Albert Szent-Györgyi Medical University of Szeged

Department of Dermatology

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Ph. D. Thesis

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

OTTÓ Iringó Ágnes M.D.



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# INDEX

# PUBLICATIONS

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*: March 15, 1999.

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

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

# ABBREVIATIONS

| | |
|---|---|
| **BCC** | **basal cell carcinoma** |
| **SCC** | **squamous cell carcinoma** |
| **UV** | **ultraviolet radiation** |
| **XP** | **xeroderma pigmentosum** |
| **NER** | **nucleotide excision repair** |
| **CPD** | **cyclobutane pyrimidine dimer** |
| **6-4 PP** | **(6-4) pyrimidine pyrimidone** |
| **ROS** | **reactive oxygen species** |
| **TCR** | **transcription-coupled repair** |
| **GGR** | **global genome repair** |
| **TTD** | **trichothiodystrophy** |
| **CS** | **Cockayne syndrome** |
| **PCD** | **programmed cell death** |
| **UDS** | **unscheduled DNA repair** |
| **PBS** | **phosphate buffered saline** |
| **FITC** | **fluorescein-iso-thiocyanate** |
| **TUNEL** | **TdT-mediated dUTP nick-end labeling** |
| **DAB** | **diaminobenzidine** |
| **WT** | **wild type** |
| **CFE** | **colony forming efficiency** |
| **LSP** | **large with smooth perimeter** |
| **W** | **wrinkled** |
| **ST** | **small terminal** |
| **SBC** | **sunburn cell** |
| **CK** | **cytokeratin** |
| **RPA** | **replication protein A** |
| **RFC** | **replication factor C** |
| **PCNA** | **proliferating cell nuclear antigen** |

# I. INTRODUCTION

Skin cancers are the most frequent neoplastic afflictions in human beings. Their incidence increases with sunny climates and has been dramatically exacerbated by the increased enthusiasm for the beach and sun-tans (1-3). 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). UVB radiations reach the basal epidermal layer which contains keratinocytes with high proliferative potential (4). UVA radiations penetrate throughout the epidermal compartment and reach fibroblasts in the underlying dermis as well (5-8). The skin penetration properties of UVs are shown in *Figure 1.*.

The potential catastrophic effects of ultraviolet radiations are mimicked in some hyperphotosensitive genodermatoses, such as in the xeroderma pigmentosum, a pathological condition where skin cancer incidence is very much increased. Xeroderma pigmentosum (XP) is a rare, autosomal disease characterized by hyperphotosensitivity and a very high (2000 fold) predisposition for developing skin cancers on sunlight exposed areas, and in some cases, neurological disorders (5). XP has a worldwide distribution, with the incidence varying from about 1:250 000 in Europe and the USA to as high as 1:40 000 in Japan, North Africa and in Egypt. Consanguinity of the patients' parents was reported in about 30%, an elevated frequency often seen in recessive disorders. Nearly 20% of the patients had neurologic abnormalities. The cellular phenotype of XP has increased sensitivity to killing following exposure to a wide variety of DNA damaging agents, including UV radiation and UV mimetic chemicals.

*The observation that skin fibroblasts in culture from an XP patient are unable to carry out nucleotide excision repair (NER) following exposure to UV established the relationship between DNA repair defect and skin photocarcinogenesis in man (6).*

## I.1. Ultraviolet light-induced DNA damage and nucleotide excision repair (NER)

UVC and UVB radiations are known to be photochemically active and potent genotoxic agents. These radiations are absorbed by DNA and cause lesions, mainly at neighboring pyrimidines. Lesions generated by short UVB wavelengths and by UVC (7) are mostly cyclobutane pyrimidine dimers (CPDs). CPDs are formed by the covalent interaction of two adjacent pyrimidines in the same polynucleotide chain. In addition to CPDs, the 6-4 pyrimidine-pyrimidone photoproducts (6-4 PPs) may be formed (*see Figure 2.*). 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. Indeed, molecular epidemiology studies have clearly demonstrated that more than 50 % of the DNA of the *p53* tumor suppressor gene in epidermal tumors harbors DNA mutations characteristic of UV irradiation (i.e. CC->TT tandem mutations and C->T transitions) (8). Nearly all (95%) of the mutations in XP are located on the non-transcribed strand while internal or non-XP skin tumors do not show this strand bias. Hence, the mutation spectrum analysed in XP skin tumors also demonstrates the existence of preferential repair in humans (9). Furthermore, in vitro experiments using shuttle DNA reporter vectors transfected in fibroblasts from XP patients have established with certainty the highly mutagenic and distinctive properties of UV induced lesions (10, 11). The absorption of DNA drops off steeply for wavelenghts over 300 nm, and it becomes vanishingly small in the longwave UVA region (wavelength > 340 nm). The induction of DNA damage drops off for wavelengths over 300 nm. However, in the longwave UVA region it plateaus, where molecules other than DNA absorb the UV radiation and radicals, particularly reactive oxygen species (ROS), are generated.

At least three different biochemical repair systems operate in damaged cells to safeguard DNA from permanent damage. These are excision repair, postreplication repair, and photoreactivation. Excision repair is extremely versatile and can mend a large variety of UV-light, X-ray, and chemically induced forms of damage to DNA. Excision repair may be subdivided into nucleotide excision repair and base excision repair.

NER is of central importance in the recovery of cells from radiation damage. Nucleotide excision repair 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. The NER machinery has very broad specificity, being able to recognize a wide variety of chemical alterations to DNA that result in large local distortions of the DNA structure. A major impetus to the study of NER was the discovery in 1968 (Cleaver et al) that NER was defective in patients with the rare, highly cancer prone genetic disorder, xeroderma

pigmentosum. This suggests that NER is a highly effective cancer avoidance mechanism and that DNA-repair genes can be classed as tumor-suppressor genes.

## I.2. History, clinical features of XP and genetic background

*Xeroderma*, or parchment skin, was the term given by Moritz Kaposi to the condition he observed in a patient in 1863 and reported in the dermatology textbook he wrote with Ferdinand von Hebra in 1874. In 1882 the term *pigmentosum* was added to emphasize the striking pigmentary abnormalities. Eye involvement, including cloudiness of the cornea, was recognized by Kaposi. DeSanctis and Cacchione in 1932 described three sibs with cutaneous XP associated with microcephaly, progressive mental deterioration, dwarfism, and immature sexual development - the DeSanctis-Cacchione syndrome. Defective nucleotide excision repair (NER) in UV-irradiated cultured skin fibroblasts from some XP patients was reported by Cleaver in 1968. The form of the disease in the first XP patient with normal excision repair described by Burk *et al* was subsequently named the variant form of XP and was found to have an abnormality in another DNA-repair system - postreplication repair.

In 1972, De Weerd-Kastelein *et al* using cell-fusion techniques, demonstrated genetic heterogeneity in the DNA excision repair defect of XP. Cells from different XP patients can be hybridized in culture that produces multinucleated cells with nuclei from each patient (heterokaryons). These heterokaryons may exhibit complementation and increased repair, survival, or recovery of RNA synthesis. If complementation occurs, it is an indication that cell types contain defects in different genes and each supplies what the other is lacking. Other combinations remain repair-deficient; in these cases, cell types contain defects in the same genes. Two forms of XP have been described depending on their clinical symptoms and biochemical defects: « classical XP » and « XP variant ». In the classical form, the systematic complementation of DNA repair defects by cell fusion assay has led to the identification of seven XP complementation groups (A to G) and the genes corresponding to groups XP-A through XP-G have been cloned and mapped to different specific chromosomal locations (12, 13). The most common complementation group is XP group C (XP-C) in Europe, North Africa and USA, while XP group A (XP-A) is prevalent in Japan. XP-C cells are exclusively deficient in the NER system that corrects the UV-induced lesions on the non-transcribed part of the genome (GGR - global genome repair ), while they repair normally the lesions on the transcribed strand of active genes by a mechanism known as transcription-coupled repair (TCR). Non-XP-C (XP-A, -B, -D, -E, -F, -G) cells are deficient for both GGR and TCR. XP

variant patients have a normal level of NER but are defective in some kind of still undefined post-replication repair, and the patients develop skin cancers at a late age (between 20 and 40 years old).

*Symptoms*

Premalignant actinic keratoses, malignant and benign neoplasms develop. The neoplasms are predominantly basal cell or squamous cell carcinomas but also include melanomas in about 5%, keratoacanthomas, and angiomas. About 90% of the BCCs and SCCs occurs on the face, head, and neck - the sites of greatest UV exposure. The median age of onset of first skin neoplasms is 8 years, nearly 50 years younger than that in the general population. *This represents one of the largest reductions in age of onset of neoplasia documented for any recessive human genetic disease.* There was an approximate 30-year reduction in survival, with a 70% probability of surviving to age 40 years. XP patients show accelerated poikiloderma-like changes or photoaging of their skin. Ocular abnormalities include photophobia, conjunctivitis, ectropion due to atrophy of the skin of the eyelids, keratitis, neoplasms of the lids. The neurologic symptoms varied in age of onset and severity, but were characterized by progressive deterioration. Diminished deep tendon reflexes and sensorineural deafness were frequent early abnormalities. In some patients, progressive mental retardation became evident.

## I.3. Trichothiodystrophy (TTD), Cockayne syndrome (CS) and the mechanism of NER

Deficiency in NER is also associated with two other rare but non-cancer-prone disorders, trichothiodystrophy (TTD) and Cockayne syndrome (CS; *see Figure 3.*). Complementation studies established that the XP phenotype could result from defects in one of seven or more genes, all of which seemed to be required at an early stage of the NER process. The initial step in the process involves recognition of the damaged DNA by the XPA protein. This protein binds to the damaged DNA via a zinc-finger domain. Another part of the XPA protein can bind to the basal transcription factor TFIIH, which was recently shown to have a role in NER as well as in transcription. TFIIH contains at least six subunits, including the XPB and XPD proteins, which both have helicase activity, but of opposite polarity. The function of these helicases is to open out the structure around the damaged site to enable the

structure-specific nucleases to incise the DNA. XPF and XPG are both structure-specific nucleases with different specificities: XPF cuts on the strand that leads off from the junction in the 5'-3' direction, whereas XPG cuts on the strand leading off in the 3'-5' direction. The result is the removal of a piece of DNA of about 29 nucleotides in length. The incision reaction required the XPC protein, whose function has not yet been established, and an uncharacterized protein fraction. It is stimulated by the product of the *XPE* gene, a protein that binds specifically to UV-irradiated DNA and is thought to assist in the early recognition step. The mechanism of NER is represented in *Figure 4.*.

*Cockayne syndrome and trichothiodystrophy: defective repair, no cancer*

Whereas the clinical features of XP are confined to the skin and, in some individuals, the nervous system, CS is characterized by dwarfism, loss of adipose tissue, mental retardation, retinal atrophy, gait defects, cataracts, dental caries and acute sun sensitivity. Very rarely, individuals have clinical features of both XP and CS. CS cells are defective in an important subpathway of NER. Following DNA damage, it is of prime importance for the cell to remove damage from actively transcribed regions of DNA, and in human cells repair is more rapid in transcribed than in untranscribed regions. It is the transcribed strand that is repaired most rapidly. The kinetics of intracellular DNA repair is represented in *Figure 5.*. This preferential repair is referred to as transcription-coupled repair (TCR), and it is this rapid, preferential repair that is specifically defective in CS cells. It is envisaged that the products of the two known CS genes fulfil some kind of transcription-repair coupling function in human cells, but the details are unknown. The model for TCR is represented in *Figure 4.*.

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. Although XP and TTD are completely different clinical entities with hardly any features in common, astonishingly, cells from XP and sun-sensitive TTD donors have indinstinguishable defects in NER. Complementation studies have assigned the defect in the majority of TTD cells to the *XPD* gene, in one family to the *XPB* gene, and in one single individual to a completely new NER gene designated *TTDA*. Fibroblasts from TTD patients without sun-sensitivity had normal UV survival and normal unscheduled DNA synthesis.

To elucidate these perplexing findings, the involvement of TFIIH in NER proved to be crucial. This basal transcription factor is involved in loading RNA polymerase II onto

promoters. All the subunits of TFIIH are involved in NER and a TFIIH core could associate either with other repair proteins to carry out NER as described above, or with different proteins to carry out transcription. The XPB and the XPD proteins are both subunits of TFIIH and it was shown that the TTDA protein was also likely to be a constituent of TFIIH. Thus, the products of the three genes in which mutations can result in TTD are all subunits of TFIIH.

*Repair and transcription syndromes*

Mutations of the *XPD* gene can result clinically in XP, TTD or, in a couple of cases, XP with CS. It is feasible that mutations in the *XPD* gene could affect the two different functions of TFIIH (repair and transcription) in different ways. A mutation affecting only the NER function will result in defective DNA repair and clinical features of XP. A mutation that abolishes the transcription function would obviously be lethal. However, a mutation that causes a slight deficiency in transcription might specifically affect the synthesis of critical proteins that are very dependent on maximal transcription rates. It has been proposed that this is the situation with TTD, in other words, it is a « transcription syndrome ». Critical proteins dependent on maximal transcription might, for example, include the sulphur-containing ptoteins that are deficient in the hair of TTD patients. The mutations in XPD that affect transcription in this way may or may not also affect DNA repair. Thus, TTD may be found with or without DNA repair defects, as is indeed the case. This very attractive hypothesis leaves unexplained the question of why the clinical picture of TTD patients with defective repair does not include the clinical features of XP, which have been attributed to defective repair. A possible explanation is that one of the proposed pathways relying on maximal transcription is involved in the development of a mutated cell into a skin tumor. The immune system affect the development of skin cancers. UV-induced damage to the immune system produces mutations in skin cells and also triggers signal transduction pathways that result in the immunosuppression in the skin. Preliminary evidence suggests that a signal transduction process involved in antigen presentation in the skin is differentially affected in XP and TTD cells following UV radiation (14).

The above hypothesis, that different mutations in the *XPD* gene can affect DNA repair and transcription differentially and thereby result clinically in XP or TTD, implies that the mutations in the *XPD* genes in XP and TTD patients are located at different sites in the gene. From the data available so far we can say that most of the patients are compound

heterozygotes, i.e. they have different mutations in the two copies of the gene; in no case do an XP and a TTD patient have the same mutations; and at a few sites in the gene, mutations have been found in several patients. These may be « XP-specific » and « TTD-specific » mutations.

CS might be also a « transcription syndrome ». The multisystem nature of the clinical features make this an attractive hypothesis. A coupling of nucleotide excision repair with transcription was first recognized through the increased repair of DNA lesions in the transcribed strand of active genes. Venema *et al* demonstrated a defect in this pathway in cells of CS patients, and the defective gene in the CS complementation group B. This gene likely encodes one of the transcription repair coupling factors and is the first human gene cloned that is specifically involved in TCR. CS is a growth deficiency syndrome progressively manifesting from the second year of life and leading to dystrophic dwarfism with microcephaly, mental retardation, neurological and ocular defects, and other anomalies. Inheritance is autosomal recessive, patients usually have early onset of sun-sensitivity, with marked redness on minimal sun exposure. In about one third of cases cataracts are formed. Pigmentary retinal degeneration often develops.

Patients in CS groups A and B are deficient in repair of actively transcribed genes. This defect is reflected in delayed recovery of DNA and RNA synthesis following UV radiation which has provided the bases for prenatal diagnosis. Cultured CS cells have similar hypersensitivity to killing by UV as is found with XP. CS differs from XP in that the usual assays of DNA excision repair in the total genomic DNA are normal. However, the increased rate of repair of active genes usually seen in repair-proficient cells is absent in CS cells. CS shows a repair deficit that is not associated with cancer.

A couple of patients have been identified with clinical features of both XP and CS. These patients had the cutaneous pigmentary and neoplastic features of XP with dwarfism, mental retardation, and retinal degeneration typical of CS. Complementation studies have revealed that these patients have different excision repair defects: they are the members of complementation groups B, D, and G. The elucidation of the biochemical bases of these disorders should provide clues to understanding the genetic changes involved in carcinogenesis by many physical and chemical agents.

Cells from different complementation groups of XP show characteristic ranges of hypersensitivity to killing and mutagenesis by UV radiation and by certain chemical carcinogens such as benzpyrene or nitroquinoline oxide. Concerning the clinical features and cellular characteristics of XP, TTD, and CS *see Appendix I. and II.*.

## I.4. Apoptosis and programmed cell death (PCD)

Apoptosis and PCD are often mistakenly used as synonyms. However, the term apoptosis only applies to the morphological changes that occur during this cell death process, while the term PCD describes the underlying biochemical mechanism that drives these changes. PCD is an inducible mechanism that is dependent on macromolecular synthesis, because inhibitors of either transcription or translation interfere with this death process (15). Withdrawal of growth factor (GF) stimulation or decreased ligation of β1 integrin by matrix molecules are two major signals for keratinocyte differentiation and are triggers for PCD. Immunologic damage induced by activated lymphocytes or cytokines is an important trigger for apoptosis.

Another inducer of apoptosis is DNA damage, or oxidative stress. Both of these are frequently induced in the normal epidermis by ultraviolet radiation, which also induces release of pro-inflammatory cytokines. In skin cells appearing after UV exposure that have a histologic appearance that has been called « sunburn cells » were demonstrated to be apoptotic cells (16). Cell damage results in increased stabilization of p53 protein that slows down the cell cycle to permit repair of DNA damage and turns cells sustaining unrepaired DNA damage toward apoptosis rather than to normal squamous differentiation. UV radiation dependent apoptosis requires the presence of the p53 proto-oncogene; inactivating mutation of p53 prevents formation of apoptotic sunburn cells. UV induction of p53 arrests cycling cells in G1. *This is a protective mechanism that rids the skin of severely damaged cells.* Cells with defective DNA repair of transcribed genes, such as those from XP-A or CS induce nuclear accumulation of p53 and apoptosis at much lower UV doses than normal.

