DNA REPAIR AND CELL SURVIVAL PROPERTIES AFTER UVA AND UVB IRRADIATIONS OF EPIDERMAL KERATINOCYTES FROM NORMAL AND HYPERPHOTOSENSITIVE XERODERMA PIGMENTOSUM AND TRICHOTHIODYSTROPHY INDIVIDUALS

Ph.D. Thesis Summary

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I. INTRODUCTION AND AIMS

Skin cancers are the most frequent neoplastic afflictions in human beings. The large majority of skin cancers can be divided into melanomas and nonmelanoma neoplasms. Two types of nonmelanoma skin cancers are known, the basal cell carcinoma (BCC) and the squamous cell carcinoma (SCC). BCCs and SCCs develop from epidermal keratinocytes, whereas melanomas originate from melanocytes. Melanocytes and keratinocytes can be considered as the primary cellular targets of solar ultraviolet radiations that reach the Earth's surface (UVB, 290-320 and UVA, 320-400 nm).

The potential catastrophic effects of ultraviolet radiations are mimicked in some hyperphotosensitive genodermatoses, such as in the xeroderma pigmentosum (XP), a pathological condition where skin cancer incidence is very much increased.
UVC and UVB radiations are known to be photochemically active and potent genotoxic agents. Lesions generated by short UVB wavelengths and by UVC are mostly cyclobutane pyrimidine dimers (CPDs). In addition to CPDs, the 6-4 pyrimidine-pyrimidone photoproducts (6-4 PPs) may be formed. In the absence of efficient DNA repair, e. g. in XP cells, these lesions persist in the genome and may lead to the introduction of deleterious mutations after DNA replication. Nucleotide excision repair (NER) is of central importance in the recovery of cells from radiation damage. NER is the process whereby DNA damage is removed as part of an oligonucleotide fragment, followed by replacement with new DNA using the intact strand as template. NER is defective in patients with XP.

Deficiency in NER is also associated with two other rare but non-cancer-prone disorders, trichothiodystrophy (TTD) and Cockayne syndrome. Complementation studies established that the XP phenotype could result from defects in one of seven or more genes (*XPA - XPG)*, all of which seemed to be required at an early stage of the NER process. The defining feature of TTD is sulphur-deficient brittle hair, which is associated with reduced size, mental retardation, unusual facial features, ichthyosis, and in many, but not all cases, sun sensitivity. Complementation studies have assigned the defect in the majority of TTD cells to the *XPD* gene. Prolonged UV exposure results in inhibition of DNA synthesis and subsequent resumption of the cell cycle following *repair* or *apoptosis*. To date, only few studies have analyzed the responses of non-transformed keratinocytes and dermal fibroblasts from normal individuals to UV radiations.

To date almost all efforts devoted to the characterization of the XP phenotype have been focused on the molecular mechanisms of DNA repair itself rather than on the physiological response of epidermal cells after UV irradiations. Paradoxically, most studies have been performed using dermal fibroblasts, but only very few have included the study of DNA repair properties and UV survival of epidermal keratinocytes, those cells from which epidermal cancers originate.

The specific aims of this thesis were:

1. To compare the repair capacities of UV-induced DNA lesions of keratinocytes from normal, XP (XP-C, XP-D) and TTD/XP-D individuals (by unscheduled DNA repair synthesis).

2. To compare the post-UV survival of these keratinocytes after seeding at clonal density using UVA and UVB rays which reach the Earth's surface and penetrate into the skin.

3. To study the responses of the different types of keratinocyte colonies after increasing doses of UV (UVA and UVB) irradiation.

4. To evaluate the differences in the post-UV response of fibroblasts and keratinocytes derived from the same individuals (cell survival and DNA repair capacities).

5. To determine differences related to UV irradiation-induced DNA lesion numbers between fibroblasts and keratinocytes , which may contribute to the better understanding of skin photocarcinogenesis in man.

