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PHYSICAL VARIABLES IN EXPERIMENTAL PHOTOCARCINOGENESIS AND QUANTITATIVE RELATIONSHIPS BETWEEN STAGES OF TUMOR DEVELOPMENT

Frank R. de Gruijl [1] and Jan C. van der Leun [2]

[1] Dermatology, Leiden Univ. Med. Ctr./LUMC, Sylvius Lab., Wassenaarseweg 72, NL-2333 AL Leiden, Netherlands, [2] EcoFys, PO Box 8408, NL-3503 RK Utrecht, Netherlands

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1. ABSTRACT

Solar ultraviolet (UV) radiation is a prominent environmental carcinogen, but it does not penetrate any deeper than the skin. The UV-related skin cancers are by far the most common form of cancer among white Caucasians in the USA and Australia, and this poses a serious public health problem. Chronic UV exposure of hairless mice is a well established model for squamous cell carcinomas in man. It is important to identify the essential physical variables, and explore fully how photocarcinogenesis evolves in dependence of these variables. The 3 main physical variables in photocarcinogenesis are (i) the wavlength of the radiation, (ii) the exposure and (iii) time. A good quantitative description of tumor induction and precursing stages can be given in terms of these variables. An analysis of this description shows us that the early induction of clusters of epidermal cells that over-express mutant p53 ('p53 patches') are closely and, most likely, causally linked to the eventual tumors. These p53 patches may thus serve as early indicators of tumor risk. The induction of an immune-tolerance toward the UV-induced tumors precedes the actual occurrence of the tumors at high daily doses, but extrapolation indicates that this order of events may be reversed at low daily doses. This disparity between the dose-time relationships for the tumor tolerance and the tumors needs to be investigated further. It could imply a shift to non-immunogenic tumors at low daily doses.

2. INTRODUCTION

The sun's ultraviolet (UV) radiation is arguably the most prominent environmental carcinogen. Cancer is now understood to be largely caused by changes (mutations) in genes whose products control the cell cycle, cell differentiation and apoptosis (programmed cell death). UV radiation damages the DNA from which genes are comprised and may thus cause gene mutations, most likely by errors in replication of damaged DNA. Because of the limited penetration of UV radiation, the cancer formation is restricted to the skin. Clearly, the skin needs to be well adapted to this continuous UV challenge. Research has shown that the skin is equipped with a great variety of

defense mechanisms. These include 'simple', enhanced absorption of the UV radiation by thickening of the epidermis and increases in pigment, a plethora of cellular defenses (most notably, anti-oxidants, DNA repair and apoptosis), and immune reactions (for reviews see (1, 2)). Judging by the fact that most skin cancers only develop late in life (even after decades of excessive sun exposure), it must be concluded that the skin does remarkably well in defending against UV-induced damage. How important these defense mechanisms are can, for example, be inferred from the dramatic increases in skin cancer risk in immune-suppressed patients (e.g., with renal transplants) and in patients with Xeroderma pigmentosum who lack effective DNA repair mechanisms.

The easily observable skin tumors and easy access to the skin have made experimental skin carcinogenesis a much studied and convenient model for epithelial cancers in general. Over the last century experimentation on skin carcinogenesis has evolved from the phenomenology of tumor occurrences to analyses of the underlying molecular-genetic and immunological processes (2). More so than chemical skin carcinogenesis, UV-induced skin carcinogenesis is of practical relevance because solar UV radiation is the main exogenous factor of risk for white Caucasians in the etiology of the three main forms of skin cancer: *viz*., basal cell carcinomas (BCC) (the most common form), squamous cell carcinomas (SCC), and cutaneous melanomas (CM), the latter the most aggressive and lethal form (3). Only recently have specific models with transgenic mice begun to generate experimental data on the relation between UV radiation and BCC (4), and between UV radiation and CM (5). Most of the earlier experiments in wild-type (hairless) mice concern the induction of SCC and precursor lesions, *viz.* actinic keratoses (AK) and intra-epidermal neoplasias (Bowenoid tumors) (6). Here, we focus on the large body of experimental data on UV carcinogenesis in relation to SCC.