The cells of the human epidermis are constantly turning over. Cells in the basal layer divide and daughter cells migrate upward and differentiate into squamous cells. Thus, the human epidermis is in a process of constant regeneration. In order for the skin to remain viable, some cells must remain in the basal layer. These putative stem cells divide infrequently but produce other rapidly dividing cells that then differenciate forming an « epidermal proliferative unit ». The epidermal proliferative unit originates from two kinetically distinct subpopulations of basal epidermal cells: (i) keratinocyte *stem cells*, which represent a minor subpopulation of relatively quiescent cells, defined by their great proliferative potential and an unlimited capacity for self-renewal, identified as slow-cycling. ($^3$H )-thymidine label retaining cells; and (ii) *transit amplifying cells* - the progeny of stem

cells, with a limited proliferative capacity identified as a pool of rapidly proliferating cells that are lost from the basal layer to terminal differentiation within 4-5 days (17).

When regarding the role of apoptosis, it is important to make a distinction between apoptosis and necrosis, the two major forms of cell death. Morphological changes are used as the basic criteria for segregating these two processes (*see Table I.*).

| | NECROSIS | APOPTOSIS |
|---|---|---|
| **1. Size of cells** | Cells become larger (swell) | Cells become smaller (shrink) |
| **2. Lysosomal release** | Rupture of lysosomes and release of hydrolytic enzymes | No lysosomal release |
| **3. Intracellular organelles (e.g. mitochondria)** | Destroyed | Remain intact |
| **4. Size of nuclei** | Swell | Condense (pyknosis) |
| **5. Nuclear DNA** | Karyolysis | Chromatin marginates along the nuclear envelope and the nucleus fragments |
| **6. Agarose gel electrophoresis** | Smear (random digestion) | DNA ladder (multiples of 180-200 base pair units) |

**Table I. Apoptosis and necrosis**

One of the best studied classes of cell surface molecules expressed by keratinocytes are the integrin superfamily of cell adhesion receptors. Integrins are heterodimeric cell surface glycoproteins that primarily mediate the attachment of basal keratinocytes to extracellular matrix proteins found in the basement membrane, but can also mediate intercellular adhesion. *In vivo*, basal keratinocytes express the β1-integrins α2β1 and α3β1 as well as the integrin α6β4. Recently, it has become clear that integrin-mediated cell anchorage has a vital role in the control of apoptosis; indeed a new term « anoikis » has been coined to describe PCD caused by loss of anchorage (18). Epithelial cells are very subject to anoikis. In contrast, fibroblasts normally do not undergo apoptosis upon loss of anchorage; rather they arrest in $G_1$ (19).

*In vivo* studies suggest that epidermal stem cells constitute between 1 to 10% of the basal layer, depending on the methodology used. Because approximately 40% of the basal layer in human foreskin exhibits high levels of β1-integrin *in vivo*, it is highly likely that basal keratinocytes with this phenotype contain both the keratinocyte stem cell population and a significant number of transit amplifying cells. In view of functional data demonstrating the role of integrin α6β4 in mediating adhesion of basal keratinocytes to the basement membrane via hemi-desmosomes, this integrin may provide a suitable marker for epidermal stem cells because these cells are permanently anchored to the basement membrane.

Bayerl *et al* (20) showed that sunburn cells (= apoptotic keratinocytes) express only a limited range of differentiation markers, including keratin 5 (K5), but they are entirely negative for late differentiation markers (e.g. filaggrin, loricrin, involucrin - these are precursor proteins of the cornified cell envelope). It was suggested that SBCs retain a basal-cell-like level of differentiation, probably as a result of cell death and migration through the epidermis without further differentiation.

*Apoptosis and terminal differentiation*

Although the process of terminal differentiation has been studied extensively, nothing is known about the events that regulate the transition from the stem cell to the transit amplifying cell compartment. Until recently, there were no biochemical markers to distinguish stem and transit amplifying cells, but it is now known that stem cells express twofold higher surface levels of β1-integrins than transit amplifying cells (21, 22).

c-Myc belongs to the basic helix-loop-helix / leucine zipper family of DNA-binding proteins and regulates transcription. The experiments of Gandarillas and Watt (23) have shown that, in normal human epidermal keratinocytes, constitutive activity of c-Myc does not stimulate proliferation or apoptosis, but suppresses growth and stimulates terminal differentiation by promoting transition from the stem to the transit amplifying cell compartment. This is in contrast to the roles established for c-Myc in other cell types, namely stimulation of proliferation, suppression of differentiation, induction of apoptosis, and neoplastic transformation.

These findings may seem to be unexpected but they might explain why there are no reports of frequent c-Myc amplification or overexpression in spontaneous or chemically induced epidermal SCCs. The major significance of these findings that increased terminal differentiation *is not correlated* with increased apoptosis, even though it has been argued

frequently that keratinocyte terminal differentiation is a form of apoptosis (24). This way, constitutive activity of c-Myc affects the balance between stem cell renewal and terminal differentiation, rather than causing a complete block of either process. The effect of Myc activation on integrin levels is of particular interest, because it is possible that integrin genes are subject to transcriptional suppression by c-Myc and that high integrin levels are required for maintenance of the stem cell phenotype. The level of c-Myc in the epidermis is very low and an increase in c-Myc activity in individual stem cells would be sufficient to induce those cells to become transit amplifying cells and, thence, to undergo terminal differentiation. Identification of c-Myc target genes in keratinocytes, therefore, offers the exciting prospect of gaining further understanding of the control of stem cell fate.

According to the results of Bernerd *et al* (25) - who identified apoptotic keratinocytes in the basal layer - the apoptotic process is engaged to allow a beneficial removal of severely damaged cells thus avoiding mutations potentially involved in carcinogenic transformation, especially in the basal compartment which is the epidermal proliferative layer.

# II. AIMS

Xeroderma pigmentosum (XP) and trichothiodystrophy (TTD) are hyperphotosensitive, DNA repair deficient genodermatoses. 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).

# III. MATERIALS AND METHODS

## III.1. Patients

The **XP-C** patient has developed multiple skin tumors from the age of 9. Surgical excision of eight BCC-s and six SCC-s in sun-exposed areas were documented. A melanoma, a histiocytofibroma, a neurofibroma and two cavernous angiomas (one cerebral) were diagnosed as well. The patient died at the age of 17, presumably of a metastatic neuro-endocrine tumor of the thyroid gland. The complementation group was determined as XP-C in our laboratory by the heterodikaryon complementation test.

The **XP-D** patient developed four BCC-s and one SCC in sun-exposed areas and suffered from melanomas as well. The age of appearance of the first skin tumor was 12 years. His elder brother suffered also from XP group D which, in both cases, was diagnosed on the bases of a heterodikaryon complementation test. In addition to it, identical mutations were found in the *XPD* gene. Apart from skin manifestations, he presents a marked, bilateral hearing impairement combined with speech impediment.

**TTD** cells used in this study were obtained from back skin of a fetus expulsed at estimated gestation age of 29 weeks (weight: 1,250 kg). Both parents were heterozygous for the *XPD* gene. This couple have already had a child suffering of the same disease who died at the age of 14 and another aborted fetus diagnosed as TTD. Antenatal, the diagnosis was established by UDS analysis of amniocytes and by the examination of fetal hair with polarizing microscopy. It was confirmed after expulsion by the microscopical examination of skin and of scalp. We also showed that these TTD cells were mutated on the *XPD* gene.

## III.2. Cell culture

Biopsies were obtained after approval of individuals from non-exposed skin areas. Fibroblasts and keratinocytes were derived from the same donors except for normal cells. Normal human skin was obtained from baby foreskin.

Human epidermal keratinocytes of normal, XP and TTD individuals were obtained and cultured as described by Rheinwald and Green (1975) (26) on a feeder layer of X-ray irradiated (60 Gy) Swiss 3T3 fibroblasts.

Human diploid fibroblasts were grown in DMEM medium (Gibco Laboratories Inc.) supplemented with 10% fetal calf serum (Dominique Dutscher, Mulhouse, France), 1mM sodium pyruvate, 0,1 mM non-essential amino acids, 10,000 IU penicillin-streptomycin for 100 ml culture medium and with 2mM L-glutamine.
Cells were cultured at 37°C in a 9.5% $CO_2$ atmosphere. All experiments were performed using keratinocytes at passage 2 or 3 and fibroblasts at passages 3 to 5.

### III.3. UV radiation sources

UVA radiation was provided by a metal halide lamp (UVASTAR, OSRAM Rosny/s/bois, France) equipped with anticaloric filter KG1 (Schott France, Clichy, France) and UVB cut-off filter WG 335 (Schott). The proportion of UVB (310-320) was reduced to $6.4 \times 10^{-6}$ of the total fluence measured at 310-400 nm. The fluence rate given in this study (HBW 357-370 nm), at the chosen distance, was 165 $W/m^2/s$.

The UVB radiation source was the Transilluminator banc, Spectroline (Spectronics Corporation, Westbury, NY), model TR-312, equipped with cut-off filter WG 305 (Schott). The fluence rate given in this study (HBW 307-319 nm) was 20 $W/m^2/s$ at 14 cm from the lamp and was 9.4 $W/m^2/s$ at 32 cm from the light source (distance used at doses smaller than 500 $J/m^2$).

UVC irradiations were performed at 254 nm from a germicidal lamp, at a fluence rate of 0.21 $W/m^2/s$.

### III.4. 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. To avoid excessive warming of the cells during UVA irradiation, dishes were placed on a cooling metal plate with an inside flow of temperate water.

### III.5. 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 (BioMedia). From the fourth day after plating the medium was supplemented with epidermal growth factor at 10 ng/ml (EGF; Euromedex). The culture medium was changed in every 4 days. After 12 days, keratinocyte colonies were fixed in 3.7% formaldehyde in PBS and coloured by 1% rhodamine B (Sigma). Numbers of colonies were determined by eye and under binoculars to ensure that all, including small, abortive colonies, were counted. 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 cloning efficiency (CFE) was comprised between 7.5-10.5 %. The quantification of the three types of keratinocyte colonies were carried out following the criteria cited in the *Result* section.

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, changed once at day 8 after seeding. Fibroblast colonies were fixed in a mixture of methanol/formaldehyde (9:1) and were stained by crystal violet. The relative survival was calculated as for keratinocytes. The CFE was comprised between 9.5 - 10.5%.

At least two independent experiments were performed in duplicate dishes for each experimental point.

### III.6. UDS analysis

After UV irradiation keratinocytes and fibroblasts were seeded on glass coverslips, DNA repair was followed by $^3$H-thymidine incorporation as described previously (27) except that hydroxyurea and fluorodeoxi-uridine were both omitted. UDS of keratinocytes was performed in keratinocyte culture medium containing 1% dialysed FCS. UDS of fibroblasts was carried out in DMEM supplemented with 1% of dialysed FCS. 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 control experiments UDS of both cell types was carried out using the same culture medium (DMEM or F10 medium), the same dialysed fetal calf serum (Dominique Dutscher) and the same exposition time (72 hours) at 4°C. In any case, the mean number of grains per nucleus was obtained by counting 80 non-S-phase nuclei for each UV dose. All UDS experiments were performed at least twice. All experimental points were made in duplicate dishes.

### III.7. 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 as described previously (28). Purified DNA was loaded on PVDF membrane (Amersham, Hybond-P). 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. TDM-2 specifically recognises CPD photoproducts and was diluted to 1:2000, 64M-2 antibodies recognise the (6-4) PP lesions and were diluted to 1:500. Chemifluorescent detection was carried out using the ECF western blotting kit (Amersham). The secondary antibody was linked to fluorescein-iso-thiocyanate (FITC), the tertiary anti-FITC antibody was linked to the alkaline phosphatase enzyme. Precise quantifications of signals were obtained after scanning the blots on a FluorImager (Molecular Dynamics) using the ImageQuant software (Amersham).

### III.8. TUNEL method

**T**dT-mediated d**U**TP **n**ick **e**nd **l**abeling (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. In negative controls the TdT enzyme was omitted. 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 (DAB). TUNEL was used according to the manufacturer's instructions with the following

modification: the Converter-POD solution was diluted at 1:1 with the Tris-HCl-NaCl buffer (100mM-150mM). With this modification the non-apoptotic cell coloration was practically eliminated. 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 (Sony). Values represent percentages from at least 1000 counted apoptotic and non-apoptotic cells on each coverslip. Morphological findings typical for apoptosis were also considered (i.e. marked condensation of chromatin and cytoplasm, cytoplasmic fragments with or without condensed chromatin and intra - and - extracellular chromatin fragments).

### III.9. DNA fragmentation analysis

Cells were washed in PBS, trypsinized, resuspended in STE buffer (NaCl 0.1M, Tris-HCl 10mM pH 8, EDTA 1mM pH 8) with 0.1% SDS and 20 mg/ml of proteinase K (Boehringer Mannheim). Samples were incubated overnight at 56°C. DNA was extracted with phenol, chloroform/isolamyl alcohol (24:1; Fluka, Saint-Quentin Fallavier, France) 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 (Tris base 2M, boric acid 2M, EDTA 10mM).

### III.10. 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 *(see Table II).* FITC (fluoro-iso-thio-cyanate)-conjugate rabbit anti-mouse or anti-rat immunoglobulins or TRITC-conjugate swine anti-rabbit immunoglobulins (DAKO, Denmark) were used as second antibodies (in 1:100 dilution).

*Procedure* : 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 in the ready-to-use DAKO fluorescent mounting-medium, before being observed under a fluorescence microscope.

| Specificity | Reference | Marker of | Dilution used | Supplier |
|---|---|---|---|---|
| *β1 integrin* (CD 29) | IMO 790 mouse | cell adhesion | 1:75 | Immunotech |
| *α6 integrin* (CD 49f) | IM 0769 rat | cell adhesion | 1:75 | Immunotech |
| *involucrin* | BT-601 rabbit | terminal differentiation | 1:1 | Cliniscience |
| *K10* | MON 3010 mouse | suprabasal keratinocytes | 1:5 | Genzyme |
| *K14* | - mouse | basal keratinocytes | pure | a gift from L'Oréal |
| *β1 integrin** (CD 29) | AB 1937 rabbit | cell adhesion | 1:45000 | Euromedex (Chemicon) |

** polyclonal for Western blotting*

**Table II. List of antibodies used for immunostaining and Western blotting**

### III.11. Western blotting

Total proteins were extracted in: 8 M urea; 50 mM TRIS-HCl, pH:7.6; 0,1 M β-mercaptoethanol; 1mM dithiotreitol (DTT); 100 µg/ml paramethyl sulfonyl fluoride (PMSF). Protein samples were separated by 7,5% SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membrane (Amersham). Anti-β1 integrin antibodies were diluted *(see Table II.)* in PBS supplemented with 0,1% bovine serum albumine (BSA) 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).

### III.12. 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.

# IV. RESULTS

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

DNA repair properties of fibroblasts and keratinocytes from normal, XP-C, XP-D and TTD-D/XP-D were measured by UDS *(Figure 6.a.)* after irradiation of cells with increasing doses of UVA, UVB and UVC wavelengths. Irradiation with UVA doses up to 500,000 J/m$^2$ did not result in a grain number above the background in any cell strain. Data obtained after irradiation with increasing doses of UVB and UVC are presented in *Figure 6.b.* and 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. This observation suggested either that fewer lesions were present in keratinocytes or that repair of lesions was more efficient in fibroblasts than in keratinocytes. To decide which of these hypotheses was correct, UV-induced DNA-lesions (6-4 PPs and CPDs) present in the DNA from keratinocytes and from fibroblasts were quantified by immunoblotting using specific antibodies against these lesions (29, 30).

*Figure 7.* shows that 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$^2$. Repair kinetics measured as the decrease of both types of DNA-lesions were found similar in keratinocytes and in fibroblasts (data not shown).

## IV.2. Cell survival measurement as the colony forming efficiency (CFE) after UV irradiation

In the absence of UV irradiation, the CFE of fibroblast and of keratinocyte strains ranged between 7.5 and 10.5 %.

### (a) Fibroblasts

Nonconfluent cultures of fibroblasts from normal, XP-C, XP-D and TTD/XP-D were irradiated with either UVA (335 nm) or UVB (305 nm) wavelenghts. Cells were then

dissociated and plated at increasing densities according to the UV dose as described in *Materials and Methods*. This procedure was to be used because irradiation using increasing doses of UV of fibroblasts seeded at constant densities could not give enough colony for their reliable counting and the statistical analysis of results.

