Mutations in Cancer Genes of UV-Induced Skin Tumors of Hairless Mice

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Ultraviolet (UV) radiation is a very common carcinogen in our environment. Epidemiological data on the relationship between skin cancers and ambient solar UV radiation are very limited. Hairless mice provide the possibility to study the process of UV carcinogenesis in more detail. Experiments with this animal model have yielded quantitative data on how tumor development depends on dose, time and wavelength of the UV radiation. In addition, at the molecular level the interactions between UV, specific cancer genes-like the Ras oncogene family and the p53 tumor suppressor gene, together with the role of DNA repair in this process have been addressed recently. In wildtype hairless mice mutations in the p53 gene are clearly linked to UVB but not to UVA radiation. Furthermore, the p53 alterations seem to be essential early in tumor development. However, in Xpa-deficient mice this dependency on p53 alterations appeared to be different as is the tumor type induced by UVB. Research using genetically modified hairless mice should enable us to further unravel the mechanisms of UV-induced skin cancer.


UV, skin, cancer, genes, mice

Ultraviolet (UV) radiation is natural to our environment and it's main beneficial effect is vitamin D3 production in our skin. However, UV radiation is also one of the carcinogens to which we are most commonly and abundantly exposed. Fortunately, like most organisms exposed to solar UV radiation, the human skin has several very efficient defense mechanisms to protect itself from the damaging effects. These primary lines of defense, of which several are believed to be triggered by DNA damage, include hyperplasia (thickening of the epidermis), melanin production (tanning), immunesuppression (reducing inflammatory responses) and DNA repair. The most effective DNA repair pathway dealing with UVB-induced DNA damage is the nucleotide excision repair pathway (NER). This is clearly illustrated by patients with the rare, heritable, autosomal recessive disorder xeroderma pigmentosum which results in skin cancers on sun-exposed body sites at very early age 9. The current view of multistage skin carcinogenesis assumes a process driven by carcinogen-induced genetic and epigenetic changes in susceptible cells leading to a selective growth advantage and clonal expansion of so called initiated cells 2. In this paper, we first present an introduction on skin cancer and UV-induced specific damage, followed by a summary of our results on genetic changes observed in (genetically modified) hairless mice.

SKIN CANCER

Skin cancer is one of the most common neoplasms in Caucasians worldwide 9, and the incidences in for example the USA are considerably higher in the Southern, more sunny part than in the Northern part. Three main types of skin cancer can be distinguished: basal cell carcinoma (BCC), squamous cell carcinoma (SCC) and cutaneous malignant melanoma (CMM). All of these skin tumors originate from the epidermis, i.e. the outermost viable cell layer with a typical thickness of 70μm (note that the most carcinogenic solar UV radiation hardly pen-
etrates any deeper than this). BCC are most common in Caucasians (typically about 90 cases per 10^5 people per year in NW Europe), followed by SCC (about 15 cases per 10^5 per year), and CMM (about 10 per 10^5 per year) 4. CMM originate from the pigment cells (melanocytes) and are the most aggressive type: they can rapidly metastasize. SCC and BCC originate from the keratinocytes, and these tumors are less aggressive, but they do grow invasively. When neglected, SCC can metastasize, which is quite a rare event with BCC. Mortality is about 20-25% of the CMM cases, whereas it is 1-3% for SCC and less than 1% for BCC. Adequate detection of these tumors on the body surface is clearly favorable to the therapeutic success, especially for SCC and BCC. Although CMM is a minority in the skin cancer incidence, it strongly dominates the mortality from skin cancer.