Tumor development is an intricate multi-step process, complicated *interalia* by the various defensive responses, and many of the basic steps (e.g., specific gene mutations) are still unknown. Hence, it is virtually impossible to compute an individual's tumor risk from basic principles. Even if we knew the process in detail, the computations on the sequential steps may accumulate errors to an extent that the outcome would be useless. Here, we will present an empirical approach based on mathematical descriptions and quantitative analyses pertaining to experimental UV-induced skin carcinogenesis in hairless mice. From this approach we learn the nature of the relations between the main physical variables in the process of UV carcinogenesis, i.e., mainly the relation between the delivery of radiant energy to the skin and the ultimate rate of tumor occurrence or the lapse of time till the first tumor. The prior benign stages of tumor development will also be considered. We will point out the parallels between skin cancer induction in mice and men, and how the mathematical model can be used for risk assessments for humans.

3. THREE PHYSICAL DIMENSIONS

Tumor formation is a dynamic multi-step process in which one or more steps may be driven by external carcinogens, e.g., UV radiation. Beside quantity, the specific quality, i.e., efficacy, of the exogenous carcinogen determines its impact. In UV carcinogenesis the *wavelength* (in nm) of the irradiation (the spectrum) determines the quality, i.e., the efficacy per unit radiant energy. The quantity of radiant energy is given by the *exposure* (in J/m^2), and the spatio-temporal distribution of the exposure (the exposure regimen) is also of great importance. The factor *time*, in days or years, is the final determinant for the ultimate occurrence of tumors and is expressed as the tumor latency time. Thus, *wavelength*, *exposure* (or dose) and *time* are the most important physical variables in UV carcinogenesis.

3.1. Wavelength Dependence and Action Spectra

A photo-induced biologic response has to start with the absorption of a photon by a molecule (chromophore) that can then initiate the photochemical reaction. The efficacy by which the radiation initiates a photochemical reaction is measured by the reaction cross section, $\sigma_r = 1/F_{37}$, where F_{37} is the fluence (number of photons per m^2 passing through the cross section of an infinitesimally small sphere) at which 37% of the chromophores have not reacted. The dependence of σ_r on the wavelenght (λ) is called the *action spectrum*, i.e., $\sigma_r(\lambda)$. This is the photochemical definition. The effective reaction constant, k_r, with a polychromatic spectrum with a fluence rate $f(\lambda)$, is then given by $k_r = \int \sigma_r(\lambda)\, f(\lambda)\, d\lambda$ (a summation over the wavelength of the action spectrum times the fluence rate). In this manner, the wavelength dependence of the photochemical reaction is accounted for.

For biological responses the situation is usually less well defined: the target molecules and their depth in the skin are generally unknown. Even if this were known, in situ measurements would be very difficult (F can only be measured indirectly or in vitro). What is commonly studied in photobiology is the relationship between surface exposure (H, in J/m^2, the radiant energy passing through a flat surface) and the biological response. A wavelength dependency can be ascertained by measuring the exposure, $H_e(\lambda)$, required at different wavelengths to evoke the same level of response. An action spectrum may then be defined as: $A(\lambda) = H_e(\lambda_{max})/H_e(\lambda)$, where λ_{max} is the wavelength of maximum efficacy, i.e., where $H_e(\lambda)$ reaches a minimum (compare $A(\lambda)$ with $\sigma_r(\lambda) = 1/F_{37}(\lambda)$; where $F_{37}(\lambda)$ in photons/m^2 can be divided by $5.10^{15}.\lambda$ to convert to J/m^2). Hence $A(\lambda_{max}) = 1$ and $A(\lambda)H(\lambda)$ expresses $H(\lambda)$ as an equivalent exposure at λ_{max}, i.e., $H(\lambda_{max})$. The latter inference is only generally true for any level of $H(\lambda)$ if the dose response relationship at every wavelength is the same as at λ_{max}, except for a constant ratio between the exposures at corresponding response levels (i.e., $A(\lambda)$ is then independent of the level of $H(\lambda)$). Furthermore, if the contributions from different wavelengths simply add up (i.e., additivity holds), we can meaningfully write $D = \int A(\lambda)H(\lambda)\, d\lambda$, where $H(\lambda)$ is the exposure spectrum of a polychromatic source and D is the 'biologically effective dose' in equivalents of J/m^2 of λ_{max}-radiation.