**UVB wavelengths**: Variable ranges of UV irradiation comprising four doses were determined by preliminary experiments according to the UV sensitivity of each strain. *Figure 8.b.(Panel D)* shows that irradiation of all fibroblast strains induced a dramatic decrease of cell viability. Normal fibroblasts exhibited much higher resistance to UVB irradiation than XP or TTD cells. 50 % cell survival to UVB wavelenghts of the fibroblast strains are indicated in *Table III.*.

| | Keratinocytes | | Fibroblasts | |
|---|---|---|---|---|
| **Cell Type** | **UVA dose kJ/m²** | **% of WT cells** | **UVA dose kJ/m²** | **% of WT cells** |
| **wild type** | 285 | 100 | 89 | 100 |
| **XP-C** | 122 | 43 | 56 | 63 |
| **XP-D** | 217 | 76 | 58 | 65 |
| **TTD/XP-D** | 323 | 113 | 78 | 88 |
| | **UVB dose kJ/m²** | **% of WT cells** | **UVB dose kJ/m²** | **% of WT cells** |
| **wild type** | 800 | 100 | 240 | 100 |
| **XP-C** | 260 | 33 | 30 | 13 |
| **XP-D** | 80 | 10 | 25 | 10 |
| **TTD/XP-D** | 430 | 54 | 82 | 34 |

**Table III. UVA and UVB doses leading to 50% cell survival in human WT, XP-C, XP-D and TTD/XP-D fibroblasts and keratinocytes (determined by extrapolation).**

**UVA wavelengths**: All fibroblast strains were thus exposed to either three or four doses ranging from 50,000 to 500,000 J/m$^2$ *(Figure 8.b., panel C)*. Below doses of 150,000 J/m$^2$ the four cell survival curves were very close. Following higher UVA doses, differences of UV sensitivity between normal cells on one hand, and TTD/XP-D, XP-C, XP-D cells, on the other hand, was much more marked than for low (100,000 J/m$^2$) or intermediate (250,000 J/m$^2$) UVA doses. 50 % cell survival to UVA wavelenghts of the fibroblast strains are indicated in *Table III.*

**(b) Keratinocytes**

Constant numbers of cells were seeded at clonal density following UV irradiation *(Figure 8.a.)*. As for fibroblasts, the range of UV doses delivered was adapted according to the UV sensitivity of a given cell type.

**UVB wavelengths**

*Figure 8.b. (Panel B)* shows that 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. The UVB doses leading to 50% cell survival for the keratinocyte strains are indicated in *Table III.*

**UVA wavelengths**

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. The UVA doses leading to 50% survival for the keratinocyte strains are indicated in *Table III.*.

## IV.3. UV irradiation differentially influences the types of colonies in normal and in repair-deficient keratinocyte cultures

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 (<5$mm^2$), 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. These three types of colonies (LSP, ST and W) are initiated by cells called *holoclone*, *paraclone* and *meroclone*, respectively. The discovery,

description and definition of *holoclones, paraclones and meroclones* were proposed originally by Barrandon and Green, (1987) (31).

The macroscopic examination of keratinocyte plates showed evident variations in the nature of colonies after UV irradiation. We thus quantified the relative variation of each type of colony obtained in each keratinocyte strain *(Figure 9.)*.

**Number of LSP and ST colonies following UVA radiation**: In three cell strains (WT, XP-D, TTD/XP-D), the relative numbers of LSP colonies were significantly decreased and the relative number of ST colonies were increased after UVA irradiations. The relative number of both LSP and ST colonies did not vary significantly in XP-C keratinocytes exposed at a UVA dose.

**Number of LSP and ST colonies following UVB radiation** : In three strains (WT, XP-D, and TTD/XP-D) the relative numbers of LSP colonies were significantly decreased with a maximum in XP-D keratinocytes (84.2%). The decrease of LSP colonies was accompanied by the increase of ST colonies, with the highest relative value in XP-D keratinocytes (197,8%). In XP-C keratinocytes, UVB irradiation did not induce a significant change in the relative numbers of LSP or ST colonies.

**Number of W colonies after UVA or UVB irradiation**

Variations in the relative ratio of W colonies after UVA or UVB irradiation were observed. However, their amplitudes were much lower than those observed in the case of LSP and ST colonies. It was worth noting, however, that the highest relative increase of W colonies was observed using XP-C keratinocytes after both UVA and UVB irradiation *(Figure 9., C and D)*. In the three cases where the relative numbers of wrinkled colonies decreased (UVB : XP-D and TTD; UVA: XP-D), a significant increase of ST colonies was observed.

Taken together these data demonstrate that the relative ratio of LSP colonies decreased after UVA or UVB irradiation and that in all but one case (XP-C after UVA and UVB), exposure to UV irradiation increases the relative ratio of ST colonies. *Figure 10.* summarizes the percentage of the three types of keratinocyte colonies at UVA and UVB doses resulting in 50 % survival determined by extrapolation of data presented in *Figure 9.*.

## IV.4. Differential induction of apoptosis in normal, XP-C, XP-D and TTD/XP-D keratinocytes

*The TUNEL method*

To investigate the role and rate of apoptosis in post-UV keratinocyte cell death and to determine whether apoptosis participate in the clonal transition of keratinocytes induced by UV irradiation, the TUNEL method was used, 24 and 48 hours after increasing doses of UVB irradiation (250 J/m$^2$, 500 J/m$^2$, 1000 J/m$^2$). It seemed that apoptotic cells appeared first at the periphery of clones (TUNEL-DAB staining), however, it could not be indisputably verified. We could not conclude whether the relative proportions of apoptotic cells were different in a given clone- type from each cell strain, and within the different clonal types of a given strain. *Figure 11.* shows that the number of TUNEL-positive keratinocytes was significantly higher in XP-C, TTD/XP-D and XP-D keratinocytes than in normal cells after 1000 J/m$^2$ UVB irradiation.

At both 24 and 48 hours after irradiation, the hierarchy of the cell strains studied concerning their susceptibility to apoptose was: WT < XP-C < TTD/XP-D < XP-D (*see Figures 12., 13.*). All differences were statistically different (in all cases $p < 0.02$). After 24 hours, the percentage of apoptotic cells in WT keratinocytes was only 10%, in XP-C keratinocytes it proved to be 27%, it was found to be 30% in TTD/XP-D keratinocytes and it could be determined as 50% in the case of XP-D keratinocytes. 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 (i.e. in the latter case, the large majority of the cells disappeared and only DAB-labelled cells or cellular fragments could be identified scattered through the coverslip).

*DNA fragmentation assay for verification of apoptosis*

*Figure 14.* represents the so called « DNA ladder » patterning of UVB irradiated keratinocytes, 24 and 48 hours after irradiation with 1000 J/m$^2$ . In the first lane, we used the MW VII. as a molecular weight marker; in the second, third, fourth and fifth lane the WT, XP-C, TTD/XP-D and XP-D keratinocyte DNAs were loaded, 24 hours after UVB irradiation. In the sixth, seventh, eighth and ninth lanes the WT, XP-C, TTD/XP-D and XP-D keratinocyte DNA preparations were electrophoresed, 48 hours after 1000 J/m$^2$ UVB irradiation.

In contrast with the previous method (TUNEL-DAB), 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. Note that the difference in the quantity of apoptosis between XP-C and TTD/XP-D cells is more evident than it was found previously.

## IV.5. Immunocytochemistry of early markers of keratinocyte differentiation

Keratin 10 (K10), an early marker of the epidermal keratinocyte differentiation, could be dimly identified without irradiating the cells, in our culture system. After irradiating with 500 and 1000 J/ $m^2$ UVB, in no studied cell strains a major change in the intensity of staining could be found. Keratin 14 (K14), being a marker of basal keratinocytes, could be well detected in non-irradiated cells (in all the studied cell types; data not shown). After 500 and 1000 J/$m^2$ UVB irradiation (24 hours after irradiation), the K14 signals diminished after a transient augmentation. However, differences characterizing the studied cell strains (WT, XP-C, TTD/XP-D, XP-D) could not be determined.

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 - TUNEL double labelling was used to determine whether double stained or counter-stained cells exist or not. *Figure 15.* (WT cells irradiated with 1000 J/$m^2$ UVB, 48 hours after irradiation) and *Figure 16.* (XP-C cells irradiated with 1000 J/$m^2$ UVB, 48 hours after irradiation) represent these results. Although the TUNEL labelling was not optimal (because the classical formaldehyde fixation could not be applied since it would destroy the involucrin labelling - acetone:methanol 1:1 fixation was used instead), there could be found double labelled cells as well as counterstained cells after irradiation. Note that the involucrin signal was more intense in the case of XP-C cells.

## IV.6. The immunostaining of α6 and β1 integrins

The β1 integrin staining led to artefact formation in UVB irradiated keratinocytes, thus, only the results of α6 integrin labelling is presented here. *Figure 17.* shows the α6 integrin labelling of WT *(A, B, C)* and XP-C *(D, E, F)* cells after 500 J/$m^2$ *(B, E)* and after 1000 J/$m^2$ *(C, F)* UVB irradiation at 24 hours. *A, D* show non-irradiated keratinocytes. It can

be clearly seen that α6 polypeptides were mainly found in cell-cell contact areas. The immunostaining became more intense with irradiation in both cell types; after 1000 J/m$^2$ XP-C cells began to be disorganized while WT cells preserved their integrity. *Figure 18.* shows the labelling of TTD/XP-D *(G, H, I)* and XP-D *(K, L, M)* keratinocytes after 500 J/m$^2$ *(H, L)* and after 1000 J/m$^2$ *(I, M)* UVB irradiation at 24 hours. *G, K* show non-irradiated keratinocytes. The immunolocalization of the α6 chains were the same as it was found previously. It is worth noting, that these cell types started to be disorganized earlier than it could be detected in WT and in XP-C cells.

## IV.7. Western blotting using β1 integrin, polyclonal antibodies

Hereby, a preliminary experiment is demonstrated using β1 integrin, polyclonal antibodies. Non-irradiated XP-D and XP-C keratinocytes after 250 J/m$^2$, 500 and 1000 J/m$^2$ UVB irradiation are shown in *Figure 19.*. Proteins of cells were isolated at 8, 16 and 24 hours after UVB irradiation as presented. A rainbow marker is shown on the right hand side. The expression of the β1 polypeptide seemed to increase with time after 250 J/m$^2$ UVB in both cell types. 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.

# V. DISCUSSION

Depletion of the ozone layer by chloro/fluorohydrocarbon pollutants and subsequent increase in exposure to UV light threatens to significantly increase the incidence of skin cancer. In addition, exposure to UV radiation can lead to photokeratitis, photoconjunctivitis, and permanent retinal blindness. Prolonged UV exposure results in inhibition of DNA synthesis and subsequent resumption of the cell cycle following *repair* or *apoptosis*, a mechanism to rid the organism of irreversibly damaged and potentially cancerous cells.

To date, only few studies have analyzed the responses of non-transformed keratinocytes and dermal fibroblasts from normal individuals to UV radiations (32-35). Furthermore, the study of epidermal keratinocytes from XP patients has been limited to the measure of their DNA repair capacity by UDS after short term culture and UVC irradiation (36-39). *The aim of this thesis was the characterization and comparison of the responses to physiological UV radiations (i.e. UVA and UVB) of dermal fibroblasts and epidermal keratinocytes grown from skin of normal, XP (XP-C, XP-D) and TTD/XP-D individuals.* The main objective was to determine whether specific responses to UV radiation of keratinocytes from XP patients could explain their predisposition to epidermal carcinomas in sun-exposed areas of the skin. In addition, we aimed to clarify the paradoxical question why XP-D patients are cancer-prone, whereas TTD/XP-D patients are not, although the same DNA repair gene, i.e. *XPD*, is mutated in both syndromes.

The DNA repair capacities (using the UDS technique), and cell survivals (using the clonal analysis) of fibroblasts and keratinocytes grown in parallel from each XP or TTD/XP-D patient were compared. The analysis was refined by the study of the clonal type-specific sensitivity of keratinoytes to UV irradiation and by the comparison of the numbers of UVB-induced DNA lesions in keratinocytes and in fibroblasts. All experimental data were analyzed statistically.

Concerning UV-induced apoptosis, experiments have been performed to determine the role and rate of apoptosis in WT, XP (XP-C, XP-D) and TTD keratinocytes after UVB exposure. In connection with this aspect, two members from the integrin cell surface receptor family (i.e. $\alpha 6$ and $\beta 1$) were investigated since integrin-mediated anchorage is a key regulator of apoptosis in epidermal cells.

The results presented here represent, the first set of exhaustive and comparative data between primary keratinocytes and fibroblasts from one normal individual, XP (one XP-C and one XP-D) patients, and one TTD/XP-D patient after exposure to UVB or to UVA irradiation.

## V.1. DNA repair as measured using UDS

We failed to detect any significant variation in UDS levels after the irradiation of cells (keratinocytes or fibroblasts) with UVA doses up to 500,000 J/m$^2$. This finding is in agreement with the absence of detection of 6-4 PP and CPD DNA lesions after UVA irradiation of human skin explants (40). Furthermore, CPDs are hardly detectable by immunocytochemistry after wide range (i.e. 320-400 nm) UVA irradiation of skin reconstructed in vitro (41). Because high UVA doses (250 kJ/m$^2$ ) induce tumors in rodents (42, 43) and are mutagenic both *in vivo* (44) and *in vitro* (2) one must admit that specific UVA-specific DNA damages (mostly 7,8-dihydro-8-oxoguanine), are either not repaired by NER or or that their repair by NER remains below the detection limit of UDS. In this respect, it must be considered that the repair of CPDs and 6-4 PPs by NER is a non-semiconservative insertion of 29-32 DNA desoxyribonucleotides, whereas a major mode of removal oxidative DNA damages occurs at the level of a single base by a mechanism called base excision repair. The UDS technique as performed in our experiments, therefore, is not appropriate to measure UVA-induced oxidative damages.

UVC and UVB radiations induced variable increases of UDS levels according to the complementation group of both fibroblasts and keratinocytes. Moreover, equivalent doses of both wavelengths (i.e. leading to 50% cell survival) led to comparable numbers of grains in normal cells (either fibroblasts or keratinocytes), suggesting the presence of similar numbers of DNA lesions. UDS levels could be arranged hierarchically in fibroblasts as WT > TTD/XP-D > XP-D > XP-C (in all cases $p$ <0.001), and in keratinocytes as WT > TTD/XP-D = XP-D > XP-C ($p$ values were : WT/XP-C, $p < 0.001$; TTD/XP-D/XP-C, $p < 0.01$; XP-C/XP-D, $p < 0.01$; TTD/XP-D, thus, not significant as far as the TTD/XP-D is concerned). XP-D and TTD/XP-D fibroblasts, but not keratinocytes, could be separated by their capacity to repair UVB-induced DNA lesions as observed previously in normal fibroblast cultures and in normal human skin explants (40).

These experiments thus are sufficient to explain the predisposition of XP-D but not TTD/XP-D patients, for epidermal carcinomas. However, due to their respective specific DNA repair capacities (XP-D cells are deficient in the repair of both 6-4 PP and CPD

photolesions, whereas TTD/XP-D are only deficient in the repair of CPDs (11, 28), UDS may underestimate the repair capacity of TTD/XP-D compared with XP-D keratinocytes (see next section). This hypothesis is supported by recent data showing that 25 % of CPD induced by a 30 J/m$^2$ UVC irradiation are repaired within 3 h after irradiation in normal cells (45). In addition, analysis of DNA repair by the UDS technique is not likely to reflect repair of non-CPD and 6-4 PP minor photoproducts induced by UVB irradiation (i.e. formaminopyrimidine and pyrimidine hydrates).

Indeed, contribution of these photoproducts to UDS is expected to be negligible because they represent about 1/100 of CPDs and because they are repaired by base excision repair (46).

## V.2. Same doses of irradiations lead to significantly fewer DNA lesion numbers in keratinocytes than in fibroblasts

Following irradiation at the same doses (e.g. 1000 J/m$^2$ UVB), the UDS values within a complementation group were systematically inferior in keratinocytes than in fibroblasts irrespective of whether the culture medium contained high (e.g. DMEM) or low (Ham's F10) thymidine concentrations (data not shown). Concordant observations have been reported previously (32, 38) but remained unexplained. Using WT cells, we demonstrated that there were 50% fewer both 6-4PP and CPD lesions in keratinocytes than in fibroblasts. This suggests that keratinocytes have developed a natural shielding protection against UV radiations to which they are much more exposed than dermal fibroblasts. Although differing compactness of chromatin in fibroblasts and keratinocytes could be responsible for this protection, no significant difference in the sizes of nuclei of both cell types could be measured.

We suggest that the presence of keratins (47) in keratinocytes, but not in fibroblasts, may perhaps contribute to significantly attenuate UV-induced DNA damages. Nevertheless, the potential importance of UV light-induced damage to non-DNA targets has received little attention. Zamansky *et al* (48, 49) reported that sun lamp irradiation induced the condensation of keratin intermediate filaments into the perinuclear region. Exposure to UVC appeared to disrupt keratin filaments similarly, whereas UVA had no discernible effect.

Alternatively, it would be interesting to measure DNA lesions in transgenic fibroblasts that ectopically express keratins or to investigate the UV transmission/absorbance properties of fibroblasts and keratinocytes.

The larger number of both DNA lesions (6-4 PPs and CPDs) found in fibroblasts may explain why XP-D fibroblasts could be separated from TTD/XP-D fibroblasts based on their DNA repair capacity (measured using UDS), whereas homologous keratinocytes could not. This strongly supports our hypothesis that UDS underestimates the DNA repair capacity of TTD/XP-D keratinocytes. Additional experiments will compare the number of lesions and their repair kinetics in keratinocytes and fibroblasts with those in normal, XP-C, TTD/XP-D, and XP-D individuals.

### V.3. Cell survival/clone-forming ability

Cell survival of both fibroblasts and keratinocytes was measured using clonal analysis. Determination of colony-forming ability after challenging the cells with UV light reflects the overall repair capacity and asseses the capacity of single cells to restore the integrity of their DNA to the extent necessary for cell division. Whereas this is a quite usual procedure with respect to the measurement of post-UV survival of fibroblasts (50), clonal analysis of keratinocytes from XP and TTD/XP-D patients has never been used to measure their resistance to UV radiation.

