UV-induced ablation of the epidermal basal layer including p53-mutant clones resets UV carcinogenesis showing squamous cell carcinomas to originate from interfollicular epidermis

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“Chronic ultraviolet (UV) exposure induces clones of cells overexpressing mutant p53 in the interfollicular (IF) epidermis and subsequently squamous cell carcinomas (SCCs) with similar p53 mutations. Mutated p53 may give cells growth advantage over neighbouring cells by impaired apoptosis. We tested this by UV overexposure of skin laden with p53-mutant clones and assessed the impact on subsequent tumour development. P53-mutant clones were induced in two groups of hairless SKH1 mice by daily exposures (500 J/m² UV from TL12 lamps) for 28 days. On day 29, one group was overexposed (to 10 kJ/m² UV), whereas the control group received the regular daily dose. After 1 week of recovery, the daily exposures were resumed in both groups to induce SCCs. UV overexposure forced the entire IF basal layer into caspase-3-driven apoptosis while leaving overlying layers with sunburn cells intact. No apparent regions were spared from apoptosis. Pulse-chase BrdU labelling showed the IF epidermis to be repopulated from the hair follicles (remaining p63 positive). One week after overexposure, the p53-mutant clones had virtually disappeared (0.6, 95% confidence interval 0.5–0.8 per mouse versus 102, 59–179, without overexposure). Tumour development was significantly delayed after UV overexposure (P < 0.0001) by an average of 27 days (standard error of the mean 3); a period matching that of daily exposures preceding the overexposure. Thus, we found that UV-induced ablation of the IF epidermal basal layer eliminates p53-mutant clones and resets UV carcinogenesis. Furthermore, and in contrast with earlier reports, our data show that UV-induced p53-mutant clones and SCCs originate from the IF epidermis.

Introduction

Solar ultraviolet (UV) irradiation causes cutaneous basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) in exposed skin. The increased incidence over the last decades poses an increasing burden on public health care. Estimated from annual incidences times a lifetime expectancy of 80 years, lifetime risk ranges from 6 to 8% for BCCs and from 1 to 2% for SCCs in Europe in 2004 (1,2). The combined BCC and SCC lifetime risk is predicted to rise with an additional 1% per 5 years. In SCCs and in BCCs, UV signature mutations are found in the P53 tumour suppressor gene: cytosine (C) > thymidine (T) and CC > TT transitions at dipyrimidine sites (3,4). Such mutations are also present in the benign precursor lesions of SCCs, actinic keratosis (5). In chronically UV-exposed skin, immunohistochemically detectable epidermal clones of cells overexpressing mutant p53 (p53-mutant clones or p53 patches) occur in the interfollicular (IF) epidermis. These microscopic lesions are considered to be precursors of actinic keratosis and SCCs. Several observations indicate that these p53-mutant clones are causally related to SCCs: (i) similar UV signature mutations and the very same hotspots in the p53 gene are found in both types of lesions (6–8), (ii) these lesions show comparable UV dose dependencies (7) and (iii) increased incidence of p53 clones in DNA repair-deficient Xpa-, Xpc- and Xbd knockout mice correspond with tumour risk (7). Furthermore, ~70% of SCCs bear p53 mutations which obstruct the tumour-suppressive action through cell cycle arrest, induction of DNA repair and apoptosis (9). By suppression of apoptosis and cell cycle arrest, p53 mutations can give an epidermal cell a growth advantage over its wild-type neighbours. Subsequently, clonal expansion creates an enlarged more susceptible target for the acquisition of additional mutations.

Impaired UV-induced apoptotic responses due to defective p53 signalling were found experimentally both in p53 knockout mice (10) and in cells with mutated p53 (11). P53-mutant clones in the epidermis could therefore be relatively resistant to apoptosis compared with surrounding keratinocytes carrying wild-type p53 and survive higher UV doses. Because this was never verified directly, we set out to investigate this expected contrast in apoptosis by subjecting skin laden with p53-mutant clones to high level UV exposure. Upon strong overexposure [4–6 times the threshold dose for a minimal sunburn reaction (12,13)], widespread apoptosis occurs and lost cells leave apparent vacancies in the epidermis. Under these conditions, p53-mutated cells may show a better survival than their neighbouring wild-type cells. This should then lead to a preferential outgrowth of the mutant clones at the expense of the wild-type keratinocytes and thus enhance subsequent UV carcinogenesis (14). On the other hand, if the clones become apoptotic and are replaced by wild-type keratinocytes, the number of clones will drop and subsequent UV carcinogenesis should be reduced.