The contention that SCC and BCC are related to extensive sun exposure dates back to the 19-th century, when it was noted that these tumors occurred commonly on people with outdoor professions (people in vineyards and fishermen). A possible link between CMM and sun exposure has always been a subject of debate. The common feature of people who are at the highest risk of skin cancer is their sun (UV) sensitivity, i.e., they sunburn easily and never tan (skin type I). SCC appears to have the most straightforward relationship to sun exposure: these tumors occur on regularly exposed skin areas (head, neck, back of the hands) 8 and the risk increases with the life-long accumulated exposure 6,7. For a long time the etiology of BCC was believed to be largely similar to that of SCC, but more recent epidemiological studies have pointed out distinct differences. Although BCC are also mainly located on the most exposed areas of the skin (80-90% on the head 8) and the risk has been reported to go up with the total sun exposure 7, BCC -- in contrast to SCC -- hardly develops on the backs of the hands but are relatively more frequently on the trunk 5,8, and the sun exposure in the decades prior to removal of the tumor are not related to the risk 8. Apparently, BCC shows a predilection for certain skin sites (sebaceous skin?), and sun exposure is probably related to an early event in the genesis of the tumor. Australian data from a population survey 8 show that the number of recalled sunburn episodes was positively related to the risk, especially sunburns that occurred in childhood. BCC in this respect appears to be more similar to CMM. Except for the Hutchinson Melanotic Freckel Melanoma (a minority of CMM, about 10%, with an etiology more like SCC), CMM is known not to be related to the life-long accumulated sun exposure, but more to intermittent over-exposure and to high levels of ambient UV radiation during childhood 9.

Epidemiology has provided us with valuable data on associations between skin cancer and sun exposure, but it cannot by itself establish with any certainty which factors play a causal role in the pathogenesis: e.g. which part of the solar spectrum is really causing these tumors? Clearly, to this end the epidemiological data need to be supplemented and supported with relevant experimental data on UV-induced skin carcinogenesis. Animal models, like the hairless mouse model (see below), will be essential to unravel the relative contributions of UV genotoxicity and other factors like for example immune suppression as well as to study their causal interrelationship.

**SUNLIGHT**

Wavelengths of visible radiation, i.e. light, lie in the range between 400 and 800 nm. The UV wavelength range borders at the violet end of the 'visible octave' at 400 nm. From 400 nm down to 315 nm is the UVA range, from 315 to 280 nm the UVB range (a range between 290 nm- 320 nm is also commonly used), and from 280 to 200 nm the UVC range (below 200 nm is the 'vacuum UV' which is strongly absorbed in air). Although the sun emits UV radiation of wavelengths beyond the UVC range, no radiation of wavelengths shorter than 290 nm reaches the earth surface, because of absorption in the atmosphere by oxygen (UVC) and ozone (UVC and UVB). Depletion of the ozone layer is supposed to shift the UV spectrum at the earth surface to lower wavelengths, i.e., more short wave UVB radiation would be transmitted through the atmosphere. The absorption of UV radiation by proteins and DNA increases dramatically towards these shorter wavelengths in the UBV, as does the corresponding damage to these molecules.

The skin can be subdivided into two main layers: the outer layer, the epidermis, and the underlying dermis. UVA radiation penetrates well into the dermis, but UVB radiation is more strongly absorbed, increasingly so for shorter wavelengths. Since the human epidermis (about 5 cell layers, approx. 70 μm, dorsally) is generally thicker than that of a mouse (about 2 cell layers, approx. 30 μm, dorsally), the transmissions of UV through epidermal sheets differ from each other between the two species, especially in the UVB and UVC ranges.

The epidermis is steadily renewed from its basal stem cell layer, while dead cornified cells are shed from its surface (a turn-over time of viable cells of about 3 weeks in humans, and probably of 1-2 weeks in mice). Its proliferative activity and high UV absorption make the epidermis a primary target for UV carcinogenesis: all UV-related skin tumors in humans and experimentally UV-induced skin tumors in hairless mice originate from the epidermis.

**HAIRLESS MOUSE MODEL**

The mouse has become the leading animal model for studying biological processes in mammals. Among the classical benefits of mice are their small size, easy breeding and maintenance. Moreover, due to genetic modification of the mouse
genome, a rich collection of mouse strains harboring naturally occurring, ENU-induced, or targeted gene mutations has become available. Because cancer is a genetic disease, these mice become increasingly attractive and are already by far the mostly used animals in experimental carcinogenesis, including experimental skin carcinogenesis. For the latter the fur is a handicap, especially with UV irradiation, because the hairs form an impenetrable shield. Shaving off the fur to expose the underlying skin could introduce a confounding stimulatory effect on tumor formation by the carcinogenic agent under investigation.