This method is, however, much more adequate than any other in the case of keratinocytes. First, in the epidermis, renewing keratinocytes, which schematically belong either to the stem or to the transient amplifying cell compartment, are mostly located in the basal layer (4, 51). Second, their capacity to divide can be anticipated *in vitro* since they can initiate colonies whose morphology and lifespan quite faithfully reflect their intrinsic growth potentials (see *Results*). Third, epidermal tumors (BCCs and SCCs) are likely to develop from basal cells with high growth potential that have escaped growth control (for instance loss of a tumor suppressor gene, such as *p53*, *patched*). Therefore, clonogenic cells with high growth potential, presumably correspond to those cells susceptible of initiating an epidermal tumor (4, 52).

The survival rates of the studied fibroblasts (each complementation group compared with one another) after UVA irradiation were all significantly different only for doses > 250,000 J/m$^2$. In general accordance with previous reports (53, 54), this allowed us to draw a survival hierarchy : WT > TTD/XP-D > XP-C > XP-D ($p$ values were : WT/XP-C, WT/XP-D, $p < 0.001$; WT/XP-C, XP-C/TTD-XP-D, $p < 0.01$). The survival hierarchy obtained after UVB irradiation was WT > TTD-XP-D > XP-C > XP-D ($p$ values were : WT/XP-C, WT/TTD-XP-D, WT/XP-D, XP-C/TTD-XP-D $p < 0.001$).

The survival rates of the WT, TTD/XP-D and XP-D keratinocyte strains studied here could not be separated from each other after UVA irradiation below 250,000 J/m$^2$. Conversely, our XP-C keratinocytes were clearly more sensitive to UVA radiation than those from other complementation group studied because their survival decreased at UV doses as low as 100,000 J/m$^2$. Other XP-C keratinocyte strains studied in the laboratory exhibited a similar behaviour (date not shown).

This observation suggests that, under a threshold of 250,000 J/m$^2$ UVA, oxydative DNA damages are repaired efficiently in WT, TTD/XP-D and XP-D but not in XP-C keratinocytes. We have noticed that the expression of metallothionein I, a protein which has been suggested to behave as a ROS scavenger (55), is significantly lower in XP-C, than in WT, XP-D and TTD-XP-D keratinocytes (Magnaldo and Sarasin, unpublished data). Low metallothionein levels in XP-C keratinocytes might thus explain their increased sensitivity to UVA radiations. Further experiments will measure in the keratinocyte strains the expression of enzymes (e.g hemeoxygenases and superoxide dismutases) that participate in the elimination of reactive oxygene species (ROS) (56).

### V.4. 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. This demonstrates that despite their apparent severe DNA repair deficiency (which did not allow us to distinguish TTD/XP-D from XP-D keratinocytes), the behavior of the studied TTD/XP-D keratinocytes determined by clonal analysis could be clearly separated from that of the studied XP-D keratinocytes. This observation supports our hypothesis that DNA repair capacities are underestimated when measured by UDS in TTD/XP-D cells and in keratinocytes. The XP-C keratinocyte strain displayed an intermediate survival level to UVB although they DNA-repair was the lowest.

The fact that UDS does not give much information on the transcription-coupled repair but rather preferentially reflects the repair of the global genome (45) - i.e. the repair process specifically altered in XP-C cells - may explain their relatively high UVB survival despite their severe DNA-repair deficiency as estimated by UDS. Our finding thus support the idea that the capacity of a cell to efficiently perfom TCR is accompained by high survival rates to UV radiation. This also explains that TTD/XP-D keratinocytes (whose repair capacities of 6-4 PP is presumably normal with respect to both the global genome and to preferential repair),

exhibit significantly higher survival after UVB radiation than XP-D keratinocytes (whose repair deficiency of 6-4 PP and CPD presumably concerns both global and preferential repair pathways; (28).

## V.5. Does the UV-induced transition from proliferative to abortive colony reflect a protection against cancer?

Exposure to both UVA and UVB radiation induced changes in the relative representations of the different colony types *(Figure 9.)*. In general, the numbers of LSP colonies decreased whereas those of abortive colonies (ST) increased. In this respect, it would be interesting to measure the effects of UV radiations on the expression of the protooncogene *c-myc* in the different clonal types, whose expression was recently shown to promote the differentiation of epidermal stem cells (57).

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. It was worth noting that the ST colonies did not increase significantly after UVB radiation; relative ratios of LSP and W colonies remained constant following UVB radiation, and W colonies increased following UVA radiation. The relative resistance to UV of cells with high growth capacities (i.e. cells forming LSP and W 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. We made the hypothesis that apoptosis could be selectively responsible for this transition. However, differences in the process of UV-induced differentiation might also be responsible.

## V.6. UDS values following UV doses leading to 50 % survival

In this study we experimentally compared the capacities of keratinocytes and fibroblasts grown *in vitro* to repair their DNA following UV irradiation. We found that equal doses of UVB induced twice as many lesions in WT fibroblasts as in WT keratinocytes when both cell types were grown as monolayers *(see Figure 7.)*. Furthermore, our survival curves demonstrated that keratinocytes resist UV radiation better than do fibroblasts. We thus compared the respective DNA repair capacities measured by UDS of normal keratinocytes and fibroblasts after UVB irradiation leading to 50% survival *(see Table III.)*. Under these

conditions, UDS values obtained from the cell strains studied were about two to three times lower in fibroblasts than in keratinocytes *(see Figure 6.b.)*. This indicated that in normal cells the ratio of repair capacity to cell viability is much better in keratinocytes than in fibroblasts (i.e. 15 UDS grains/nucleus *versus* 5 UDS grains/nuclei for 50% cell survival in keratinocytes and fibroblasts, respectively). In this respect, it would be interesting to measure the repair kinetics of UV-induced DNA lesions present in both keratinocytes and fibroblasts in the skin of normal and DNA-repair-deficient patients following UVA and UVB radiation as performed previously on human skin *ex vivo* (58) and *in vivo* (59).

## V.7. UVB-induced apoptosis and the role of integrins

The skin recognizes and eliminates aberrant keratinocytes - a supplement to the protection afforded by routinely shedding keratinocytes during differentiation. The elimination mechanism has been termed « cellular proofreading » because the mistake is erased rather than repaired. Failure of proofreading may have devastating consequences: because the mutated cell's normal neighbors still undergo apoptosis when damaged, they will leave room for the mutant to expand clonally into other stem-cell compartments (3). Thus, sunlight might act as a selection pressure favoring the clonal expansion of p53-mutated cells, each beach visit giving the clone a nudge. Sunlight appears to act in two ways: mutating genes and then, afterwards, selecting for clonal expansion of mutated cells. An expanding cell death defective clone will be more likely to accumulate additional mutations. Clonal expansion, however, can easily increase the probability of each subsequent mutation a thousandfold.

Apoptosis is a relatively high-frequency physiological event that can facilitate the expansion of many p53-mutated cells simultaneously. UVB is generally regarded as the activating spectrum for the initiation of BCCs and SCCs as well as for the induction of sunburn cells (= apoptotic keratinocytes). A reduction in the number of SBCs has been used as a marker indicating increased photoprotection of keratinocytes. SBCs exhibit less DNA-repair activity than normal keratinocytes, thereby suggesting that DNA damage or unrepaired DNA damage is involved in SBC formation (60). Soter demonstrated (61) that the ratio of disulfide (S-S)/sulfhydryl(-SH-)-positive SBC cell counts increases with time after exposure, thereby indicating that premature S-S crosslinking most probably takes place in SBCs. Protein cross-linking and chain breaks lead to cell death if repair processes are overloaded.

In our experiments, we found that WT keratinocytes were relatively resistant to apoptose after physiological doses of UVB irradiation. This might be explained by the fact

that these cells being repair proficient, are capable of the elimination of bulky DNA photolesions under normal conditions.

We studied apoptosis-induced DNA degradation following exposure to UV light in keratinocytes derived from patients with inherited defects in NER. Cells were irradiated with 1000 $J/m^2$ (this dose can be considered as the twofold dose of the, so called, minimal erythema dose - thus, it is most probably within the range of the « physiologically possible » acquired UVB dose) of UVB light and harvested 24 and 48 hours later. We found that all the different cells studied were capable of undergoing DNA degradation following UV irradiation (*see Figure 14.*). The electrophoresis pattern of the fragmented DNA was indicative of apoptotic degradation. These results show that of the cell strains tested, XP-D was by far the most sensitive to the induction of apoptosis following UVB irradiation. To further establish that XP and TTD/XP-D cells are induced to undergo apoptosis at lower exposure of UV light than normal cells, we examined the apoptosis by another method, the TUNEL.

We found that deficient DNA repair of the transcribed strand of active genes correlated to an increased susceptibility to UV-induced apoptosis. It was the case in TTD/XP-D and in XP-D cells. Previous studies reported on that cells deficient in preferential repair of the transcribed strand induce p53 at lower doses of UV light compared to repair-proficient cells and that they have a more severe inhibition of RNA synthesis following UV radiation (62). A higher rate of apoptosis correlates to neurological abnormalities in XP-D and in TTD/XP-D patients. Perhaps a higher rate of DNA damage-induced apoptosis during the neurological development contributes to these abnormalities. Nevertheless, the easily triggered apoptotic pathway may protect TTD/XP-D patients against the formation of skin cancer by the elimination of damaged, pre-mutagenic cells. However, if apoptosis suppresses the formation of cancer, how is it that the XP-D patient experienced such a high incidence of skin cancers despite the fact that he had cells that are even more sensitive to the induction of apoptosis than TTD/XP-D cells? This apparent paradox may be explained by the fact that TTD/XP-D cells are proficient in the repair of 6-4PPs both in the GGR and in the TCR as well, while XP-D cells are totally defective in the repair of UV-induced lesions (*see Appendix I. and II.).*

XP-C cells are proficient in TCR, this might explain why they were more resistant to undergo apoptosis than the previous two cell strains. However, they are defective in GGR, thus, if repair processes became overloaded, PCD occurs more frequently than in normal cells.

Notwithstanding, the explanation for these findings must be more complex. Gniadecki *et al* (63) showed that in addition to the known p53-dependent pathway, UV was able to induce a p53-independent apoptosis that could be blocked by integrin-mediated cell attachment (the integrin-sensitive pathway). The p53-independent integrin-sensitive apoptotic pathway may provide an additional mechanism counteracting UV carcinogenesis in the skin. In this thesis, some results of preliminary experiments are shown using anti-α6 and β1 integrin antibodies. We could demonstrate differences in the immunostaining and Western blotting among the cell strains studied after UVB irradiation, thus, these experiments are worth of going further.

According to the data Larjava *et al* (64), distribution of β1 integrins in BCCs closely resembled that seen in the basal layer of normal epidermis (i.e. they are localized around the entire periphery of basal cells). Staining of SCCs revealed, either an absence or a variable expression of β1 integrins. How keratinocytes express and coordinate the β1 integrin in cell adhesion, in the development of cancers, are questions which wait for answers. These proteins may not only mediate the interactions between the cells or adhesion of a cell to extracellular matrix but may also have signalling functions to initiate other regulatory processes (65).

It should also be noted that patients, with several diseases characterized by defective DNA repair show *premature aging*. In particular, XP patients are very sensitive to the effects of UV light and show accelerated poikiloderma-like changes or photoaging of their skin (66,67,68). When regarding involucrin, which is an intermediate differentiation marker, we observed that XP and TTD/XP-D cells labelled more intensively with using anti-involucrin immunostaining after UVB irradiation than WT 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. It supports the idea that differentiating keratinocytes are not the only cells, which apoptose and basal keratinocytes (involucrin negative cells) may undergo apoptosis as well. However, this statement has to be confirmed by further experiments.

These findings have put forward important clues regarding the predisposition of keratinocytes to develop as tumor cells following solar irradiation. It opened up a new prospect in the study of keratinocyte protection against solar radiation, including transfer of adequate DNA repair genes in cells from XP patients.

# VI. SUMMARY AND SIGNIFICANCE 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.

# VII. FIGURES

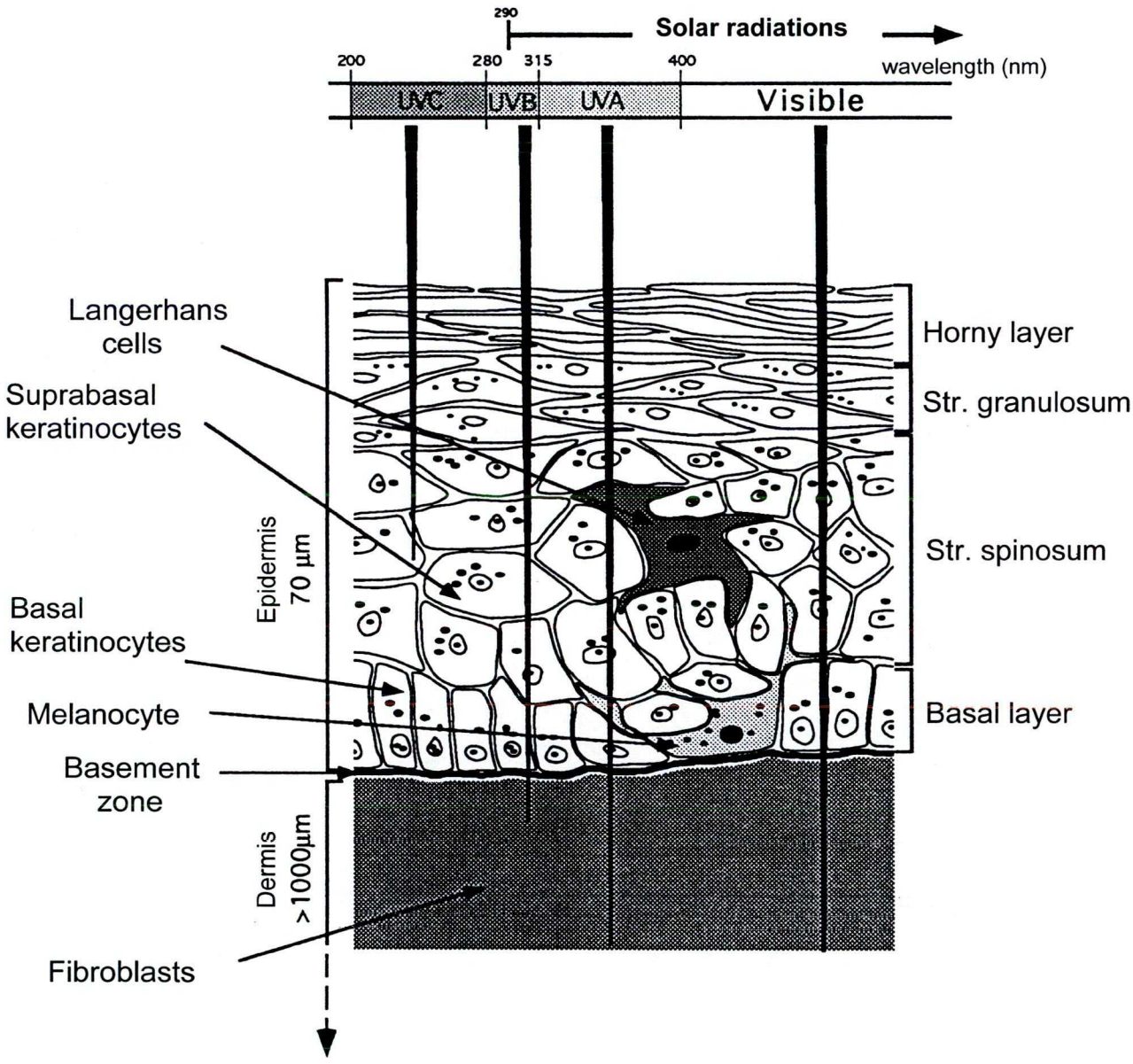
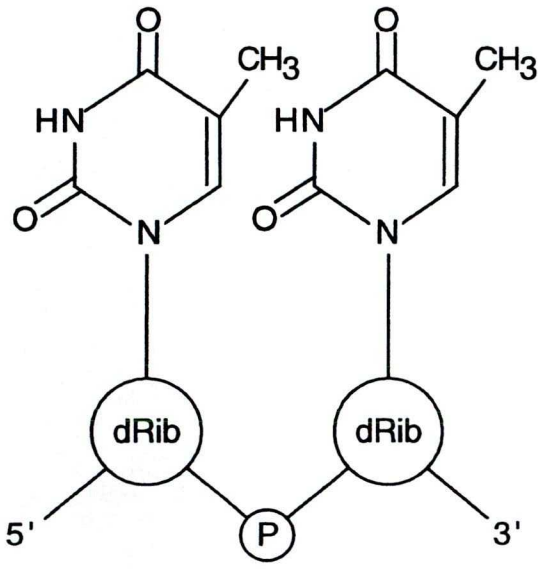
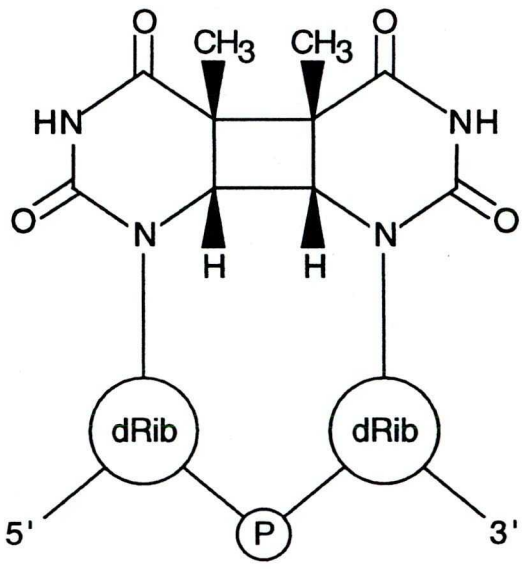