Against experimental evidence that IF p53 clones are precursors of SCCs, Faurschou et al. (15) reported that laser ablation of IF epidermis of hairless SKH1 mice that were chronically UV exposed did not impair the tumour induction time of SCCs [also discussed in (16)]. This would imply that p53 clones should be considered more as epiphenomena than real precursors of SCCs. However, p53 clone densities before and after the laser ablation were not determined in this study, and adequate elimination of the IF p53-mutant clones was therefore not verified. Our study allowed us to investigate this issue more closely and to assess the corresponding effect on UV carcinogenesis.

The epidermis harbours three stem cell niches: the bulge region, the isthmus of the hair follicle and the basal layer of the IF epidermis (17). Stem cells in these niches have the ability to self-renew and to generate daughter cells that differentiate in the cells populating the hair follicle, sebaceous gland or IF epidermis, respectively. There is growing evidence for the conjecture that BCCs originate from stem cells in the bulge region of hair follicles (18,19). Our experiments provide evidence on which stem cell pool is targeted in the UV-driven genesis of SCCs.

In this study, we found that UV overexposure at the dose we used predominantly ablated the entire IF epidermal basal cell layer, including p53-mutant clones, by caspase-3-driven apoptosis, leaving overlying epidermal layers with sunburn cells and hair follicles largely intact. Such eradication of p53-mutant clones resulted in an apparent restart of UV carcinogenesis when the regimen of daily UV exposure by which the p53-mutant clones were induced was continued. Hence, the UV-induced SCCs in hairless mice appear to originate from the IF epidermal basal layer.

Materials and methods

Mice

Legal approval for the UV experiments with hairless SKH1 mice was received from the animal ethical commission. Hairless Crl:SKH1-HR mice

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P53-mutant clones. Epidermal sheets were fixed in PBS-buffered 4% formaldehyde and stained with mutant p53-specific antibody basically as described earlier (7,23), with two modifications: antigen retrieval in 0.01 citrate buffer in a graded series of ethanol solutions. Endogenous peroxidase was blocked by incubation for 20 min incubation with 1.5% H2O2 in methanol. Antigen retrieval was performed in 0.01 M citrate buffer pH 6.0 in an autoclave for 5 min at 110°C. After a triple PBS wash, the slides were incubated with 2% normal rabbit serum and 1% bovine serum albumin in PBS and subsequently with anti-p53 antibody clone 4A4 (1:50, M7247; DakoCytomation, Glostrup, Denmark) for 1 h. After washing rabbit anti-mouse (IgG2a) biotin (1:200, 61-0240; Zymed, San Francisco, CA) was incubated for 1 h. The third step was incubation with avidin-biotin-HRP complex (PK-6100; Vector Laboratories) for 45 min. After a triple wash, peroxidase was visualized with 20 mg of 3,3’-diaminobenzidine dissolved in 100 ml of PBS.

Counter staining was performed with haematoxylin (Klinipath, Duiven, The Netherlands) and sections were mounted in Kaisers’ glycerine (Merck, Darmstadt, Germany).

Active caspase-3 p63 double staining. It was performed on paraffin sections with similar procedure as for p63 staining as outlined above and with the red chromophore 3-amino-9-ethylcarbazol instead of 3,3’-diaminobenzidine. Anti-active caspase-3 was visualized by a second step goat anti-rabbit IgG fluorescein isothiocyanate alkaline phosphatase (1:400, 1426338; Roche, Mannheim, Germany) and finally with Fast blue (F-3378; Sigma) as the chromophore. Fast blue (25 mg) was dissolved in 100 ml of 0.1 M TRIS/0.05 M MgCl2 buffer containing 12.5 ng naphthol AS-MX phosphate (N-4875; Sigma) and 35 mg levamisol (L-9756; Sigma).