Hairless skin is exceptionally well suited for experiments on carcinogenesis in general: the animals need not be sacrificed to assess the tumor load as is necessary with internal tumors. The tumors are easily detectable at very small sizes (a trained observer has no problem in spotting tumors smaller than 1 mm across), and multiple tumors per mouse can be followed in their progression without any serious discomfort to the animals. Moreover, carcinogenesis by low level chronic UV exposure of hairless skin has a direct analogy in every day human life.

In the 1960s a hairless stock of albino mice, called SKH-1, became available, and proved to be well suited for studying experimental UV carcinogenesis. The SKH-1 mice are immunocompetent (in contrast to the 'nude' mice), and they go without hair in their adult life due to a retroviral insertion at the 'haired' locus on chromosome 14, the recessive hairless allele is designated as 'hr'. In the homozygous (hr/hr) mice UV radiation almost exclusively induces skin carcinomas and precursors, all originating from the epidermis (as is true for UV-related human skin cancer), whereas in (shaved) haired mice also fibrosarcomas are found, which originate from the dermis. At present, there are various kinds of hairless mouse strains with different genetic origins, including a hairless C3H strain. In addition, we have been crossing a variety of transgenic mouse strains into the SKH-1 background to study the interactions of UV-radiation with these different genetic alterations. Up till now, genetically modified strains of mice with inactivated genes important for SCC like p53, Xpa, Xpc, Csb, and Msh2 have been selected.

UV-PHOTOPRODUCTS

It is generally accepted that DNA damage, causing mutations in genes, plays a pivotal role in carcinogenesis. UVB and UVC radiations are very genotoxic through direct absorption by DNA. The dominant DNA damage caused by UVC and UVB exposures is targeted at neighboring pyrimidines that become dimerized; either through a four-carbon cyclobutane ring at the 5 and 6 positions in the successive bases or a binding of the 6-th position to the 4-th in the next base, where the former adduct (the cyclobutane pyrimidine dimer, CPD) is formed approximately three times more frequently than the latter (the 6-4 pyrimidine-pyrimidone photoproduct, 6-4PP). CPD distorts the DNA helix less than 6-4PP. The wavelength dependencies (action spectra) of induced CPD and 6-4PP run parallel, and follow the DNA absorption spectrum (measured unto 313 nm). The action spectra of HPRT mutation induction and cell killing run parallel to the induction of CPD in human fibroblasts unto 313 nm, but at higher wavelengths in the UVA band mutation and cell killing become more frequent per induced CPD lesion than in the UVB band (well over 10 times more frequent at 365 nm; at 385 nm and higher wavelengths CPD are virtually undetectable). The latter result indicates that DNA damage other than CPD and 6-4PP becomes more important in the UVA band. In the UVA spectral region (315-400 nm) the DNA absorption becomes vanishingly small for longer wavelengths, but the DNA can still be damaged by radicals generated through absorption of the radiation by other (unidentified) molecules ('endogenous sensitizers', possibly riboflavins) in the cell. Reactive oxygen species are known to play an important role in cellular damage by UV exposure (evidenced by oxygen dependency), which includes lipid peroxidation (membrane damage) and oxidation of single bases in the DNA (e.g., possible formation of 7,8-dihydro-8-oxo-2'-deoxyguanosine, 8-oxo-G for short, thyminglycerol and of 2'-deoxyadenosine N-1 oxide).

UV-INDUCED MUTATIONS

As previously mentioned, UV radiation causes DNA damage primarily at sites of adjacent pyrimidines. The most frequent photoproducts are cyclobutane dimers that are formed at adjacent thymidines (TT), with thymine-cytosine (TC) and cytosine-cytosine (CC) dimers occurring less frequently. Collectively, cyclobutane dimers represent about three-quarters of the photoproducts. The remaining non-CPD photoproducts consist mostly of 6-4 pyrimidine-pyrimidone lesions at TC, CC, or TT bases on the same DNA strand. All these photoproducts are removed in normal cells by DNA excision repair . Mutations are usually C→T, resulting from insertion of A opposite the damaged C during subsequent DNA replication (Thymines in photoproducts are less often mutagenic). Ten percent of the mutations are tandem CC→TT transitions resulting from replacement of both cytosines. This unique specificity of UVB mutagenesis- about 70% C→T at dipyrimidines and 10% CC→TT- has been known for many years and is commonly referred to as UV fingerprint or UV signature.