Shortwave UV radiation is directly absorbed by DNA and causes the formation of dimers at dipyrimidine

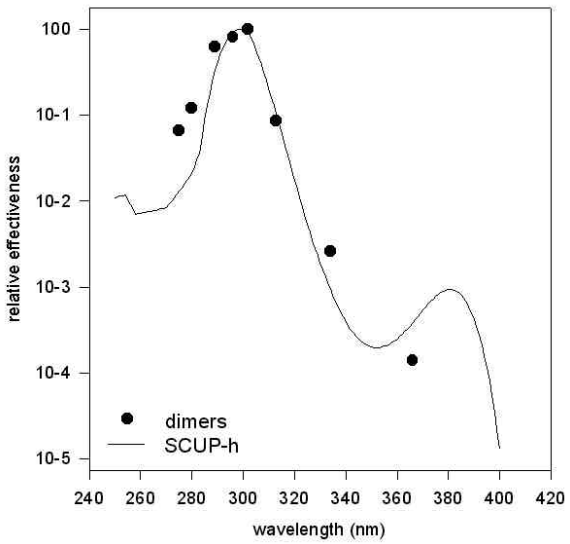

Figure 1. Comparison of wavelength dependencies of skin carcinoma induction (SCUP-h, solid line spectrum (11)) and cyclobutane pyrimidine dimers in human skin (points (12)).

sites. This kind of DNA damage is clearly associated with (*p53*) gene mutations found in human SCC and UV-induced murine SCC (7,8). Above 300 nm the efficiency of induction of these DNA adducts drops off steeply, and correspondingly, the mutation rate per J/m^2 drops off. A series of skin carcinogenesis experiments on hairless SKH-1 mice with chronic (daily) exposure to various broadband UV spectra has provided the data to establish the wavelength dependency of UV carcinogenesis: the result was dubbed the SCUP-m action spectrum (*S*kin *C*ancer *U*trecht-*P*hiladelphia-*m*urine) (9). The SCUP-m action spectrum has a maximum at 293 nm and, at a level about 10,000 times lower, a minimum around 350 nm. This minimum could be attributable to a transition from direct photochemical DNA damage (pyrimidine dimers) at shorter wavelengths to relatively more indirect DNA damage (e.g., 8-hydroxyguanine) from reactive oxygen species (ROS) at longer wavelengths; i.e., the SCUP-m action spectrum can be considered as the composit of two action spectra, one for each of the two types of DNA damage (see (10)).

The carcinogenic action spectrum for SCC in humans was estimated by correcting the SCUP-m action spectrum for differences in transmission of murine and human epidermis. The resulting action spectrum was dubbed the SCUP-h action spectrum (h stands for human) (11). This correction for UV transmission through human epidermis was largest for wavelengths under 300 nm, and shifted the maximum from 293 to 299 nm. The SCUP-h action spectrum is depicted in Figure 1 together with the measured action spectrum for the induction of pyrimidine dimers in the human skin by Freeman et al. (1989) (12). The two action spectra resemble each other remarkably well, especially in the UVB, which confirms that the UV-induced genotoxicity is indeed the major driving force behind UV carcinogenesis.