BrdU staining. It was performed on paraffin sections as described above (22,23): antigen retrieval was performed with 60 mg pepsin/100 ml of 0.1 M HCl during 30 min. The subsequent steps for immunostaining were anti-BrdU (1:100, M0744; Dako (Mantel Cox), rabbit anti-mouse (IgG1) biotin (1:50, 61-0140; Zymed), avidin-biotin-HRP complex (Vector Laboratories), 3,3’-diaminobenzidine and haematoxylin counter staining. Sections were photographed with a digital camera (AxioCam; Zeiss, Sliedrecht, The Netherlands) mounted on a light microscope (Axioplan 2, Zeiss, equipped with a x10 objective).

Results

UV overexposure ablates the basal layer of the IF epidermis

P53-mutant clones could be readily induced by the sub-sunburn regimen of daily UV exposures for 4 weeks (Supplementary Figure 1, available at Carcinogenesis Online). On day 29, we overexposed a group (n = 20) to a UV dose of 10 kJ/m2 (about five aMEDs to a UV-adapted skin). In the first days after the UV overexposure, the dorsal skins showed oedema, erythema, scaling and some microscopic foci of bacterial infection (indicating a compromised barrier function of the stratum corneum) but remained largely intact and recovered almost completely after 1 week (Supplementary Figure 2, available at Carcinogenesis Online). To examine the precise time course and extent of the apoptotic response, we stained active caspase-3 (25) on skin biopsies that were taken of mice at 6, 24, 48 h and 7 days after UV overexposure (n = 4 per time point, representative sections in Figure 1, first column of panels). Control mice received one additional daily UV dose (500 J/m2) instead of the UV overexposure. Specific cytoplasmatic active caspase-3 staining was found in UV-overexposed mice at 6, 24 and 48 h. The epidermis of control mice showed no staining of active caspase-3 (Figure 1, upper left panel).
The caspase-3-positive cells were predominantly located in the basal layer of the IF epidermis at 6 and 24 h, interspersed at 6 h and contiguous at 24 h. At 48 h, positive cells remnants were found in a suprabasal layer halfway up in the epidermis (an apoptotic front). At 24 h, virtually all of the epidermal cells in the differentiated layers above the active caspase-3-positive basal layer had become sunburn cells (first column of panels in Supplementary Figure 3, available at Carcinogenesis Online). None of the K10-positive differentiated cells showed activation of caspase-3 (Supplementary Figure 4, available at Carcinogenesis Online). Active caspase-3-positive staining was absent in hair follicles, except for the rim of the infundibula at 6 h where some cells in the outer root sheet could be positive to at most a depth of three cells under the IF basal layer (arrows in second panel of first column of Figure 1). At 24 h and later, cells with activated caspase-3...
were exclusively found in the IF epidermis. The caspase-positive apoptotic cells apparently migrated from the basal layer outward in a front as a new epidermis grew underneath. The overlying layers remained intact.

Our attempt to visualize apoptosis directly in p53-mutant clones by double staining with anti-mutant p53 (Pab240) and anti-active caspase-3 at 6 and 24 h failed because Pab240 staining became false positive in most of the overexposed epidermis (Supplementary Figure 5, available at Carcinogenesis Online). This was probably due to partial degradation of p53 after the applied UV overexposure, whereby the Pab240 epitope became also accessible in wild-type p53 proteins.

P63 is essential for homeostasis of the epidermis; mice knockout or mutant in p63 are severely compromised in skin development (26,27). P63 also relays an anti-apoptotic signal (28). Earlier reported immunostaining for p63 with antibody 4A4 revealed p63 expression in the basal cells of the epidermis and in the bulge of the hair follicle, which include adult stem cells (29). Immunostaining with this p63 antibody on sections at different time points after UV overexposure (Figure 1, second column) revealed positive nuclear staining throughout the outer root sheet of the hair follicles and the basal layer of the IF epidermis at all time points, except at 24 h after overexposure. At this time point, p63 expression was completely lost in the IF epidermis, whereas the hair follicles remained positive. At 48 h, a renewed p63-positive basal layer was observed underneath the apoptotic caspase-positive 'front'.

Double staining with anti-active caspase-3 and anti-p63 showed no double-positive cells (Figure 1, third column), except at 6 h where a few double-positive cells were observed, on average 4.0 (SEM 1.2) % of basal cells.