NUCLEOTIDE EXCISION REPAIR

As mentioned previously, the central role of DNA damage in skin carcinogenesis and the importance of an efficient DNA
repair mechanism to eliminate the UV-induced DNA damage, is best illustrated by the heritable disease xeroderma pigmentosum (XP)\(^1\). Approximately 80% of all XP patients who have been classified today have a defect in the nucleotide excision repair (NER) pathway. In these so called "classical" XP patients, seven complementation groups exist (XP-A to XP-G), each characterized by a defect in a different gene involved in NER. Due to the inability to repair UV-induced lesions, cells isolated from XP patients are highly sensitive to UV radiation. Furthermore, XP patients develop skin tumors with an extremely high frequency (>1000 fold increase compared to normal individuals).

The NER pathway is involved in the removal of a wide range of DNA damage, including that caused by photoproducts and that caused by chemicals giving rise to bulky DNA adducts and DNA crosslinks. NER appears to operate with different kinetics depending on the function of the damaged DNA. DNA damage encountered in DNA that is actively transcribed by RNA polymerase II (RNAPII) is repaired more rapidly and completely than DNA damage in the genome overall. These two different sub-pathways are known as transcription coupled repair (TCR) and global genome repair (GGR), respectively. All the genes involved in XP (XPA to XPG), with the notable exception of the XPC gene (and possibly XPE), contribute to both sub-pathways of NER. The XPC gene product seems to be essential in the recognition (and repair) of DNA damage in the genome overall and in the non-transcribed strand of active genes\(^\text{18}\).

The first steps in NER involve the recognition of DNA damage and local unwinding of the DNA double helix around the lesion. In GGR the initial recognition step is thought to take place via binding of the XPC protein at the site of the lesion. XPC is found in complex with the human homologue of yeast RAD23B protein (HHR23B). Binding of the XPC/HHR23B protein complex to damaged DNA is thought to induce a conformational change\(^\text{19}\). Subsequently, the basal transcription initiation factor IIH (TFIIH) and the XPA protein are recruited. Interactions between XPC/HHR23B and TFIIH have been described and it has been proposed that these two components initiate the local opening of double-stranded DNA around the lesion. Thereafter, the XPA protein, which is found \textit{in vivo} in complex with replication protein A (RPA), is thought to interact to form a so called "pre-incision complex"\(^\text{20}\). The function of the XPA/RPA protein complex is still unclear, but it has a preferential affinity for damaged (NER-specific) double-stranded DNA, it is essential in both GGR/NER and TCR/NER. Possibly, XPA/RPA control the NER substrate specificity\(^\text{19}\). Further details about the proposed functions of the other XP proteins as well as several other issues about XP and UV-induced skin cancer are discussed in a recent review about this topic\(^\text{21}\).

As a first step to permit the functional analysis of the XPA gene in vivo, H. van Steeg and co-workers in our laboratory have generated XPA-deficient mice by gene targeting through homologous recombination in embryonic stem cells\(^\text{22}\). The XPA\(^{-}\) mice are phenotypically normal up till 18 months, when a limited fraction is starting to develop liver tumors. XPA\(^{-}\) mice are highly susceptible to UVB-induced skin and eye tumors and to DMBA-induced skin tumors. It was concluded that these XPA deficient mice strongly mimic the human phenotype of xeroderma pigmentosum.

**THE P53 TUMOR SUPPRESSOR GENE**

The p53 tumor suppressor gene is involved in many cellular functions including cell cycle inhibition, regulation of differentiation, transcription, DNA repair, and apoptosis of cells sustaining DNA damage\(^\text{23,24}\). Mutations in the p53 gene have been found in about half of all sporadic human cancer cases examined in the general population\(^\text{25}\). These p53 mutations have been used in studies of molecular epidemiology because many mutations can alter its function\(^\text{25}\). Characteristic UV fingerprints were indeed also found in the p53 tumor suppressor gene in a majority of SCC and BCC\(^\text{26,27}\); the most direct evidence so far that solar UV radiation is the causative agent. Also in SCC and BCC from XP patients the UV fingerprint mutations were found, with exceptionally frequent CC to TT double mutations\(^\text{28}\).