6. To investigate the role and rate of apoptosis in post-UV keratinocyte cell death and also to determine the participation of apoptosis in the clonal transition of keratinocytes induced by UV irradiation (by the TUNEL and the DNA fragmentation assay methods).

II. MATERIALS AND METHODS

Biopsies were obtained after approval of an XP-C, an XP-D, a TTD/XP-D and a normal individual from non-sun exposed skin areas. Fibroblasts and keratinocytes were derived from the same donors except for normal cells. Human epidermal keratinocytes of normal, XP and TTD individuals were obtained and cultured as described by Rheinwald and Green (1975) on a feeder layer of X-ray irradiated (60 Gy) Swiss 3T3 fibroblasts.

1. UV radiation sources

UVA radiation was provided by a metal halide lamp equipped with anticaloric filter KG1 and UVB cut-off filter WG 335.

The UVB radiation source was the Transilluminator banc, Spectroline, equipped with cut-off filter WG 305. UVC irradiations were performed at 254 nm from a germicidal lamp.

2. UV cell irradiation

Irradiations were performed on 60-70% confluent cells. The cells were first rinsed twice with phosphate-buffered saline (PBS) prewarmed at 37°C and irradiated under a film of PBS for UVA irradiation and without PBS for the short periods needed for UVB and UVC irradiations.

3. Determination of cell survival

Clonal analysis of keratinocytes: cells were grown in 60 mm Petri dishes for about five days and then irradiated under UVA (doses ranging from 100,000 to 500,000 J/m^2) or UVB (100 to 1200 J/m^2). Immediately after irradiation cells were trypsinized (in 0.1% trypsin and in 0.02% EDTA) and counted. Keratinocytes were seeded at clonal density (35 $cells/cm^2$) onto

previously irradiated 3T3 Swiss fibroblasts. Cultures were maintained in keratinocyte medium containing 10% FCS. After 12 days, keratinocyte colonies were fixed in 3.7% formaldehyde in PBS and coloured by 1% rhodamine B. The relative cell survival was calculated as the number of colonies obtained after UV irradiation per total colony number obtained from unirradiated cells x 100.
The colony-forming ability of fibroblasts was determined by seeding after irradiation increasing cell numbers as a function of UV doses (from 1 x 10^3 to 8 x 10^3 cells per 100 mm dish). Cells were maintained in culture for 14 days and were stained by crystal violet.

4. UDS analysis

After UV irradiation keratinocytes and fibroblasts were seeded on glass coverslips, DNA repair was followed by ^{3}H-thymidine incorporation. Coverslips with cells were mounted onto glass slides, dipped in Amersham EM-1 photoemulsion. The exposure time was one week at 4°C for keratinocytes and three to four days for fibroblasts. In any case, the mean number of grains per nucleus was obtained by counting 80 non-S-phase nuclei for each UV dose.

5. Immuno slot-blot analysis of CPD and 6-4 PP lesions in genomic DNA

Exponentially growing fibroblasts and keratinocytes were UVB irradiated at a dose of 1000 J/m^2. Following irradiation DNA was prepared and purified DNA was loaded on PVDF membrane. Membranes were then immersed in PBS containing 5% low-fat dried milk and 0.1% Tween 20 for 1 hour at room temperature and washed in the same buffer containing either anti-(6-4) PP or anti-CPD monoclonal antibodies for 1 hour at 37°C in an orbital shaker. Chemifluorescent detection was carried out using the ECF western blotting kit (Amersham).
Precise quantifications of signals were obtained after scanning the blots on a FluorImager (Molecular Dynamics) using the ImageQuant software (Amersham).

6. TUNEL method

TdT-mediated dUTP nick end labeling (TUNEL) reaction was carried out in duplicate using the *In Situ* Cell Death Detection Kit (Boehringer Mannheim, Germany) on 4% formaldehyde fixed keratinocytes grown on coverslips. The kit uses fluorescein-dUTP to label DNA strand breaks generated preferentially during apoptosis. TUNEL-positive cells can be detected directly after the enzymatic reaction by fluorescence microscopy or after an amplification reaction using a secondary anti-fluorescein antibody conjugated with peroxidase. In this latter case the TUNEL-positive cells are visualized by light microscopy after incubation of cells with diaminobenzidine. Samples were observed with a Zeiss Axiovert 135 microscope (magnification, 80X and 100X for quantification of labeled cells) and prints were obtained using a Mavigraph color video printer. Values represent percentages from at least 1000 counted apoptotic and non-apoptotic cells on each coverslip.