The IF basal layer is repopulated from hair follicles after UV ablation
To study repopulation of the basal cell layer of the IF epidermis after UV ablation, we performed a BrdU pulse-chase experiment in which we injected BrdU at 0, 5, 23 and 167 h after UV overexposure and used chase periods of 17 and 23 h counting from 2 h after injection (Figure 2). In skin that was not overexposed, epidermal basal cells showed clear BrdU incorporation, but at 5–6 h after UV overexposure, the BrdU uptake was strongly diminished. No BrdU-positive cells were observed in the IF epidermis after a subsequent chase period up to 24 h after overexposure. At 23–24 h after overexposure, no BrdU uptake was detectable in the IF epidermis, whereas abundant labelling was found in the hair follicles. After a subsequent chase period up to 48 h after the overexposure, BrdU-labelled daughter cells had apparently moved from the hair follicles to the newly formed IF basal cell layer. Seven days after UV overexposure, the BrdU uptake was normal and closely resembled what was observed in skin that had not been overexposed.

Interestingly, p63 staining revealed channels of cells that connected cysts located deep down in the dermis with the rudimentary hair follicle of the hairless mouse (Supplementary Figure 6, available at Carcinogenesis Online). The channels were only two cells in width and therefore went unnoticed earlier. The dermal cysts remained BrdU positive at all time points (Supplementary Figure 7, available at Carcinogenesis Online).

P53-Mutant clones eliminated by UV ablation
To study whether p53-mutant clones disappeared after UV ablation of the IF basal cell layer or persisted due to their possible resistance to apoptosis, we first measured the median number of 258 (95% CI 188–354) in dorsal epidermal sheets of six mice killed after the 4 weeks regimen of daily sub-sunburn exposures (i.e. the number just before UV overexposure). The p53-mutant clones appeared to be exclusively located in the IF epidermis (i.e. no staining in hair follicles; Supplementary Figure 1, available at Carcinogenesis Online). After the UV overexposure, all mice were left unexposed for 1 week for recuperation of the epidermis. At the end of that week, at day 35, the p53 clones in the overexposed group had almost completely disappeared: only 0.6 clones per sheet (95% CI 0.5–0.8) were found, whereas the p53 clones in the control group had dropped in number to 102 per sheet (95% CI 59–179) (Figure 3). Compared with these control mice at day 35, 99.5% of the p53 clones were thus eliminated by UV ablation. To ascertain whether the loss of p53-mutant clones was not merely temporary (possibly by survival of stem cells with mutated p53), the UV regimen of daily sub-sunburn exposures was resumed in both groups at day 36 till day 42. In both groups, the p53 clones were found only slightly increased by day 42 [ablated mice: 2.9 per sheet (95% CI 1.4–6.0) and control mice 168 per sheet (95% CI 128–221)]. Hence, the clearance of the p53-mutant clones after UV ablation was virtually complete and permanent.

Fig. 2. BrdU immunostaining of murine skin sections at several time points after UV overexposure (+OE) and control skin (−OE) at 1 h after one MED. The mice were killed 1 h after BrdU injection at 0, 5, 23 and 167 h after overexposure or after a chase period of up to 19 h after injection at 5 h or up to 25 h after injection at 23 h.
Ablation of basal layer ‘resets’ UV carcinogenesis

To assess the effect of the ablation of the IF epidermal basal layer, including p53 clones, we performed a UV carcinogenesis experiment by resuming daily sub-sunburn exposures (500 J/m² UV/day) 1 week after UV ablation in one group of 26 hairless SKH1 and in a corresponding control group that had not been overexposed (one mouse in the latter group died from unknown cause). The vast majority of the tumours consisted of SCCs and precursor lesions. Very few (<5%) exophytically growing papillomas (30) were observed, which were not included in the analysis. UV carcinogenesis in the UV-ablated group was significantly delayed for all tumour sizes (P < 0.0001 Mantel Cox test), Figure 4. The delay was 19, 24, 33 and 31 days for tumours ≥0.1, ≥1, ≥2 and ≥4 mm in diameter, respectively, with an average of 27 (SEM 3) days. Blinded histopathologic analysis showed similar tumour types in both groups, 80% SCCs and 20% Bowenoid tumours, i.e. in situ SCC (samples of n = 10). However, two of the eight SCCs from the ablated group showed sarcomatoid morphology with spindle-shaped cells, which lost p63, K5 (data not shown) and pan-keratin staining (Supplementary Figure 8, available at Carcinogenesis Online).