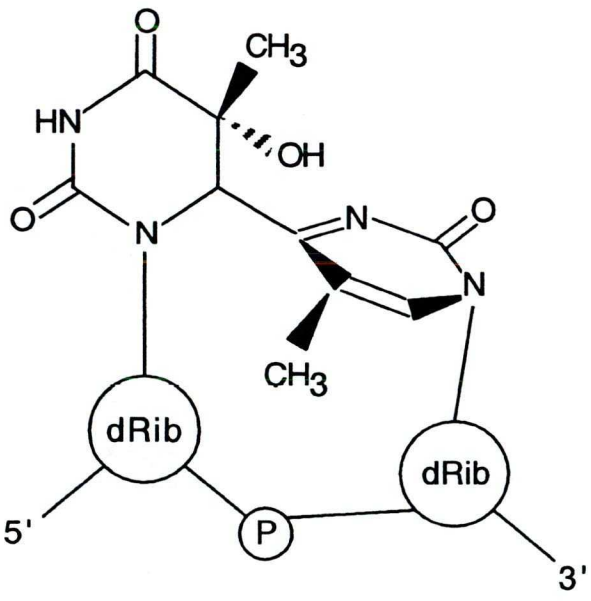

**Figure 1. Skin penetration properties of ultraviolet radiations**



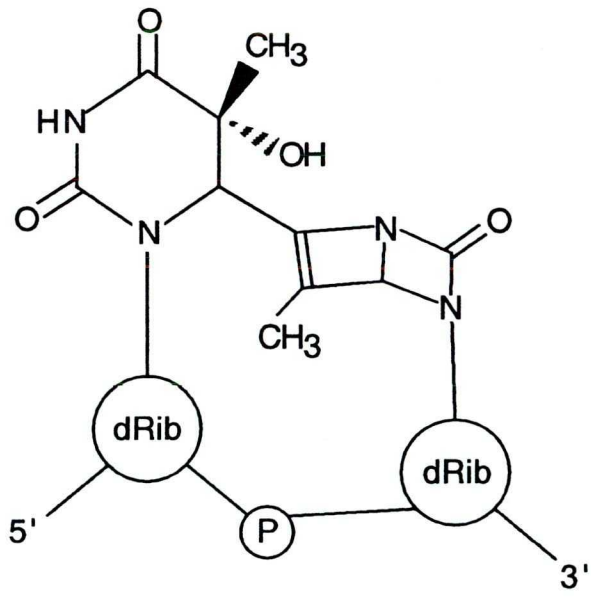

Bipyrimidine sequence




Cyclobutane pyrimidine dimer (CPD)




(6-4) pyrimidine-pyrimidone dimer ((6-4)PP)




Dewar isomer of thymine

**Figure 2. UV-induced DNA lesions (cyclobutane pyrimidine dimers and (6-4) pyrimidine pyrimidone photoproducts)**

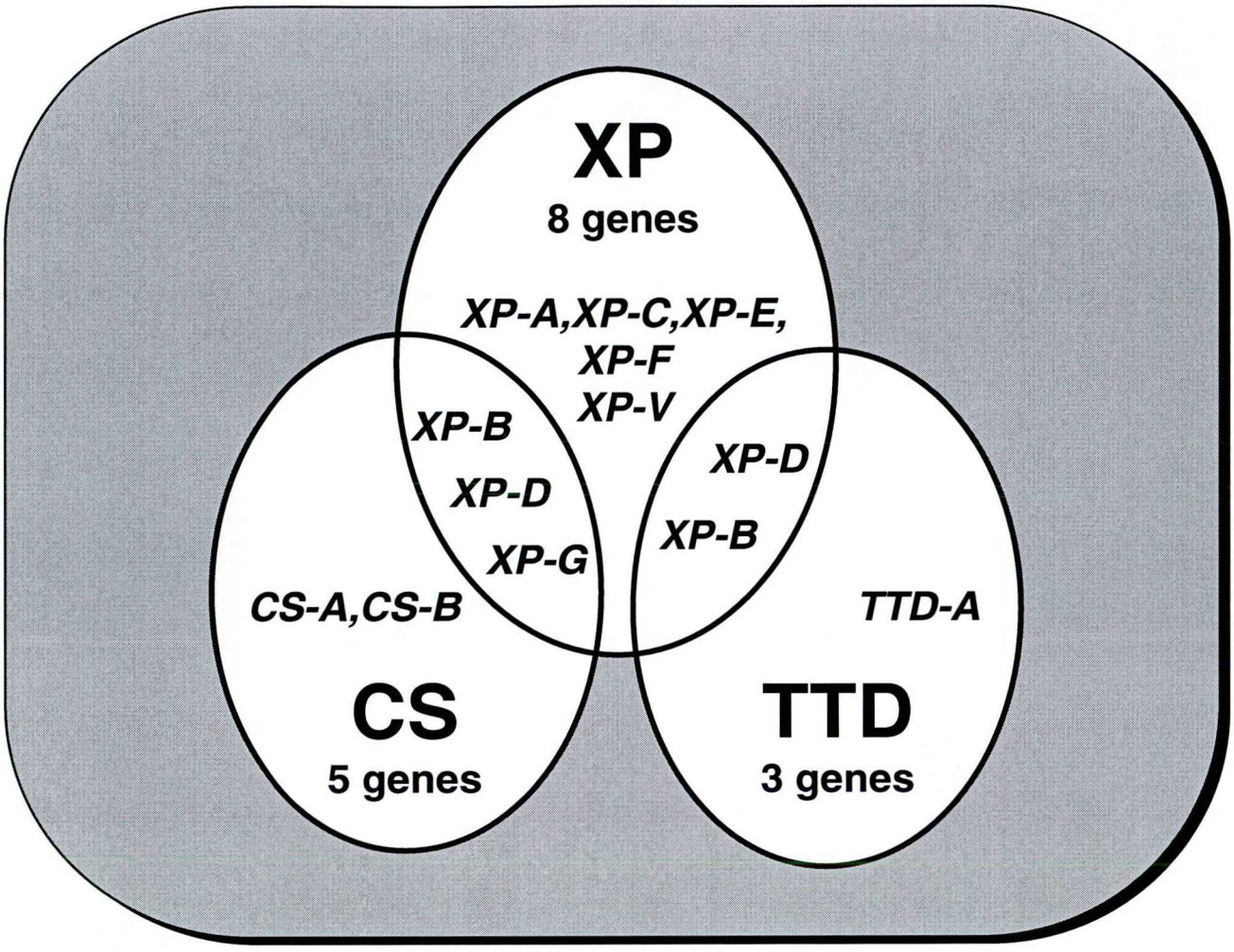



**Figure 3. DNA repair-deficient diseases**

XP - xeroderma pigmentosum,
CS - Cockayne syndrome,
TTD - trichothiodystrophy

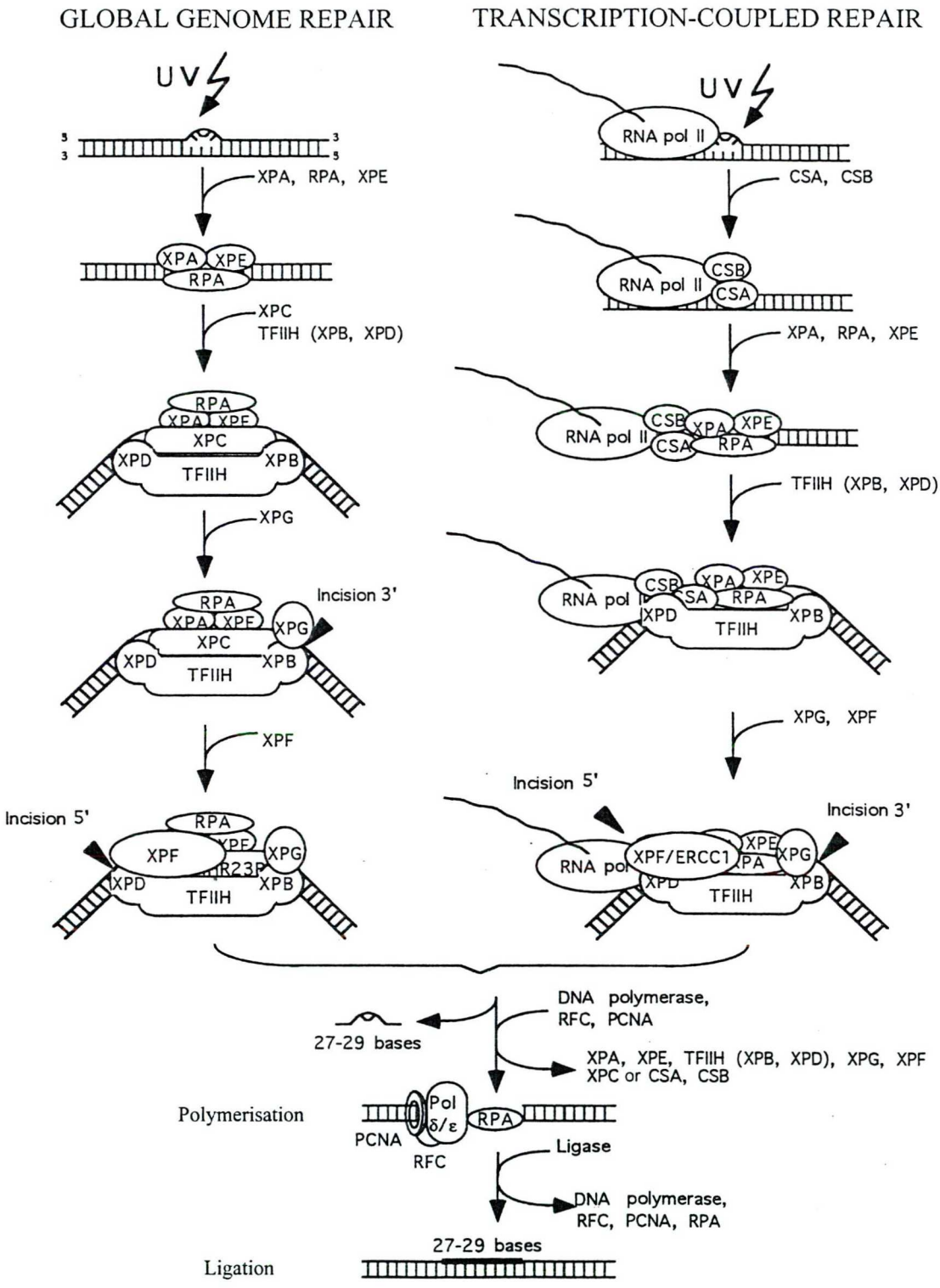



**Figure 4. Model of the nucleotide excision repair (NER) process - global genome repair (GGR) and transcription-coupled repair (TCR)**

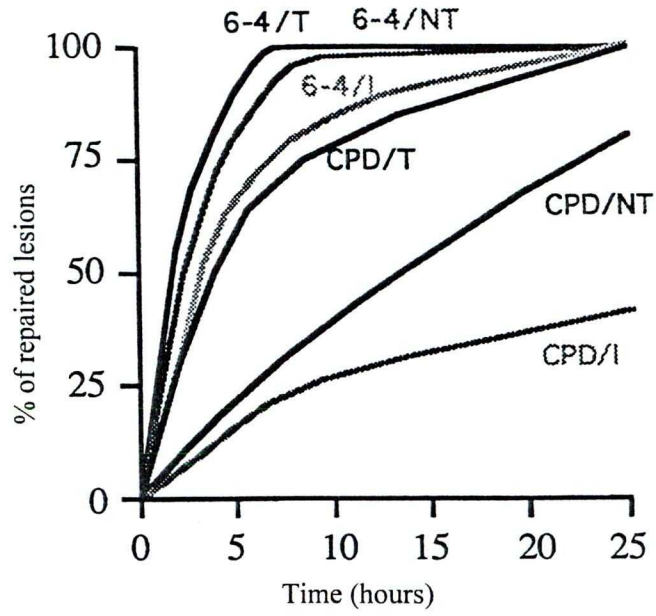



**Figure 5. Kinetics of intracellular DNA repair after UVC irradiation in human fibroblasts.**

T - Transcribed strand, NT - Non-transcribed strand,

I - Inactive part of the genome (van Hoffen, et al.)

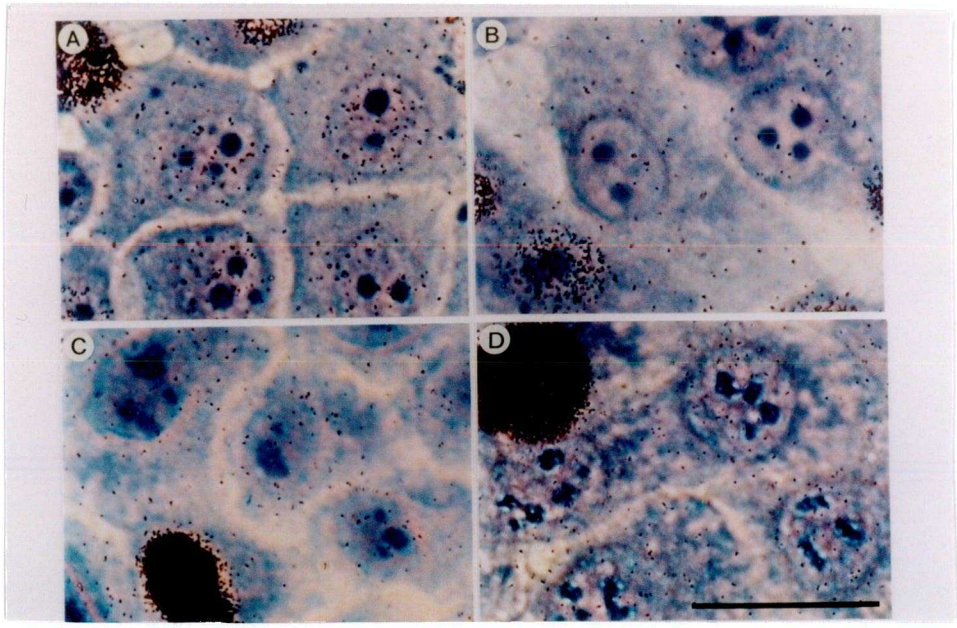



**Figure 6.**

**Figure 6.a. Unscheduled DNA repair synthesis (UDS) of human epidermal keratinocytes after UVB irradiation at 2000 J/m $^2$.**

A, wild type cells. B, XP-C cells. C, XP-D cells. D, TTD/XP-D cells. Bar, 14 mm.

**Figure 6.**

**Figure 6.b. Dose responses of unscheduled DNA-repair synthesis (UDS) curves of normal, XP-C, XP-D and TTD/XP-D fibroblast and keratinocyte strains after UVB and UVC irradiations.**

Wild type cells (□), XP-D (◊), XP-C (Δ) and TTD/XP-D (O) cell strains.

Panels A and B, results obtained after UVC treatment of cells. Panels C and D, results obtained after UVB treatment of cells. Numbers at right hand side of each curve: maximal number of grains obtained in a given cell strain. Percentages: ratio of the maximal number of grains in a given cell strain to the maximal number of grains in wild type cells at maximal doses x 100. Mean grain counts of dose zero were substracted throughout.

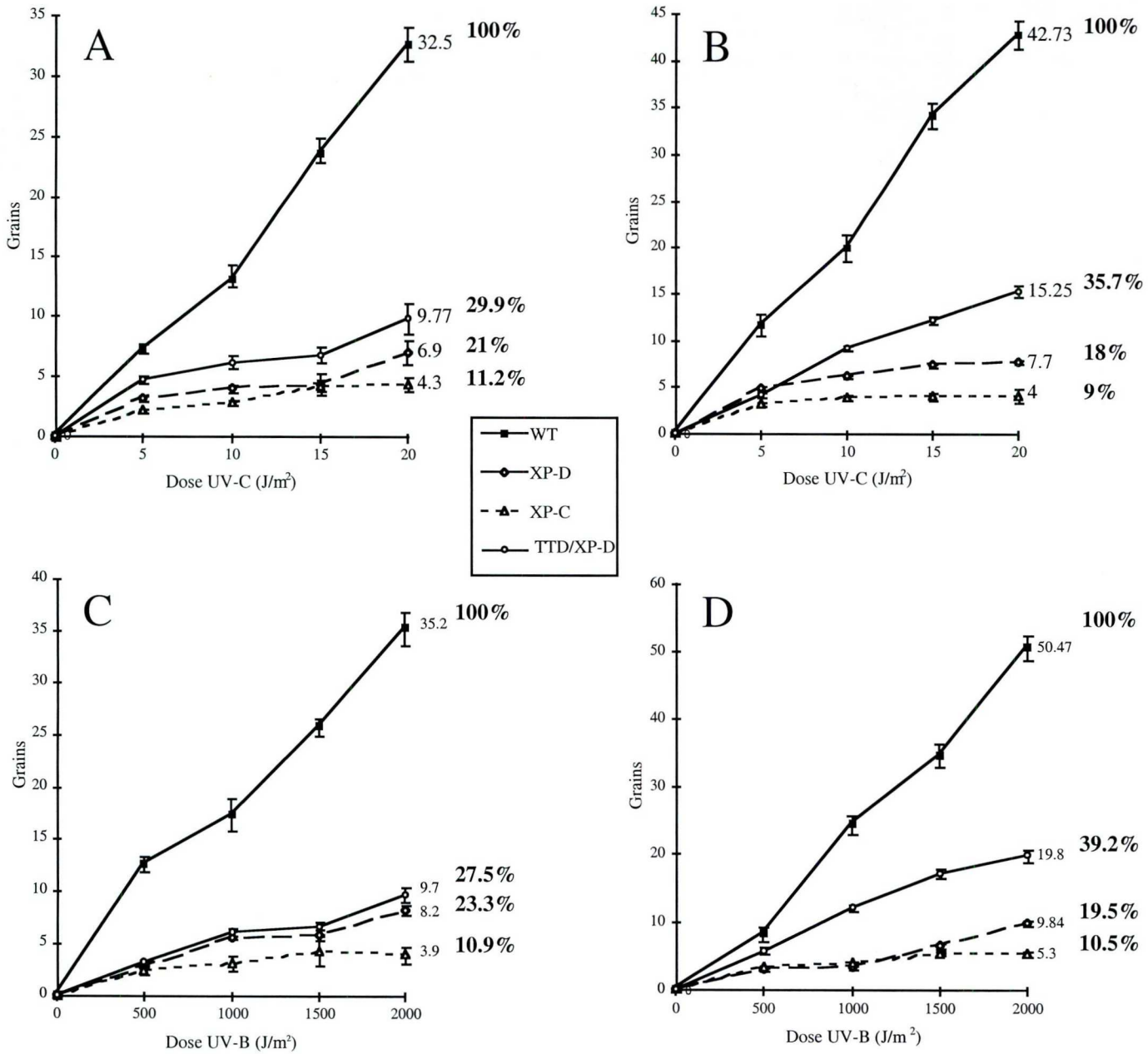

A
Grains
Dose UV-C (J/m²)
32.5
100%
9.77
29.9%
6.9
21%
4.3
11.2%
WT
XP-D
XP-C
TTD/XP-D
B
Grains
Dose UV-C (J/m²)
42.73
100%
15.25
35.7%
7.7
18%
4
9%
C
Grains
Dose UV-B (J/m²)
35.2
100%
9.7
27.5%
8.2
23.3%
3.9
10.9%
D
Grains
Dose UV-B (J/m²)
50.47
100%
19.8
39.2%
9.84
19.5%
5.3
10.5%

**Figure 7.**

**Quantitative assesment of CPD and 6-4PP lesions in normal (WT) fibroblasts and keratinocytes, after UV-B irradiation.**

**Figure 7.a.** Immuno slot-blot experiments carried out with increasing amount of genomic DNA prepared from irradiated normal fibroblasts or keratinocytes (UVB, 1000 $J/m^2$), and revealed using either anti-CPD (TDM-2) or anti-64PP (64M-2) antibodies.