7. DNA fragmentation analysis

Cells were washed in PBS, trypsinized, resuspended in STE buffer with 0.1% SDS and 20 mg/ml of proteinase K. Samples were incubated overnight at 56°C. DNA was extracted with phenol, chloroform/isolamyl alcohol and precipitated at -20°C with ethanol and with sodium acetate 3M. DNA pellet was resuspended in TE buffer pH 7.4. Characterisation of DNA cleavage was obtained by submitting the samples to electrophoresis on 1.5% agarose gel in TBE buffer

8. Immunostaining

Antibodies : Mouse monoclonal antibodies were against human keratin 10, human keratin 14, human β1 integrin. A monoclonal rat anti-human-α6-integrin IgG was used as well. A monoclonal rabbit antibody was against human involucrin. Fluoro-iso-thio-cyanate-conjugate rabbit anti-mouse or anti-rat immunoglobulins or TRITC-conjugate swine anti-rabbit immunoglobulins were used as second antibodies. Samples were fixed in acetone at 4°C for 5-10 minutes then incubated at room temperature for 30 minutes with the first antibody, rinsed with PBS, incubated with the second conjugated antibody for 30 minutes, washed, and mounted before being observed under a fluorescence microscope.

9. Western blotting

Total proteins were extracted in 8 M urea. Protein samples were separated by 7,5% SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membrane.
Anti-β1 integrin antibodies were diluted and samples were incubated for 1 hour. Membranes were then incubated with secondary anti-rabbit antibodies coupled to peroxidase (dilution used was: 1:5000 in PBS-Tween) for 1 hour. Finally, membranes were revealed using the ECL reagent (Amersham).

10. Statistical study

Means were compared using Mann-Whitney and Student's-t tests. A result was considered as not significant when its associated P-value *(p)* was above 0.05.

III. RESULTS

1. DNA repair capacity and quantitative assessment of UV-induced DNA lesions

Data obtained after irradiation with increasing doses of UVB and UVC show that the increase of grain numbers in normal cells was virtually linear. The lowest UDS values were found in XP-C (about 10 % of those from WT cells) and in XP-D (about 20 % of WT) cells. Intermediate UDS values were found in TTD/XP-D cells (about 27-39 %). In all cell types, UDS values obtained in fibroblasts were significantly higher than those measured in the

corresponding keratinocytes. The number of both 6-4PP and CPD DNA-lesions in keratinocytes is about 50 % of that found in fibroblasts for a UVB dose of 1000 J/m^2.

2. Cell survival measurement as the colony forming efficiency (CFE) after UV rradiation

The survival rates of the studied fibroblasts after UVA irradiation allowed us to draw a survival hierarchy : WT > TTD/XP-D > XP-C > XP-D. The survival hierarchy obtained after UVB irradiation was WT > TTD-XP-D > XP-C > XP-D. Cell survival following UVA irradiation in keratinocytes: all keratinocyte strains were exposed to three irradiation doses from 100,000 J/m^2 up to 500,000 J/m^2. The survival curves of normal, TTD-XPD and XP-D keratinocytes were very similar for UV doses below or equal to 250,000 J/m^2. In the same dose-range, the resistance of XP-C keratinocytes was significantly lower than that of other strains.
Cell survival following UVB irradiations: XP-D keratinocytes were the most sensitive cell type. The resistance to UV of TTD/XP-D cells was similar to that of normal cells up to a dose of 400 J/m^2, and then abruptly decreased. The survival curve of XP-C keratinocytes was intermediate between TTD/XP-D and XP-D cells.