Discussion

Since UV-induced apoptosis can be impaired after loss of functional p53 (10,11), one might expect that p53-mutant clones would resist apoptosis after overexposure. However, we found apoptosis, marked by active caspase-3 expression, in the entire basal layer of the IF epidermis laden with p53-mutant clones after strong UV overexposure. We did not see any areas devoid of active caspase-3 staining in the IF epidermis. This indicated that p53-mutant clones were not resistant to apoptosis at the applied overexposure of 10 kJ/m² UV (five aMED). Wijnhoven et al. (11) reported impaired UV-induced apoptosis in heterozygous mutant p53 mice but at the high UV dose we applied, all basal cells became apoptotic.

Apoptosis induced by UV overexposure in the skin of hairless mice was virtually completely restricted to the basal layer of the IF epidermis and was accompanied by a complete loss of p63 protein expression at 24 h after UV ablation. This is in line with the observation that p63 down regulation allows apoptosis induction (28). Double staining revealed an outward moving active caspase-3-positive and p63-negative apoptotic front of cells and cell remnants at 24 and 48 h, whereas a new layer of p63-positive and active caspase-3-negative cells developed underneath; at these time points, no double-positive cells were found.

At 6 h, 96% of the basal cells were either caspase+ or p63+: only a low percentage of 4% double-positive cells was found. In the double-positive cells, p63 might have been already degraded with its remnants still detectable by antibody staining. The reason that p63 expression persisted in hair follicles could be that the UVB radiation happened to be attenuated below a critical level at these depths or it might be that the follicles were intrinsically less sensitive, perhaps maintaining a pool of (stem) cells for rapid repopulation of the damaged IF epidermis (the latter premise appears to be less likely as a loss of Bcl-2-positive cells after UV overexposure included the hair follicles, only the bulge area and deeper down remained positive; data not shown).

Interestingly, the abundance of sunburn cells in the layers overlying the (active caspase-3 positive) apoptotic basal layer did not show any sign of caspase-3-driven apoptosis or any disassembly and remnants of cells like in the basal layer (Supplementary Figure 3, available at Carcinogenesis Online). These sunburn cells appear to lose keratins (Supplementary Figures 3 and 4, available at Carcinogenesis Online), become dyskeratotic and their cytoplasm becomes eosinophilic, like the stratum corneum (haematoxylin and eosin staining in Supplementary Figure 3, available at Carcinogenesis Online) (31). Hence, these sunburn cells do not appear to be truly apoptotic (although they meet some of the morphological criteria) but they seem to be forced into a premature state of terminal differentiation to speed up their removal from the ranks of functional epidermal cells. Genuine caspase-3-driven apoptosis appears to be limited to cycling cells (in Ki-67-positive layers; Supplementary Figure 3, available at Carcinogenesis Online).

During normal homoeostasis, IF stem cells renew the IF epidermis, whereas in wound healing, hair follicle stem cells repopulate the IF epidermis (32). Our BrdU pulse-chase experiment showed rapid replacement of the apoptotic IF epidermis from hair follicle keratinocytes with increased proliferation at 24–48 h after UV overexposure. Epidermal repopulation after UV ablation is thus similar to epidermal repopulation from hair follicle cells after abrasion wounding. BrdU labelling was absent in the entire IF epidermis at 5–6 h, which was the prelude to a complete apoptotic response in which neither normal IF epidermal cells nor p53-mutant clone cells survived the applied UV overexposure.

A review report on hairless SKH1 mice suggested that the rudimentary hair follicles and dermal cysts were not connected (33). However, our p63 staining revealed narrow channels of keratinocytes connecting dermal cysts and rudimentary hair follicles (Supplementary Figure 6, available at Carcinogenesis Online). An occasional hair follicle in anagen phase in skin sections from SKH1 mice illustrated that the depth of the cysts in the dermis is indeed comparable with that of the bulbs of normal hair follicles. This revealed a more complete image of the physiology of the remnants of hair follicles in hairless mice; these follicles are seemingly ‘frozen’ in catagen with bulbs growing into cysts. In line with our findings, an earlier report (34) identified dermal keratinizing cysts as remnants of the external root sheath in which proliferation takes place during UV irradiation. We found that proliferation in the dermal cysts remained unaffected upon UV overexposure.