In contrast to UVB the situation for UVA is quite different. No clear-cut relations between the types of DNA damage and resulting mutations, i.e. mutation spectra, have been observed so far, although several studies using mice and cultured cells have demonstrated that broad band UVA sources are carcinogenic in rodents and mutagenic to mammalian cells\(^\text{16}\).

To further address the relevance of UV-induced p53 mutations in non-melanoma skin cancer, we started to analyze SCCs induced by UVB or UVA in wildtype hairless mice, but also in UVB-induced XPA or p53 deficient mice. The results will be summarized in the following sections.

**UVB-INDUCED P53 MUTATIONS IN (WILDTYPE) SKH MICE**

Before presenting any data on p53 mutations in UVB-induced SCCs from hairless mice, it should be noticed that in these same SCCs the presence of activating mutations in any of the three Ras genes is a very infrequent event\(^\text{29}\).

To compare the mutation spectrum in the p53 gene of human with murine SCC, we have initially compiled an extensive mutation spectrum of the p53 gene in UVB-induced squamous cell carcinomas from hairless mice. This should substantiate in more detail the mutagenic effects of UVB radiation in vivo. In a collaborative study (The group of Dr A. Sarasin, Lab of Molecular Genetics, Villejuif, France) tumors from albino
hairless mice, random bred SKH:HR1 as well as inbred SKH:HRA strains, were analyzed for mutations in the conserved domains of the p53 gene. The observed mutation frequency after chronic UVB irradiation in the p53 gene ranged from 54% (SKH-HRA) to 73% (SKH-HR1) among the 160 tumors analyzed. In 17% of the tumors two independent mutations in the p53 gene were detected. Over 95% of the mutations are found at dipyrimidine sites located in the non-transcribed strand, and the majority are C→T transitions and some 5% CC→TT tandem double mutations. Within the p53 mutation spectrum, four distinct hotspots can be identified; two major ones at the (murine) codons 267 (33%) and 272 (19%) and two minor ones at the codons 146 (10%) and 173 (4%). The major hotspot at codon 267 consists of a CpG preceded by a pyrimidine confirming in vivo an important role for this UVB mutable site. By comparison with mutation spectra from human (skin) carcinomas the presented data further support the validity and merits of the hairless mouse model for studying the molecular mechanisms of skin carcinogenesis. Most notably, the human equivalent of the murine codon 267 lacks the dipyrimidine site and therefore fails to be a pronounced hotspot in human skin carcinomas; however, this site is one of the major hotspots in internal cancers (evidently not induced by UV radiation but probably by deamination of 5-methylcytosine). In contrast, the murine hotspot at codon 272 does have a full (also mutated) equivalent in human skin carcinomas.

**UVA-INDUCED P53 MUTATIONS IN (WILDTYPE) SKH MICE**

As described in the previous section, mutations with clear UVB fingerprints have been observed in the p53 gene of human non-melanoma skin tumors and experimental murine skin tumors. Although UVA radiation is a complete carcinogen, its contribution in sunlight induced mutagenesis remains poorly characterized, however the production of reactive oxygen (ROS) species is considered to play an important role in this respect. In this section data are presented on the type and frequency of p53 alterations in UVB induced skin tumors (n=42) in albino hairless mice (n=14). The incidence of p53 alterations in these tumors is low compared to UVB induced squamous cell carcinomas (in 11 out of 14 tumors analyzed). In only 2 out of 14 squamous cell carcinomas we found similar ras gene mutations. The observed shift from squamous cell carcinomas in wild type hairless mice to papillomas in XPA-deficient hairless mice. We detected p53 gene mutations in only 3 out of 37 of these skin tumors, whereas in tumors of control UVB-irradiated wild type littermates this frequency was higher (45% in exon 8) and more in line with our previous data. Strikingly, a high incidence of activating ras gene mutations were observed in UVB-induced papillomas (in 11 out of 14 tumors analyzed). In only 2 out of 14 squamous cell carcinomas we found similar ras gene mutations. The observed shift from squamous cell carcinomas in wild type hairless mice to papillomas in XPA-deficient hairless mice, and a corresponding shift in mutated cancer genes in these tumors, provide new clues on the pathogenesis of chemically- versus UVB-induced skin carcinogenesis.