3. DNA repair and cell survival following UVB irradiation

At all doses, the XP-D keratinocyte strain was the cell strain most sensitive to UVB radiations. In contrast, the TTD/XP-D keratinocyte strain was as resistant as WT keratinocyte strain up to a threshold dose of 450 J/m^2 UVB. The XP-C keratinocyte strain displayed an intermediate survival level to UVB although they DNA-repair was the lowest.

4. Does the UV-induced transition from proliferative to abortive colony reflect a protection against cancer?

Epidermal keratinocyte cultures grown at clonal density on lethally irradiated 3T3 cells can give rise to three types of colonies i) colonies that are large (10- 30 mm^2) and have a smooth perimeter (LSP), which are rapidly growing colonies composed of cells with a proliferative potential expected from stem cells; ii) small (<5mm^2), highly irregular and terminal (ST) colonies, wich are founded by a cell with a short replicative lifespan, condemned to terminal differentiation; in these colonies all proliferation has ceased at the time of rhodamine staining (i.e. 12 days after seeding); iii) wrinkled (W); wrinkled colonies founded by a cell whose progeny has heterogeneous growth capacities, i.e. transitional between the two mentioned before. Exposure to both UVA and UVB radiation induced changes in the relative representations of the three different keratinocyte colony types. In general, the number of LSP colonies decreased whereas those of abortive colonies increased. Interestingly, the behaviour of keratinocytes from the XP-C patient was distinct from that of other cell strains since no significant changes in the relative number of each clonal type could be recorded after UVA and UVB irradiation. The relative resistance to UV of cells with high growth capacities (i.e. cells forming large with smooth perimeter and wrinkled colonies) may explain the dramatic predisposition of the corresponding patient to develop cancer.

Conversely, UV-induced transition from proliferative to abortive colonies might reflect a protection against cancer development.

5. Differential induction of apoptosis in normal, XP-C, XP-D and TTD/XP-D keratinocytes

At both 24 and 48 hours after irradiation with 1000 J/m^2 UVB, the hierarchy of the cell strains studied concerning their susceptibility to apoptose was: WT < XP-C < TTD/XP-D < XP-D. At 48 hours after irradiation the percentages were: 15% in WT cells, 65% in XP-C cells, 80% in TTD/XP-D cells and 100% in XP-D cells. The DNA fragmentation assay might be more accurate in visualising the rate of apoptosis, since DNA of cells floating in the culture medium (many of which are apoptotic), are also included in this verification test. The same hierarchy concerning the susceptibility for apoptosis could be drawn from this test than from the TUNEL-DAB method: WT < XP-C < TTD/XP-D < XP-D. We found that all the different cells studied were capable of undergoing DNA degradation following UV irradiation. In the studied cell strains, XP-D was by far the most sensitive to the induction of apoptosis following UVB irradiation.

6. Immunocytochemistry and Western blotting

The involucrin labelling became stronger and the percentage of involucrin positive cells became higher after increasing doses of UVB (500 and 1000 J/m^2 UVB; at 24 and 48 hours after irradiation) in all studied cell types. The involucrin signal was more intense in the case of XP-C cells. From the double TUNEL-involucrin labelling experiments the following conclusion could be drawn: double labelled and uniformly labelled cells could be detected as well. Nevertheless, it seemed that there were more involucrin positive-TUNEL negative cells than double-labelled cells indicating that differentiating keratinocytes are not the only cells, which apoptose and basal keratinocytes (involucrin negative cells) may undergo apoptosis as well. A preliminary experiment is demonstrated using $\beta 1$ integrin, polyclonal antibodies. Non-irradiated XP-D and XP-C keratinocytes after 250 J/m^2, 500 and 1000 J/m^2 UVB irradiation. After irradiation with 500 and 1000 J/m^2 UVB, it diminished gradually in case of XP-D cells. At 24 hours, 1000 J/m^2, the band almost completely disappeared in XP-D cells, while it was still well detectable in XP-C cells.