3.2. Time versus Dose

As mentioned earlier, skin tumor formation proceeds through multiple rate limiting steps (mutations in oncogenes and tumor suppressor genes). Some of these steps are UV driven and some are not (the latter may be caused by endogeneous metabolic processes which generate ROS). The likelihood of the occurrence of a UV-dependent step will increase with the accumulated UV dose, whereas the likelihood of a UV-independent step will simply increase with the lapse of time. Clearly, the UV-independent steps will render the whole process of carcinogenesis less dependent on the UV dose. Hence, one may expect that in experiments with chronically exposed mice, the tumor induction time will not be shortened by a factor of 2 if the daily dose is increased by a factor of 2, i.e., there will be no direct reciprocity between daily dose and tumor induction time. This reciprocity can also be modified by defensive and adaptive responses, e.g., epidermal hyperplasia which diminishes the penetration to the germinative basal cells (13). This lack of reciprocity is indeed found in experiments of SCC induction in hairless mice by daily UV exposure. The average number of tumors per mouse (see Figure 2A), the yield Y (or tumor multiplicity) can be written as

$$Y = (H/H_o)^{p1} (t/t_o)^{p} \qquad (1)$$

where H is the daily UV exposure (in $J/m^2/d$), t is time (in days), and H_o, t_o, p1 and p are constants (when $H=H_o$ and $t=t_0$ then Y=1, i.e., an average of 1 tumor per mouse). From this formula it also follows that a two-fold larger daily exposure does not induce tumors twice as fast (if $H=2H_o$ then $t=t_0/2^{p1/p}$ for Y=1, instead of $t= t_0 /2$). In a straight forward interpretation, the power of time, p, is likely to be proportional (not necessarily equal to) the total number of rate limiting steps that occur in the course of time (including those steps that depend on the daily UV exposure). The power of the daily UV exposure, p1, is likely to be proportional to the number of UV driven steps; which implies that $p1 \leq p$ (14).

If all mice in a group are comparable (in sensitivity and treatment), the probability, P, for a mouse of developing a first tumor then becomes

$$P = 1 - \exp(-Y) \qquad (2)$$

which, in absence of any intervening deaths, equals the prevalence of tumor bearing animals in a (large) group. These relationships hold rather well in the experiments with albino hairless mice, where $p = 7.2 \pm 0.8$ and $p1 = 4.3 \pm 0.5$ for early tumors of 1 mm in diameter (14,15), with pigmented hairless mice $p1 = 2.1 \pm 0.2$ ($p1/p = 0.3$ computed from data in (16); this lower dependency on the daily UV exposure is probably due to a better adaptation by pigmentation of the animals). When the daily exposure of the albino mice is discontinued after a couple of weeks, long before the appearance of tumors, then Y increases with t to the power 2.8 ± 0.2, which power equals p-p1 ($=2.9 \pm 0.3$), the power related to the UV-independent steps (14).