**UVB-INDUCED P53 MUTATIONS IN XPA DEFICIENT SKH MICE**

To further examine the role of p53 we studied UVB-induced carcinogenesis in albino hairless p53 deficient mice. Because the onset of other neoplasms like lymphomas interfered with the development of skin tumors in p53 null-mice, only the hairless heterozygous p53 (p53+/−) mice were daily exposed to two different UV-doses, 900 J/m² and 450 J/m² (Philips TL12 lamps), to induce skin tumors. The preliminary results indicate (dose-dependent) alterations in latency time, tumor progression and p53 mutation spectrum in these p53+/− hairless albino mice (manuscript in preparation) but with clear differences with regard to the results from of a similar study by Tron and co-workers, who mainly induced SCCs from XPA-deficient mice on the ears of the mice. Similar conclusions on the minor contribution of UVA to the p53 mutation spectrum were recently reported by Ananthaswamy and co-workers who analyzed SCCs from hairless mice induced by a solar simulator.
be quite different from that of allelic loss: the former is generally selected for in early stages of wildtype tumors, whereas the latter enhances tumor development only at high exposure levels (where apoptosis should set in) and it generally seems to increase progression of the skin tumors.

THE TIMING OF p53 ALTERATIONS INDUCED BY UV-RADIATION

To investigate the timing of the p53 alterations in the process of UVB carcinogenesis, we used our SKH albino hairless mice model in which the time that tumors appear is predictable from the UVB exposures 30. The mice were subjected to a series of daily UVB exposures, either for 17 days or for 30 days, which would cause skin tumors to appear around 80 or 30 weeks, respectively. In the epidermis of these mice, we detected clusters of cells showing a strong immunostaining of the p53 protein, as measured with the CM-5 polyclonal antiserum. This cannot be explained by transient accumulation of the normal p53 protein as a physiological response to UVB-induced DNA damage. In single exposure experiments the observed transient CM-5 immunoreactivity lasted for only 3 days and was not clustered, whereas these clusters were still detectable as long as 56 days after 17 days of UVB exposure. In addition, approximately 70% of these patches reacted with the mutant-specific monoclonal antibody PAb240, whereas transiently induced p53-positive cells did not. In line with similar more extensive human data 30, these results demonstrate that constitutive p53 alterations are causally related to chronic UVB exposure and that they are a very early event in the induction of skin cancer by UVB radiation. Moreover, in human actinic keratosis 50% of the patches were found to contain a mutation in the p53 gene. To obtain better quantitative estimates of the number of patches in the murine skin, a technique to isolate pure epidermal sheets was developed. At present, sequence analysis of microdissected immunostained patches is under development to find out to what extent these patches are containing p53 mutations. An intriguing preliminary observation is that in epidermal sheets of XPA deficient as well as in p53 deficient mice, the number of patches is markedly higher then in their wildtype littermates (manuscript in preparation).

CONCLUDING REMARKS

Hairless mouse model data as well as most human studies suggest a role of altered p53 very early in UV-induced non-melanoma skin cancer. In many relatively large areas of normal appearing, sun exposed skin, in precursor lesions like actinic keratosis and SCC p53 mutations are frequently detected. Although most of these "initiated" cells eventually are lost through normal differentiation or cell death, these mutations appear to confer a selective survival advantage following repeated sun exposure. However, modifying the DNA repair capacity of the skin, the p53 dependency is greatly decreased, possibly because other signaling pathways are more easily targeted for mutation by UV damage. Next to p53 and the important role of DNA repair, other factors like sunlight-induced immune suppression and mutations in other (unknown) genes are probably essential for UV carcinogenesis. Also in the future, the role of (some) of these players are expected to be elucidated by experimenting with (genetically modified) hairless mice.

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