IV. SUMMARY OF THE THESIS

The differential behavior of keratinocytes and fibroblasts from normal and DNA repair-deficient individuals toward ultraviolet A and ultraviolet B radiations was demonstrated.

1. Keratinocytes were always more resistant (about three or four times) than fibroblasts toward UVA and UVB radiations.

2. The ratio of DNA repair capacity to cell viability is much better in keratinocytes than in fibroblasts.

3. Same doses of UVB radiations induced twice more lesions in the DNA of fibroblasts compared to that of keratinocytes suggesting that keratinocytes have developed a natural shielding protection against UV radiation, to which they are exposed much more than dermal fibroblasts.

4. XP-C keratinocytes were significantly more sensitive toward UVA radiations than any other strains.

5. TTD/XP-D keratinocytes were significantly more resistant than XP-D keratinocytes toward UVB radiations.

6. UVA and UVB irradiation induced a transition from proliferative to abortive keratinocyte colonies. This phenomenon might reflect a protection against cancer since cells with high growth capacities (i.e. cells forming proliferative colonies) gradually disappeared with increasing UV doses. The nature of this transition in different strains, in part, correlated with the cancer susceptibility.

7. UVB irradiation induced apoptosis in all keratinocyte cell strains used in our experiments. The number of apoptotic, DNA repair-deficient (XP-C, TTD/XP-D, XP-D) keratinocytes was much higher than it was found in normal, wild type keratinocytes. Thus, UV radiation-induced apoptosis might be considered as an anti-cancer defence mechanism which is also present in DNA repair-deficient keratinocytes.

V. PUBLICATIONS AND PRESENTATIONS

1. Agnes I. Otto, Lydia Riou, Claire Marionnet, Toshio Mori, Alain Sarasin and Thierry Magnaldo, Differential behaviors toward ultraviolet A and B radiation of fibroblasts and keratinocytes from normal and DNA repair-deficient patients, Cancer Research, *59*: 1212-1218, 1999.

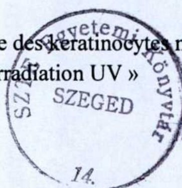
2. I. Otto: Photoaging of skin and ultraviolet radiations (Hungarian), Praxis, 1999. *in press.*

3. I. Otto: Deleterious effects of ultraviolet radiations and skin cancer (Hungarian), Orvosképzés, 1999. *in press.*

POSTERS

4. 1997. 06. 04 - 07.: « Reparation, replication, recombination » congress in Villejuif, France
« Etudes des effets des UV sur la reparation d'ADN dans les keratinocytes normaux et xeroderma pigmentosum »

5. 1998. 12. 01 - 02.: « Congres Annuel de Recherche Dermatologique (CARD)» congress in Paris, France
« Reparation de l'ADN et survie des keratinocytes normaux, de xeroderma pigmentosum et de trichothiodystrophie apres irradiation UV »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ORAL COMMUNICATION

6. 1998. 10. 07 - 11.: « European Academy of Dermatology and Venereology » congress in Nice, France « DNA repair and cell survival of normal and xeroderma pigmentosum keratinocytes after UV irradiation »

PRIZE

7. The prize of the La Roche-Posay Foundation acquired in Paris at the CARD congress (1998)

ABSTRACTS

8. Journal of European Academy of Dermatology and Venereology II. (suppl. 2.) S 163. 1998
« DNA repair and cell survival of normal and xeroderma pigmentosum keratinocytes after UV irradiation » A. Otto, A. Sarasin, T. Magnaldo

9. Annales de Dermatologie et Venereologie 125: 38243. 1998
« Reparation de l'ADN et survie des keratinocytes normaux, de xeroderma pigmentosum et de trichothiodystrophie apres irradiation UV » A. Otto, L. Riou, C. Marionnet, A. Sarasin, T. Magnaldo

10. Journal of Investigative Dermatology, April, 1999
« Keratinocytes from normal, and DNA repair-deficient patients » A. Otto, L. Riou, C. Marionnet, A. Sarasin, T. Magnaldo

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