphocytes. Thus, differences between individuals in the proportion of lymphocytes among peripheral leukocytes would be expected to influence the repair level of the total leukocyte fraction. However, no such differences in the percentage of peripheral lymphocytes or differential white cell counts were found between the two groups of controls and patients with actinic keratosis (data not shown). Neither could the individual differences with regard to UV-induced DNA repair activity recorded among subjects of the control group as well as among the patients be attributed to differences in peripheral lymphocyte counts. We therefore conclude that the significantly decreased UV-induced incorporation of [^{3}H]thymidine into leukocytes from patients with actinic keratosis compared to normal subjects very likely reflects differences in the activity of DNA repair enzymes.

Reduced repair synthesis of UV-induced DNA lesions has been reported for fibroblasts and lymphocytes of patients with xeroderma pigmentosum [6,10], for fibroblasts of patients with Fanconi's anemia [16], and for lymphocytes of patients with Down's syndrome [14]. The impairment of DNA repair functions in these conditions is probably genetically regulated. These conditions are also associated with an increased incidence of malignant disease, which suggests a relationship between defective DNA repair and malignancy. UV light-induced thymine dimers, which are not effectively excised from the DNA of epidermal cells, may give rise to accumulation of DNA damage with increased risk for somatic mutations and malignant transformation to occur [17]. The results of the present paper are in accord with this hypothesis, assuming that the decrease in DNA-repair efficiency observed for leukocytes of patients with actinic keratosis also affects the epidermal cells. Further evidence in favor of this hypothesis could be derived from similar studies on dermal fibroblasts from unaffected skin of patients with actinic keratosis. Such studies are in progress.

The present results do not, however, reveal the nature of the defect responsible for the decreased DNA-repair efficiency in patients with actinic keratosis. Although the [^{3}H]thymidine incorporation mainly reflects the polymerase function, both the incision step and the excision step of the tentative repair procedure may influence the polymerase activity. Moreover, it cannot be excluded

that the cells of the patients with actinic keratosis would ultimately reach the same level of UV-induced [^{3}H]thymidine incorporation as the controls, given sufficient time for repair synthesis. These, and other possible explanations not considered here, will have to be elucidated by other methods than that of the present study. The most likely interpretation of the decreased UV-induced [^{3}H]thymidine incorporation into leukocytes from patients with actinic keratosis is impairment or delay of the function of one or several enzymes involved in excision repair in these cells. We suggest this may be one important factor in the etiology of the disease. Subjects with a reduced DNA repair activity may therefore have an increased risk to develop actinic keratosis at higher ages. We also showed, however, that there are great individual differences in the UV-induced DNA repair synthesis which are likely to be of biologic origin. Several patients with actinic keratosis approach the mean values for the control group, and one patient was shown to have the second highest value among all subjects in the two groups. It is clear, therefore, that other previously suggested factors, such as individual pigmentation and the degree and duration of exposure to sunlight may contribute as well to the etiology of actinic keratosis.

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