After UV overexposure, we thus observed two important mechanisms working together to suppress mutagenesis: (i) apoptosis of the most exposed and damaged germinative layer located in the basal layer of the IF epidermis and (ii) replacement of this germinative layer from deep-seated (stem) cells that were more shielded from UV exposure and therefore less damaged. Overly damaged non-proliferating differentiated cells apparently became sunburn cells that showed features of termination in a stage of premature cornification.

The complete elimination of p53 clones at 7 days after UV overexposure confirms the lack of apoptotic-resistant (active caspase-3 negative) cell clusters. This is also in line with the lack of BrdU-positive and the lack of p63-positive cell clusters in the IF epidermis at 24 h after UV overexposure. Apparently, all IF epidermal basal (stem) cells, including the p53-mutant clones, became apoptotic at the high UV dose that we applied.
Our finding that the newly formed epidermis was devoid of p53-mutant clones also indicated that UV-induced p53 clones were not derived from hair follicles. Because, if they were, p53-mutant cells would have proliferated with the hair follicle cells and would have quickly formed new p53 clones in the superficial epidermis.

The fact that the applied UV ablation delays UV carcinogenesis by a period of time [27 (SEM 3) days] comparable with that of daily exposure (28 days) before UV ablation indicates that the onset of tumours was completely nullified by the UV ablation. This ‘resetting’ of UV carcinogenesis was preceded by elimination of p53-mutant clones.

As in earlier experiments, we found in the present experiment that p53 clones are predictive of tumour risk and may therefore serve as markers of risk of SCCs (35, 36).

A consequence of the fact that ablation of IF epidermal (basal) cells (including p53-clone cells) resulted in a reset of UV carcinogenesis indicates that the origin of UV-induced SCCs is in the IF epidermis and not in the hair follicles (with the possible exception of the shallow outer rim of the infundibulum). The reason that in earlier reported laser-ablation experiments on hairless SKH1 mice (15,37), no delay in UV carcinogenesis was found, is most likely attributable to a laser dose that was tuned to naive mice, while the epidermis thickens after weeks of UV exposure (38). The applied laser dose was probably not high enough to assure ablation of the deeper located cells of the basal layer in chronically exposed skin (this was apparently not checked).

We found that after 4 weeks of daily UV exposure, four MED for a naive skin was not enough to induce a clear sunburn reaction or apoptosis in the UV-adapted skin. We raised the UV dose five times more to induce similar levels of apoptosis as in a naive skin after four to six MEDs. Our data are also in contrast with earlier experiments where skin abrasion showed that SCCs induced by two-stage chemical carcinogenesis originated from hair follicles (39). This may be attributable to storage of solvent in the follicle and possible preferential exposure (40).

Although the IF ablation by a single overexposure suppresses UV carcinogenesis and gives a good cosmetic result, 20% of the SCCs in the ablated group showed features of the more aggressive spindle cell SCCs (Supplementary Figure 8, available at Carcinogenesis Online indicating epidermal–mesenchymal transition). Moreover, episodes of severe sunburn are known to increase the risk of BCCs and melanoma in humans (41,42). Therefore, it is rather unlikely that genotoxic ablation can be used as a therapeutic treatment. This investigation indicates however that treatments for SCC prevention should be targeted at the IF epidermis rather than the hair follicles.

In conclusion, UV overexposure in hairless mice induces caspase-3–driven apoptosis which is virtually completely restricted to the basal layer of the IF epidermis (not including sunburn cells in overlying spinous layers). BsuU pulse-chase experiments revealed that after this ablation, the IF epidermis is repopulated by outgrowth of hair follicle keratinocytes. Selective UV-induced ablation of the IF epidermis eliminates UV-induced p53-mutant clones and resets UV carcinogenesis. This implies that UV-induced SCCs in SKH1 mice originate from the IF epidermis.

Supplementary material

Supplementary Figures 1–8 can be found at http://carcin.oxfordjournals.org/.

Fig. 4. Kaplan–Meier plots of UV-induced carcinogenesis of successive tumour sizes. SKH1 mice of UV-overexposed group (triangles, n = 26) showed increased median tumour induction times (t50) compared with the control group (circles, n = 25), for all tumour sizes (P < 0.0001, Mantel Cox test). The differences in median tumour induction times (Δt50) are stated in the successive graphs with an average of 27 (SEM 3) days delay in the overexposed group. The UV overexposure was applied on day 29.
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Conflict of Interest Statement: None declared.

References
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