**Figure 7.b.** Densitometric quantification of immuno slot-blots shown in a: Fibroblast DNA (full circles); keratinocyte DNA (open circles).

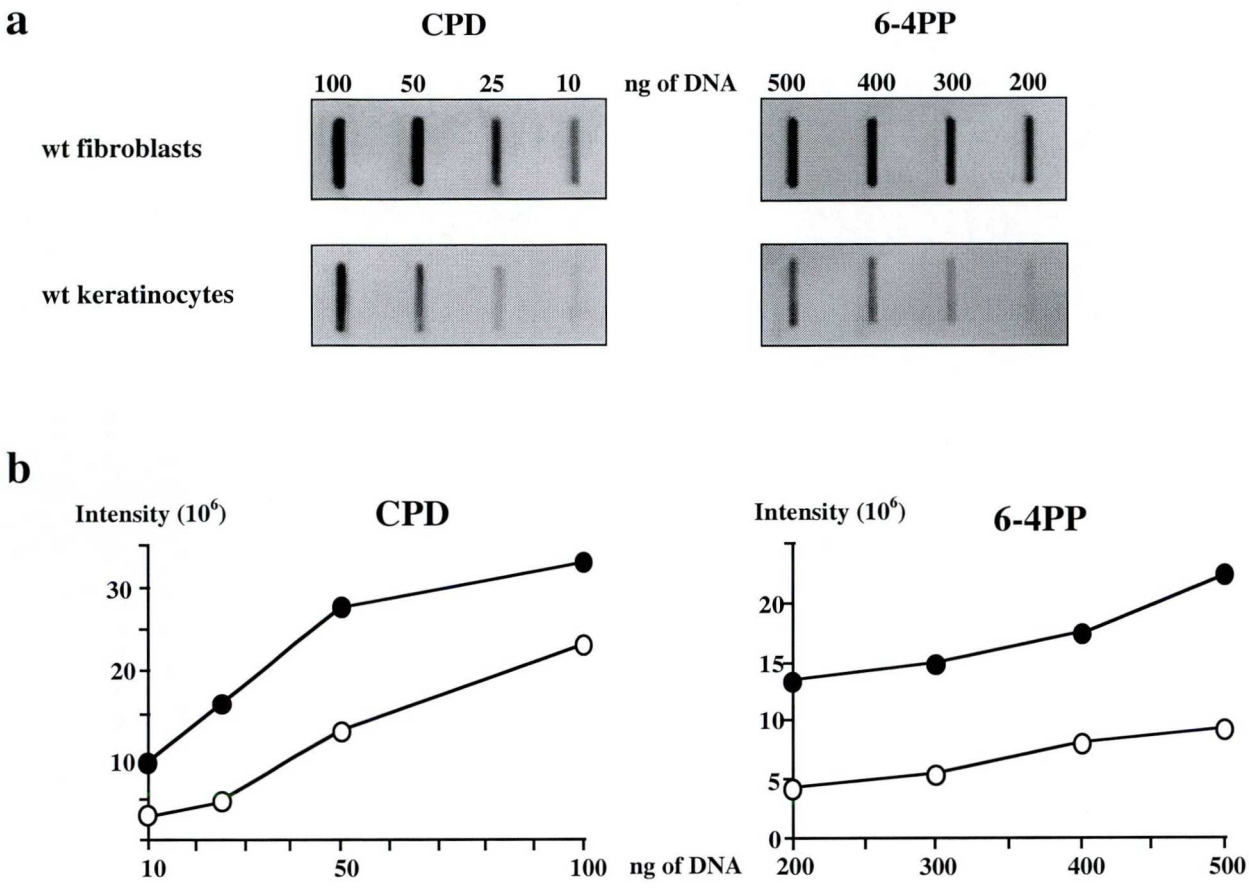

a
CPD
6-4PP
100 50 25 10
ng of DNA
500 400 300 200
wt fibroblasts
wt keratinocytes
b
Intensity ($10^6$)
CPD
30
20
10
10 50 100
ng of DNA
Intensity ($10^6$)
6-4PP
20
15
10
5
0
200 300 400 500

**Figure 8.**

**Figure 8.a. Post-UV survival of human epidermal keratinocytes assessed by colony-forming efficiency (CFE).**

Doses of UVB delivered for wild type (WT) cells, 200, 400, 800, 1200 J/m$^2$, for TTD/XP-D cells (TTD), 200, 400, 800 J/m$^2$ , for XP-C and for XP-D cells, 100, 200, 400 J/m$^2$. Doses of UVA delivered for all cell strains, 100, 250, 500 kJ/m$^2$. NO UV, non irradiated cells. For additional details see *Materials and Methods.*

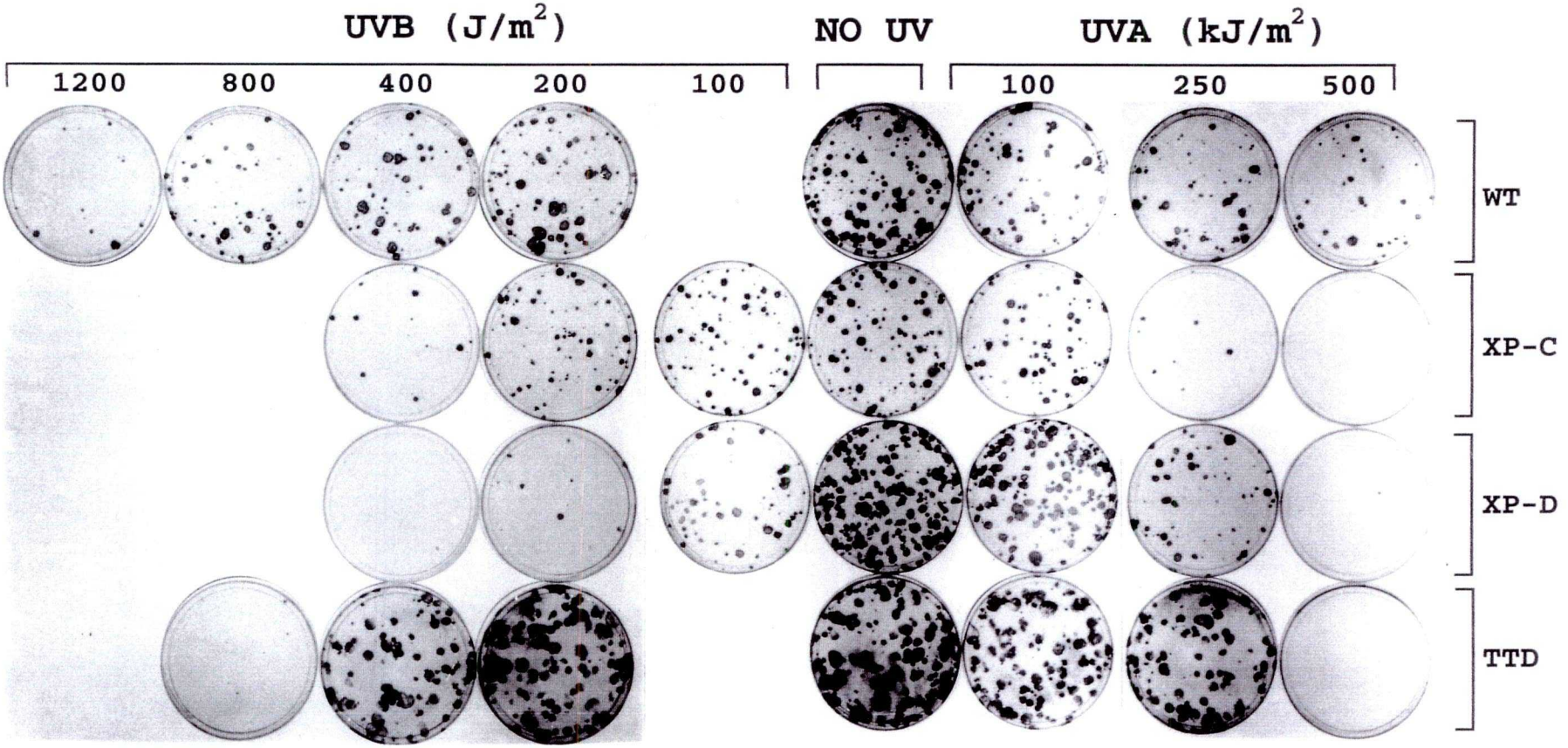

UVB (J/m²)
NO UV
UVA (kJ/m²)
1200
800
400
200
100
100
250
500
WT
XP-C
XP-D
TTD

**Figure 8.**

**Figure 8.b. Survival curves of normal WT (□), XP-C (Δ), XP-D (◊), and TTD/XP-D (O) fibroblast and keratinocyte strains after UV irradiations assessed by colony-forming efficiency (CFE).**

Panels A and B, keratinocyte strains, panels C and D, fibroblast strains. All curves were determined from at least two independent experiments, each performed in duplicate.

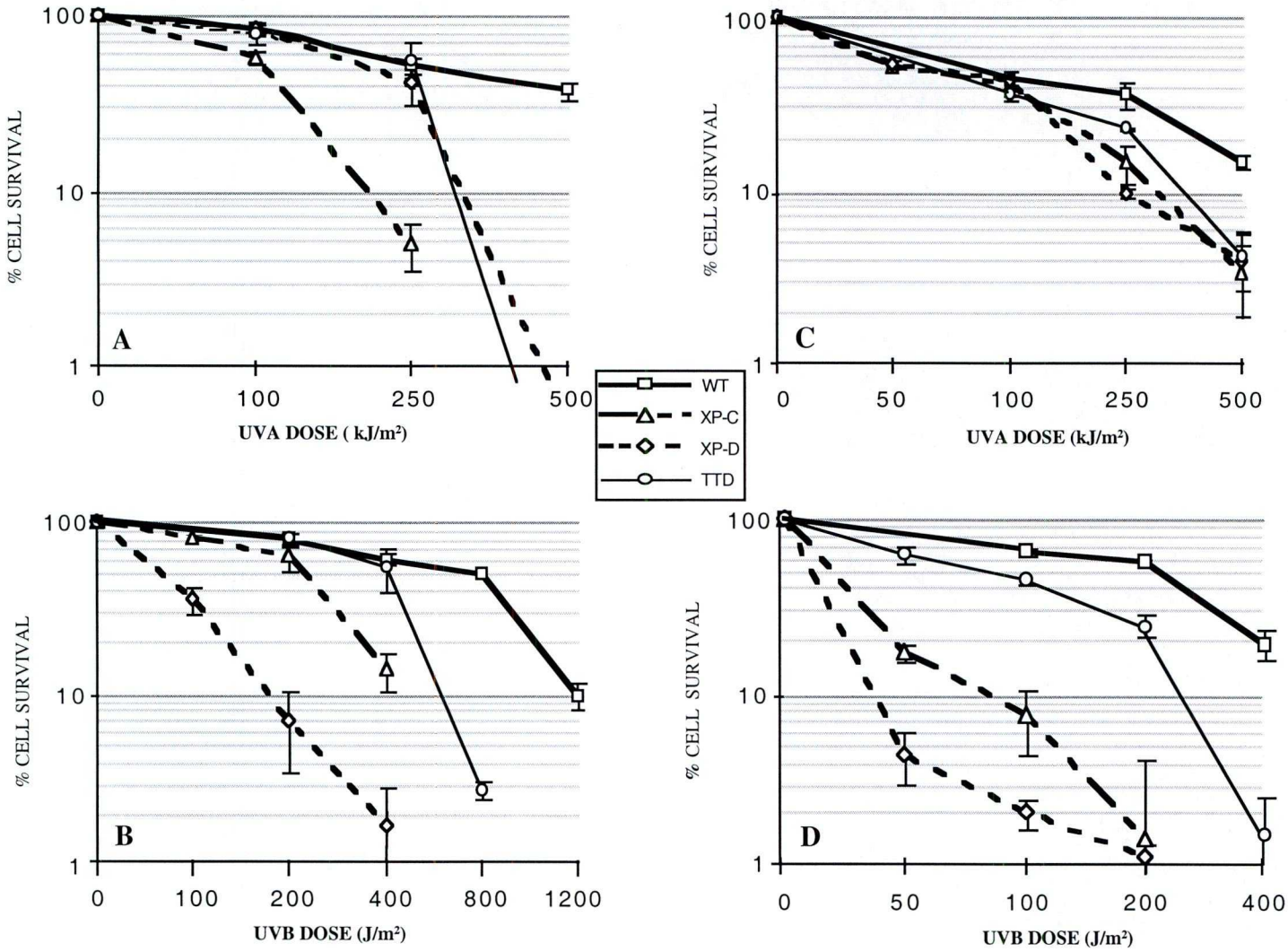

100
10
1
% CELL SURVIVAL
A
0
100
250
500
UVA DOSE ( kJ/m²)
C
50
UVA DOSE (kJ/m²)
WT
XP-C
XP-D
TTD
B
200
400
800
1200
UVB DOSE (J/m²)
D

**Figure 9. Types of keratinocyte colonies after UVA (Panels A, C, E, G) and after UVB (Panels B, D, F, H) irradiations.**

Panels A, B, wild type cells. Panels C, D, XP-C cells. Panels E, F, XP-D cells. Panels G, H, TTD/XP-D cells. Types of colonies: large with smooth perimeter, LSP (black bars), wrinkled, W (crossed bars) and small, highly irregular and terminal, ST (grey bars). Definitions of LSP, W and ST colonies are given in the *Results* section.