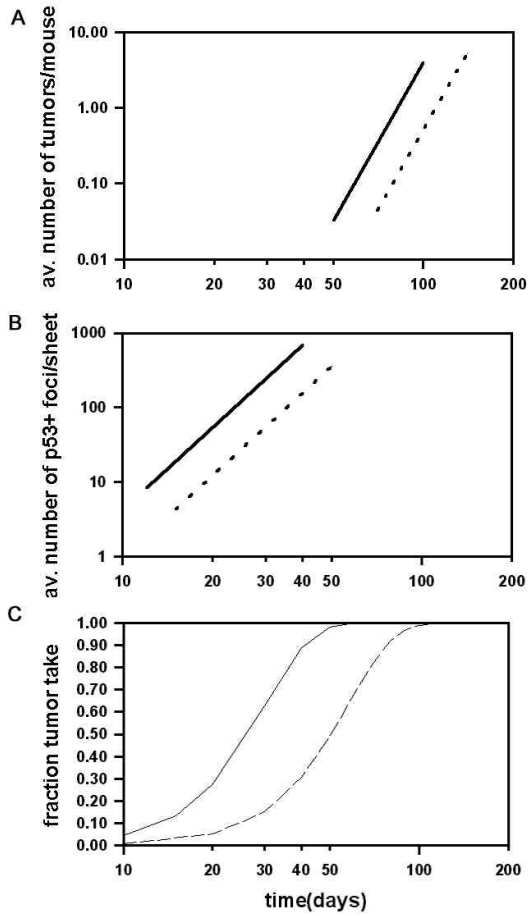

Figure 2. Comparison of the development of numbers of tumors (A) with p53-patches (B) and the fraction of mice that accept an implant of a syngeneic UV-induced skin carcinoma (C); solid lines for 1 MED/day and dashed lines for 0.5 MED/day.

In human populations the age-specific incidence of cancers is measured, which equals the increase in Y per unit time (e.g. per year) if the fraction of patients with multiple tumors is small. The probability (calculated according to eq. 2) of SCC in the Dutch population follows a time-dependence which is quite comparable to that of albino hairless mice: $p = 6.6 \pm 0.4$ for males and 8.9 ± 0.7 for females (1). Under a level of daily exposure comparable to the estimated average among Dutch males, the tumors in the mice occur about 250 times faster. This appears to indicate that the tumor kinetics are very similar, but that the developmental rate of SCC (the mutation rate) is much higher in the mice. By combining measurements of ambient UV loads and epidemiological data from the USA, it can be inferred that $p1 = 2.6 \pm 0.8$ for human SCC (17); a value of p1 that is very similar to the one for pigmented hairless mice. For BCC p ranges between 4.5 and 5.5 and $p1 = 1.4 \pm 0.5$ (17).

4. UV INDUCTION OF *p53* PATCHES AS TUMOR PRECURSORS

As discussed earlier, mutations characteristic of UV radiation have been found in the *p53* tumor suppressor gene in a majority of human skin carcinomas, and in experimentally UV-induced squamous cell carcinomas and actinic keratoses in hairless mice (8,9). Our group (18) discovered clusters of epidermal cells that over-expressed mutant p53 protein ('p53 patches') in the skin of UV-exposed hairless mice long before they developed visible tumors. In parallel, others (19) reported such p53 patches in regularly sun-exposed normal human skin. A follow-up study by our group (20) has shown a consistent and, most likely, causal relationship between the p53 patches and the ultimate skin carcinomas. Hence, the p53 patches are potential foci of tumor development.

Our group (20) has closely studied the kinetics of the UV induction of p53 patches in the hairless mice (see Figure 2B), and determined how the p53 patches are quantitatively related to the later occurring skin carcinomas (compare Figure 2 A and B). It turns out that the induction of p53 patches (i.e. the yield) can also be described by equation 1, very similar to the carcinomas, albeit p53 patches occur earlier and in much higher numbers (i.e., smaller values of H_o and /or t_0). However, the relative increase with time is less steep (p is about 2 times smaller and equals 3.7 ± 0.5). The latter indicates that the genesis of a p53 patch involves fewer (rate-limiting) steps than the genesis of a tumor.

By halving the daily UV exposure (in Figure 2 from 1 MED to 0.5 MED/day, where MED stands for minimal edemal dose), tumor induction is delayed by a factor of 1.54 ± 0.02. This factor corresponds very well with the factor of 1.49 ± 0.15 by which the induction of p53 patches is slowed down (20). This implies that the relationship between daily dose and induction time until a certain number of lesions per mouse occurs, run parallel for p53 patches and tumors. Hence, the yield of p53 patches is closely linked to the yield of tumors in dose and time, i.e., the p53 patches appear to precede the tumors in a very consistent and predictable manner.