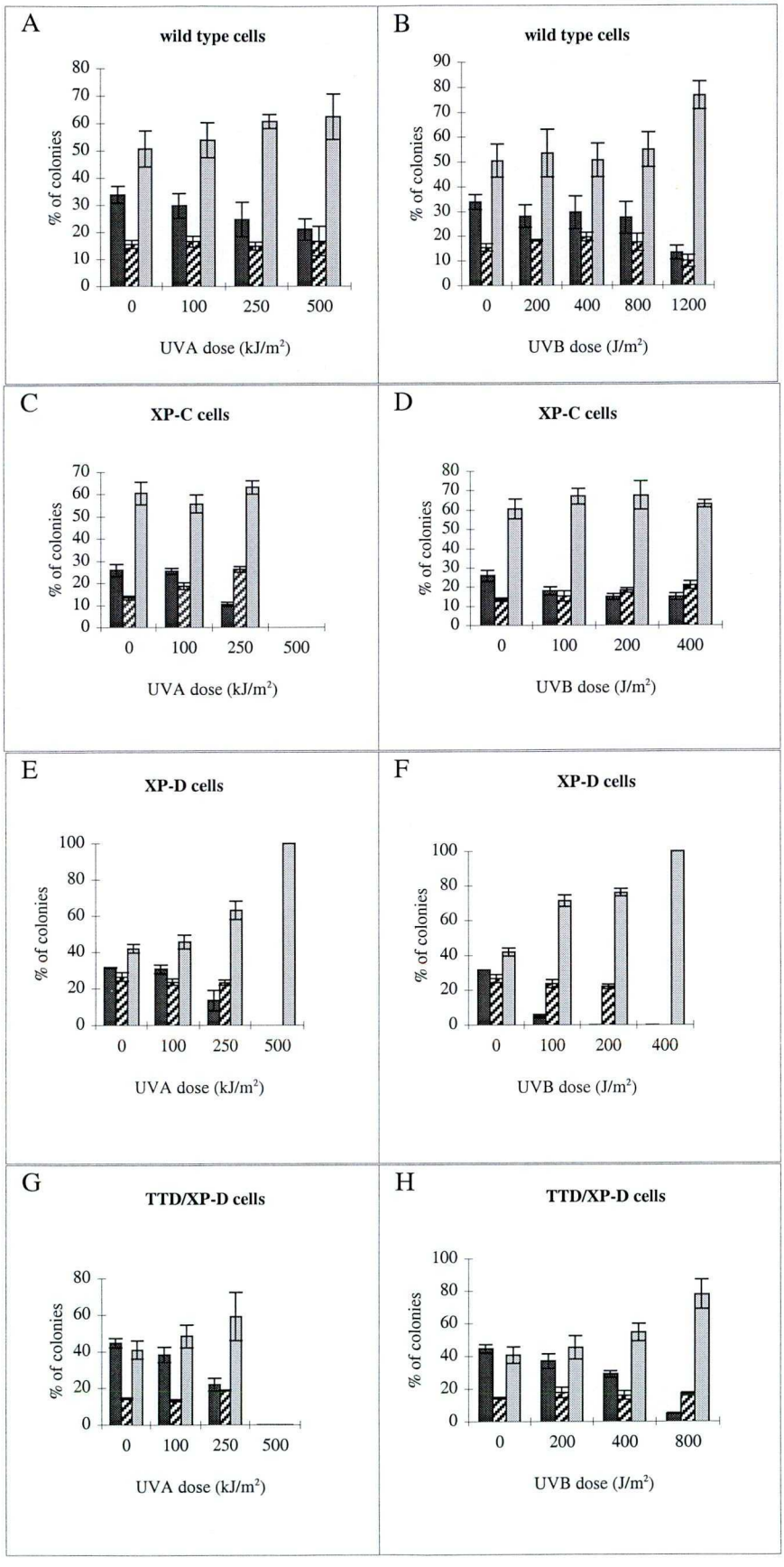
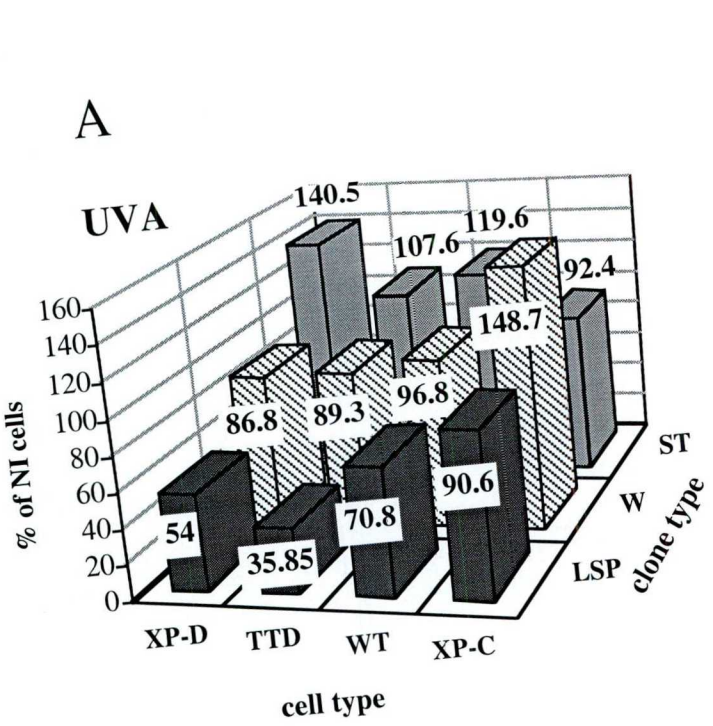

A
wild type cells
% of colonies
0 10 20 30 40 50 60 70 80
0 100 250 500
UVA dose (kJ/m²)
B
wild type cells
% of colonies
0 10 20 30 40 50 60 70 80 90
0 200 400 800 1200
UVB dose (J/m²)
C
XP-C cells
% of colonies
0 10 20 30 40 50 60 70
0 100 250 500
UVA dose (kJ/m²)
D
XP-C cells
% of colonies
0 10 20 30 40 50 60 70 80
0 100 200 400
UVB dose (J/m²)
E
XP-D cells
% of colonies
0 20 40 60 80 100
0 100 250 500
UVA dose (kJ/m²)
F
XP-D cells
% of colonies
0 20 40 60 80 100
0 100 200 400
UVB dose (J/m²)
G
TTD/XP-D cells
% of colonies
0 20 40 60 80
0 100 250 500
UVA dose (kJ/m²)
H
TTD/XP-D cells
% of colonies
0 20 40 60 80 100
0 200 400 800
UVB dose (J/m²)

**Figure 10. Types of keratinocyte colonies after UVA (Panel A) and UVB (Panel B) irradiations leading to 50% cell survival.**

Types of colonies: large with smooth perimeter, LSP (black bar), wrinkled, W (crossed bar) and small, highly irregular and terminal, ST (grey bar). UV doses leading to 50% cell survival were determined by extrapolation from survival curves (*see Table III.)*. The number of each type of colony (LSP, W, ST) at UV doses resulting in 50% survival was extrapolated from the curve representation of data presented as histograms in Figure. 9.. *Numbers in the middle of columns or at top of columns* represent the ratio of the number of a colony type at UV doses resulting in 50% survival to the number of the same colony type for the same unirradiated cell strain x 100.


A
UVA
% of NI cells
160
140
120
100
80
60
40
20
0
140.5
107.6
119.6
92.4
148.7
86.8
89.3
96.8
54
35.85
70.8
90.6
XP-D
TTD
WT
XP-C
cell type
ST
W
LSP
clone type

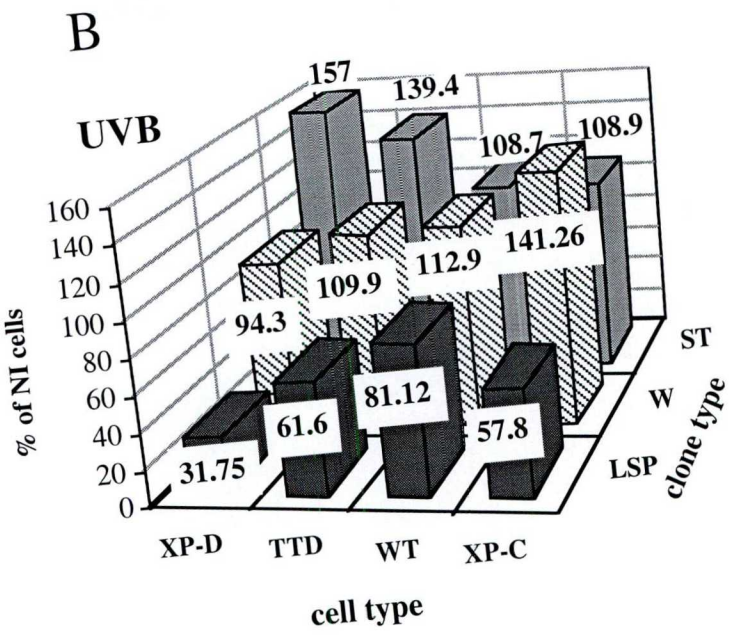
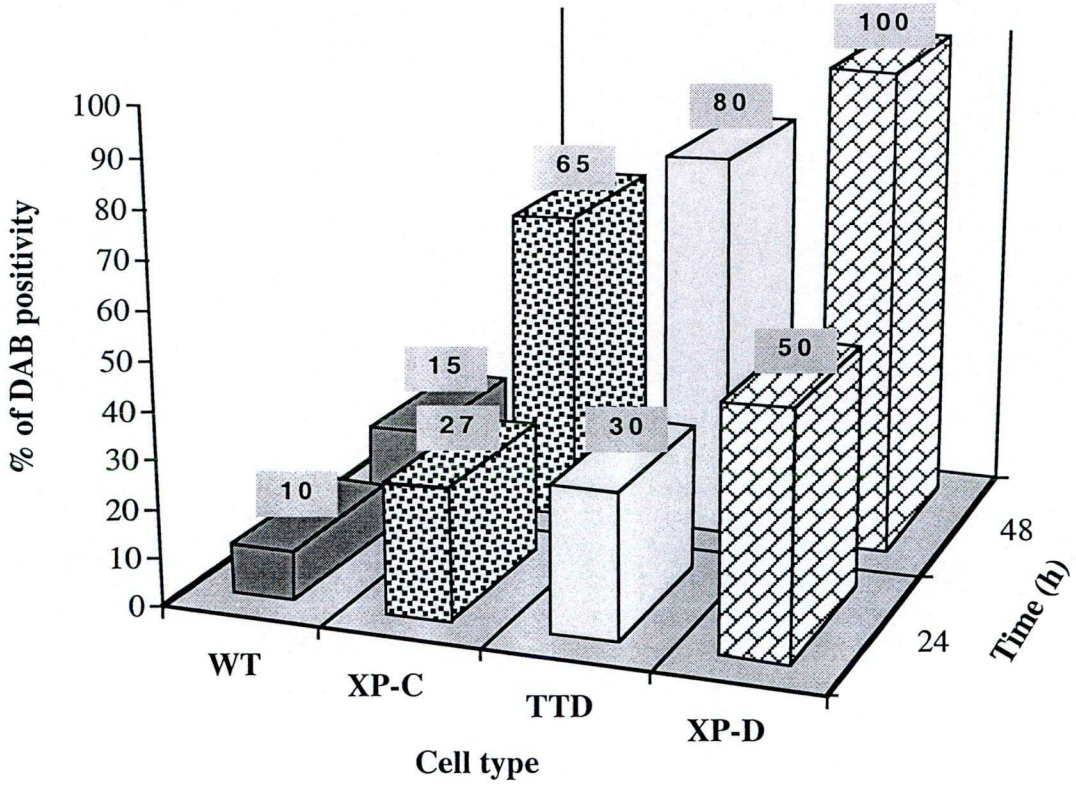

B
UVB
% of NI cells
160
140
120
100
80
60
40
20
0
157
139.4
108.7
108.9
94.3
109.9
112.9
141.26
31.75
61.6
81.12
57.8
XP-D
TTD
WT
XP-C
cell type
ST
W
LSP
clone type

**Figure 11. Apoptosis detected by TUNEL-diaminobenzidine (DAB) labelling of normal, XP-C, TTD/XP-D and XP-D keratinocytes after 1000 J/m$^2$ UVB irradiation at 24 and 48 hours.**

Normal cells (dark grey columns), XP-C cells (dotted columns), TTD/XP-D cells (light grey columns) and XP-D cells (crossed columns) show the percentage of DAB positivity after 1000 J/m$^2$ UVB irradiation at 24 (first row) and 48 hours (second row). DAB positivity was 10 (24 h) and 15% (48 h) in wild type cells, 27 (24 h) and 65% (48 h) in XP-C cells, 30 (24 h) and 80% (48 h) in TTD/XP-D cells, and finally 50 (24 h) and 100% (48 h) in XP-D keratinocytes.

**Figure 12. Apoptosis detected by the TUNEL-DAB method of wild type (a, c, e) and XP-C (b, d, f) keratinocytes after 500 (c, d) and 1000 J/m$^2$ (e, f) UVB irradiation at 24 hours.**


% of DAB positivity
100
90
80
70
60
50
40
30
20
10
0
10
15
27
65
30
80
50
100
WT
XP-C
TTD
XP-D
Cell type
24
48
Time (h)

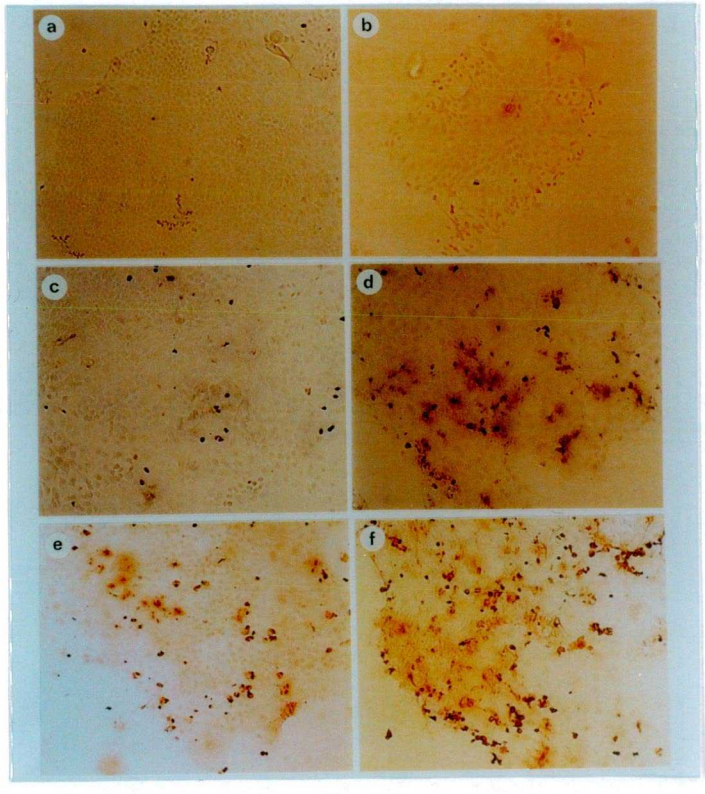
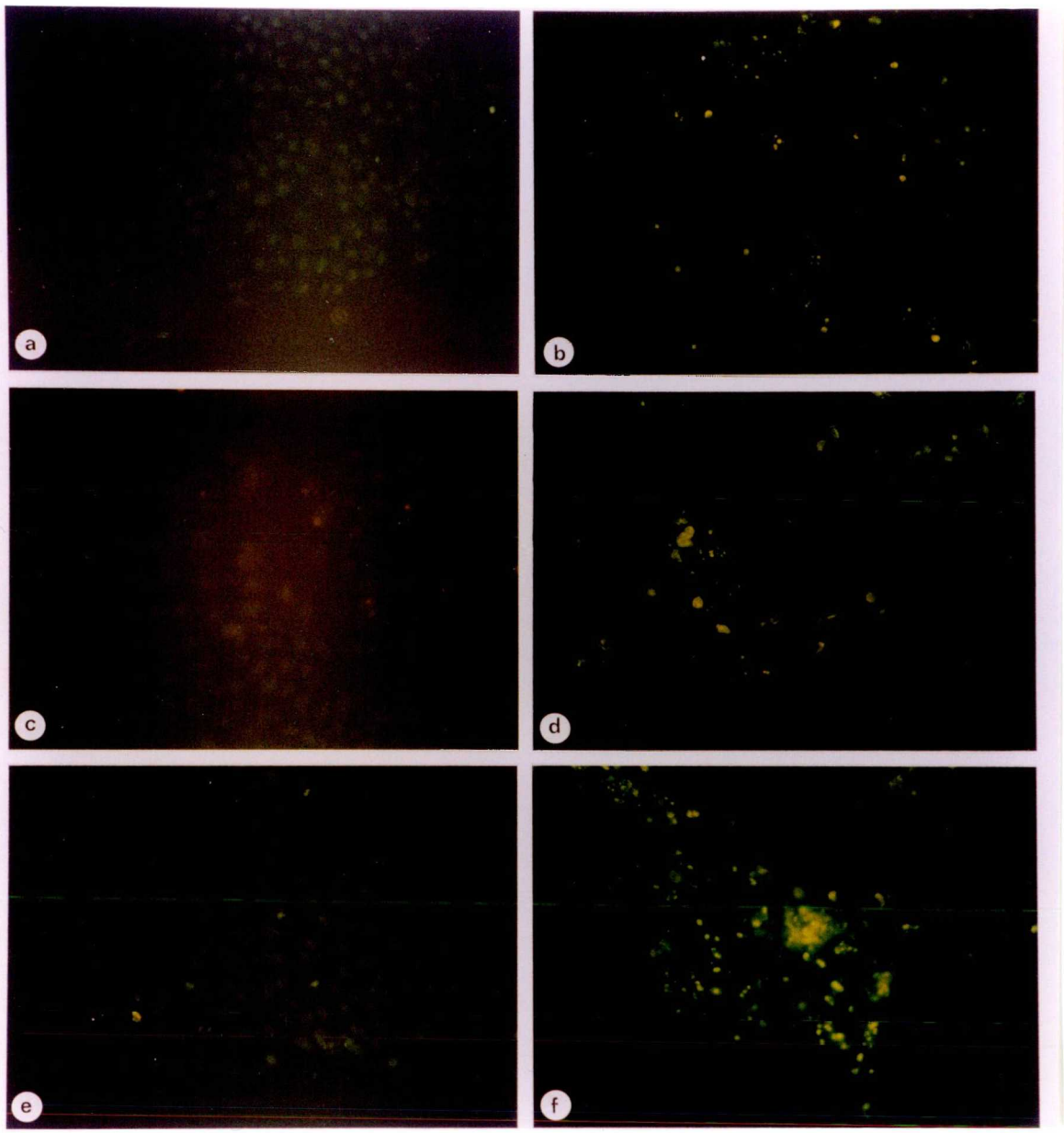

a
b
c
d
e
f

**Figure 13. Apoptosis detected by the TUNEL method of normal (a, b), TTD/XP-D (c, d) and XP-D (e, f) keratinocytes after 1000 J/m$^2$ (b, d, f) UVB irradiation at 24 hours. Non-irradiated cells are shown in a (WT), c (TTD/XP-D) and e (XP-D).**

**Figure 14. DNA fragmentation assay for the detection of apoptosis after 1000 J/m$^2$ UVB irradiation at 24 (lanes 2, 3, 4, 5) and 48 hours (lanes 6, 7, 8, 9).**

First lane: MW VII., 2., 6. lanes: WT, 3., 7. lanes: XP-C, 4., 8. lanes: TTD/XP-D and 5., 9. lanes: XP-D keratinocyte DNAs.