5. UV INDUCTION OF IMMUNO-TOLERANCE TOWARD TUMORS

Murine skin tumors induced by UV radiation were found to be highly immunogenic when transplanted into a genetically identical host, i.e., the transplants were rejected. However, the tumor transplant was accepted and grew if the host was previously subjected to a series of sub-carcinogenic UV exposures (21). UV radiation appeared to induce a specific tolerance toward UV tumors, and this tolerance was mediated by splenic 'suppressor T cells' (22, 23). Transfer of these T cells also caused an accelerated development of primary skin tumors in UV-irradiated hosts (23). The induction of this systemic tumor tolerance also explains why partial pre-irradiation of a mouse accelerates subsequent UV carcinogenesis in other skin areas (24).

Clearly, the tumor tolerance has an important bearing on UV carcinogenesis. In order to describe precisely the relationships in UV exposure and time between this tolerant state and the tumors, our group has performed experiments to determine the kinetics of

inducing tumor tolerance, and compared that to the kinetics of tumor induction (25). To this end, hairless mice were ventrally inoculated with cells from a syngeneic UV-induced tumor after different periods of daily dorsal UV exposure (either to 1 or 0.5 MED/day). Subsequently, the percentage of mice in which the tumor implant 'took' and grew was determined, and thus, percentage of mice rendered tumor-tolerant could be traced in the course of the two chronic UV exposure regimen (see Figure 2C) and compared to the eventual induction of tumors (Figure 2A). The percentage of tumor-tolerant mice could be described by equation 2, with Y as in equation 1, and $p = 2.8 \pm 0.7$. Lowering the daily dose by a factor of 2 increases the induction time of the tumor tolerant state by a factor of about 2 (from 1 to 0.5 MED/day the median induction time shifts from 26 to 50 days), i.e., here reciprocity between the daily UV exposure and induction time appears to hold, very much in contrast to the induction of p53 patches and tumors. This implies that the relationship between daily dose and median induction for tumor tolerance does not run parallel to that relationship for tumors. At the levels of daily UV exposure in use in the experiments of Figure 2, the tumor tolerant state clearly precedes the occurrence of visible tumors. However, extrapolation to lower daily doses would suggest that, with daily dose less than 0.05 MED/day, more than 50% of the mice would have developed skin tumors while less than 50% would be tumor tolerant. This would suggest that low daily doses would select for non-immunogenic tumors, whereas at high daily doses, tumor tolerance would allow the immunogenic tumors to develop.

6. CONCLUSIONS AND PERSPECTIVE

Identifying all the essential physical variables is a necessary first step for a proper quantititive analysis of carcinogenesis by exogenous agents: for UV carcinogenesis the dependence of the process on the *wavelength*, the *exposure* and *time* needs to be fully explored. Solid quantitative descriptions of the (UV) induction of tumors and precursing stages are of evident importance for mathematical modeling of carcinogenesis, but they are also crucial for a proper in depth analysis of the dynamics and possible causal relations in tumor development. The close link between p53 patches and the tumors and the similar dependence of induction times on daily doses point at the possibility of using p53 patches as a biomarker of skin cancer risk (see (21) for a more in-depth analysis and discussion). The link between the induction of tumor tolerance and the eventual tumors is less well determined in terms of dose and time. There is a marked difference in dose dependency: tumor acceptance appears to be fully determined by the accumulated UV dose, whereas induction of tumors and induction of p53 patches are not. The implications of this discrepancy have not been fully explored. One could envisage that early tumor foci, such as p53 patches, may be antigenic and, in combination with UV irradiation, may induce the specific UV tumor-tolerant state. However, the disparity between dose-time relationships for the induction of p53 patches and the induction of tumor tolerance would argue against this idea. Evidently, an understanding of the fundamentals of inducing the tumor tolerant state would be important because it may open up the possibility of preventive and/or therapeutic interventions to restore or boost the immunity against skin carcinomas.

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Send correspondence to: Frank R. de Gruijl, Ph.D., Dermatology, Leiden Univ. Med. Ctr./LUMC, Sylvius Lab., Rm 3038, Wassenaarseweg 72, NL-2333 AL Leiden, Netherlands, Tel: ++31 71 5271902, Fax: ++31 71 5271910, E-mail: F.R.de_Gruijl@lumc.nl