Susceptibility to apoptose was: WT < XP-C < TTD/XP-D < XP-D.


a
b
c
d
e
f

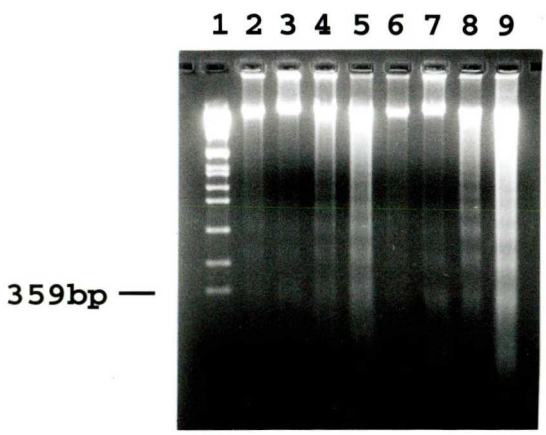

1 2 3 4 5 6 7 8 9
359bp

**Figure 15. TUNEL and involucrin double labelling of wild type keratinocytes after 1000 J/m$^2$ UVB irradiation at 48 hours.**

1., 3.: involucrin immunostaining *(red)* and 2., 4.: TUNEL immunostaining *(green)*. 1., 2. and 3., 4. photos represent the same fields.

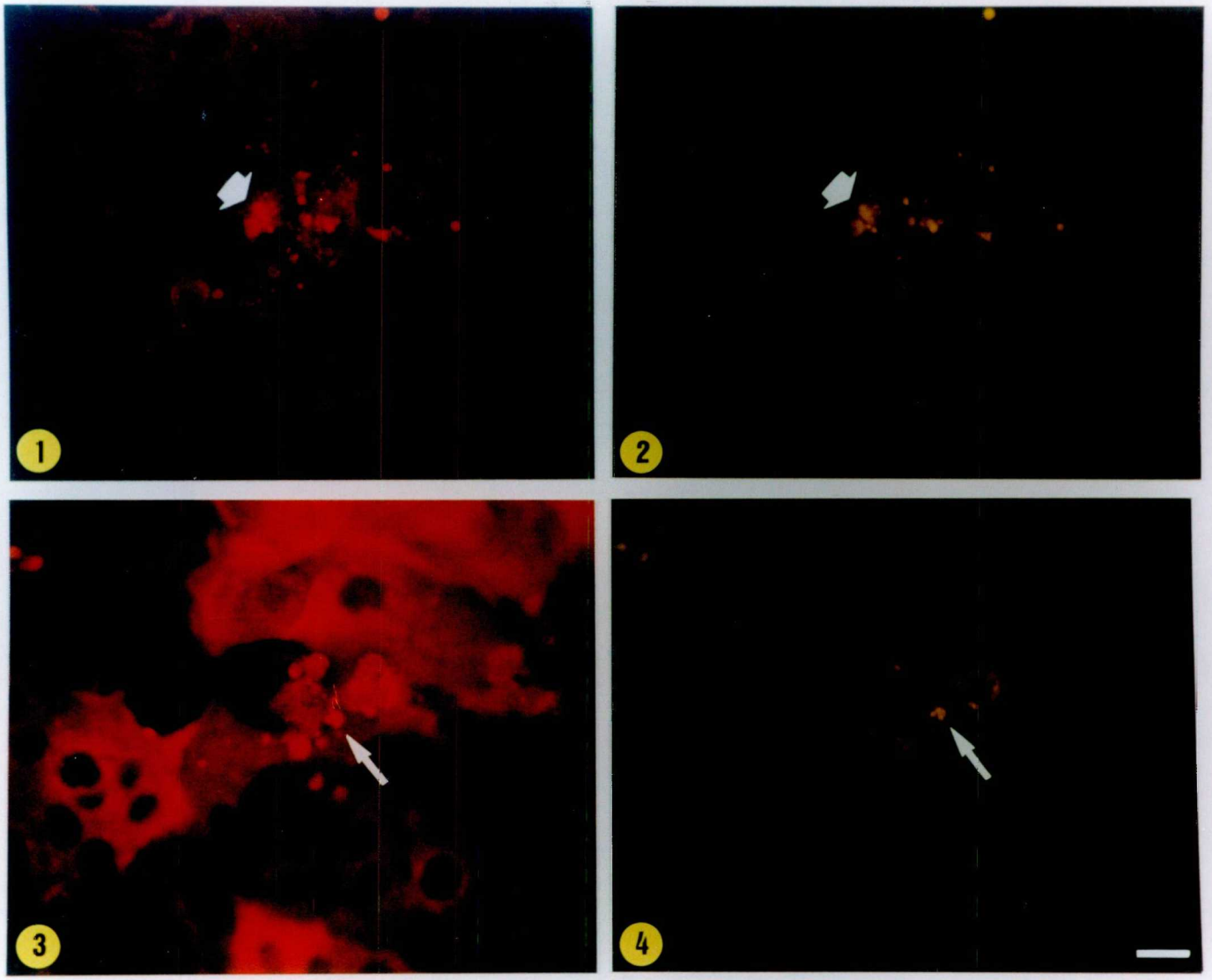
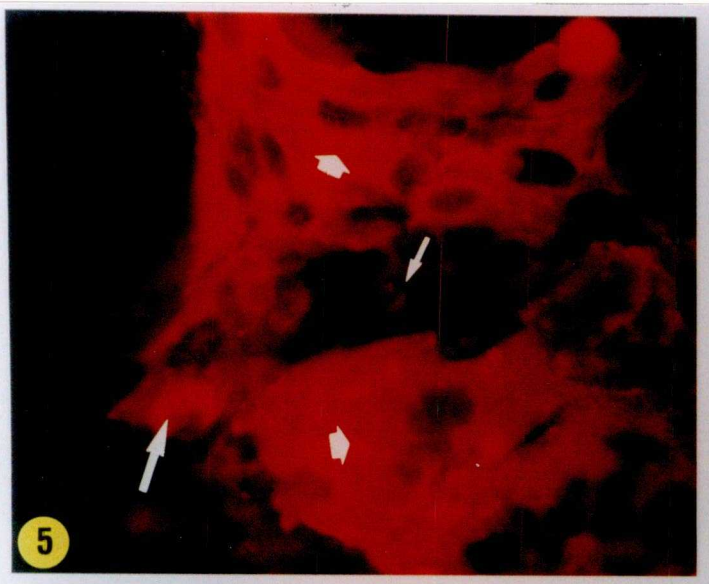
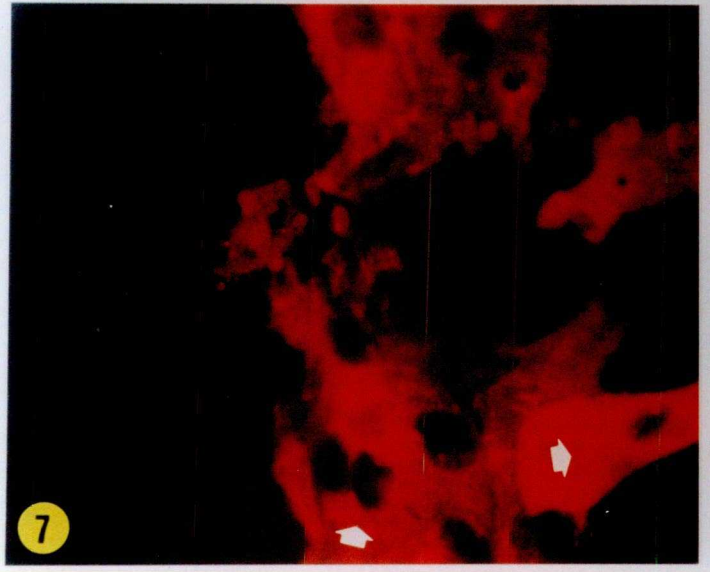

1
2
3
4

**Figure 16. TUNEL and involucrin double labelling of XP-C keratinocytes after 1000 J/m$^2$ UVB irradiation at 48 hours.**

5., 7.: involucrin immunostaining *(red)* and 6., 8.: TUNEL immunostaining *(green)*. 5., 6. and 7., 8. photos represent the same fields. Full arrowheads show involucrin positive-TUNEL negative cells, the small arrowhead shows an involucrin negative-TUNEL positive cell *(see number 6.)*; the intermediate sized arrowhead shows an involucrin positive-TUNEL positive cell *(see number 5.)*. For additional details see the *Results* section.


5

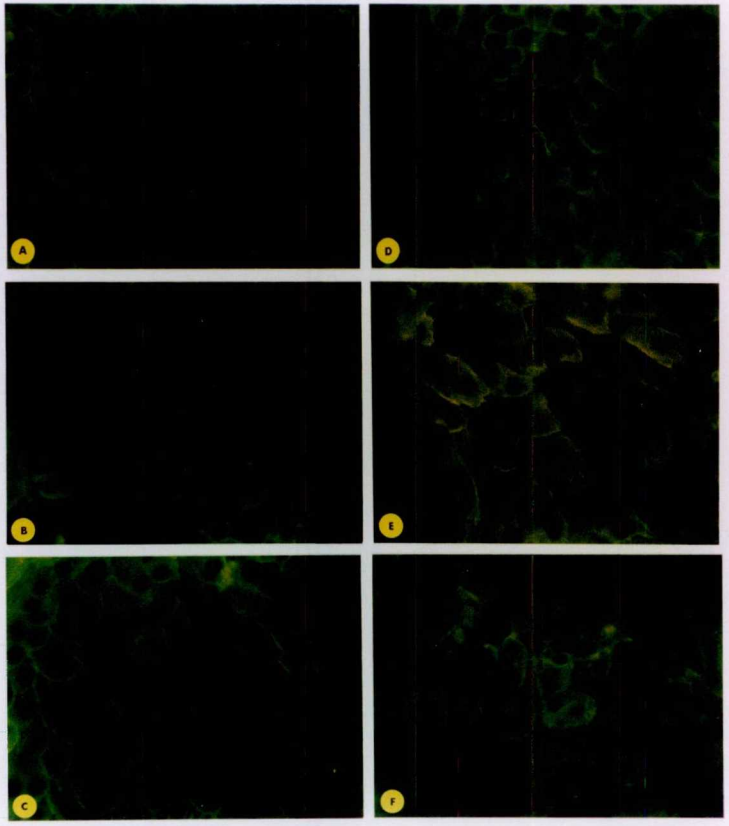

6


7


8

**Figure 17. α6 integrin immunostaining of wild type (A, B, C) and XP-C (D, E, F) keratinocytes after 500 and 1000 J/m$^2$ UVB irradiation at 24 hours.**
WT (A, B, C) and XP-C (D, E, F) cells after 500 J/m$^2$ (B, E) and after 1000 J/m$^2$ (C, F) UVB irradiation at 24 hours. A, D show non-irradiated keratinocytes.

**Figure 18. α6 integrin immunostaining of TTD/XP-D (G, H, I) and XP-D (K, L, M) keratinocytes after 500 and 1000 J/m$^2$ UVB irradiation at 24 hours.**
TTD/XP-D (G, H, I) and XP-D (K, L, M) keratinocytes after 500 J/m$^2$ (H, L) and after 1000 J/m$^2$ (I, M) UVB irradiation at 24 hours. G, K show non-irradiated keratinocytes.


A
D
B
E
C
F

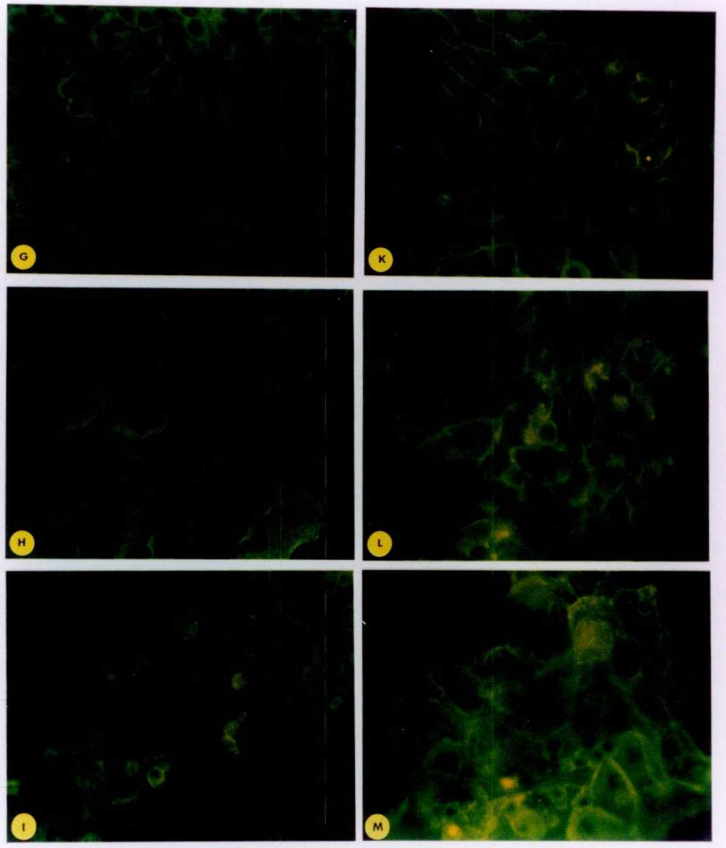
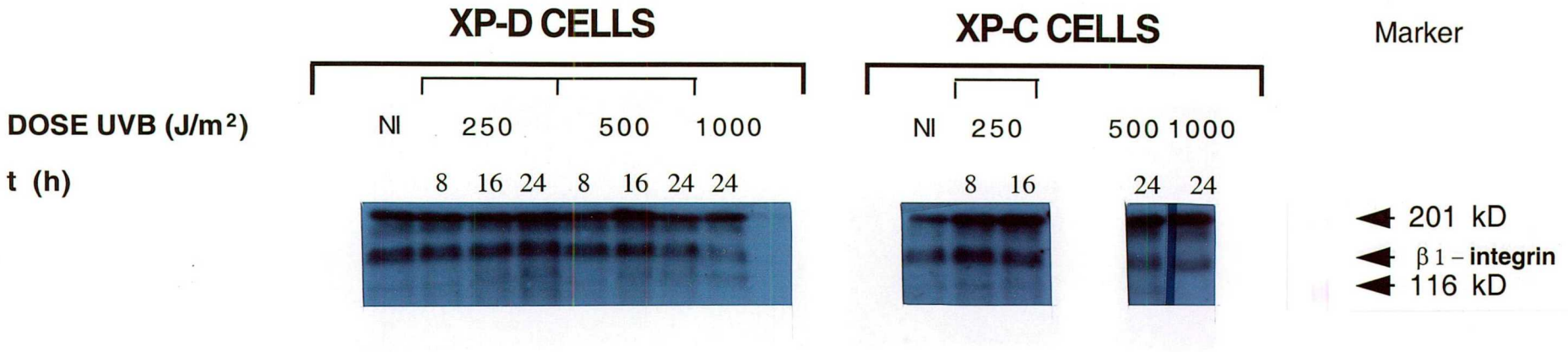

G
K
H
L
I
M

**Figure 19. Western blotting using β1 integrin, polyclonal antibodies.**

Proteins of cells were isolated at 8, 16 and 24 hours after UVB irradiation as presented. A rainbow marker is shown on the right hand side. The expression of the β1 polypeptide seemed to increase with time after 250 J/m$^2$ UVB in both cell types. 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.


XP-D CELLS
XP-C CELLS
Marker
DOSE UVB (J/m²)
t (h)
Nl
250
500
1000
Nl
250
500 1000
8 16 24 8 16 24 24
8 16
24 24
201 kD
β 1 – integrin
116 kD

# APPENDIX

## Appendix I. Clinical features and cellular characteristics of XP, TTD and CS

| | Clinical features | | Cellular characteristics | |
|---|---|---|---|---|
| Group | Neurological abnormalities | Skin cancer | UV sensitivity | UDS % |
| Normal | - | - | - | **100** |
| XP-A | ++ | +++ | +++ | 0-15 |
| XP-B | +++ | + | ++ | 5-15 |
| XP-C | - | +++ | + | 10-15 |
| XP-D | ++ | ++ | ++ | 25-55 |
| XP-E | - | + | +/- | 40-60 |
| XP-F | - | + | + | 10 |
| XP-G | ++ | + | ++ | 5 |
| XP-V | - | + | +/- | **100** |
| CS-A | ++ | - | ++ | **90-100** |
| CS-B | ++ | - | ++ | **90-100** |
| TTD-A | + | - | ++ | 10-30 |
| TTD/XP-B | + | - | + | 40-60 |
| TTD/XP-D | + | - | ++ | 20-60 |

## Appendix II. Repair of UV-induced DNA lesions of XP, TTD and CS cells

| | UV sensitivity | Global Genome Repair (GGR) | | Transcription-coupled Repair (TCR) | |
|---|---|---|---|---|---|
| Group | | CPD | (6-4)PP | CPD | (6-4)PP |
| Normal | - | + | + | + | + |
| XP-A | +++ | - | - | - | -? |
| XP-B,-D,-E,-F,-G | ++ | - | - | - | -? |
| XP-C | ++ | - | - | + | + |
| TTD/XP-B | + | - | + | - | + |
| TTD/XP-D | ++ | - | + | - | + |
| TTD-A | ++ | - | + | - | + |
| CS-A,CS-B | ++ | + | + | -* | +? |

*CS-A and CS-B cells are defective in the TCR of CPDs in the transcribed strand of active genes.

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