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Sunburn and p53 in the onset of skin cancer

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SQUAMOUS cell carcinoma of the skin (SCC) can progress by stages: sun-damaged epidermis, with individual disordered keratinocytes; actinic keratosis (AK), spontaneously regressing keratinized patches having aberrant cell differentiation and proliferation; carcinoma *in situ*; SCC and metastasis[1–3]. To understand how sunlight acts as a carcinogen, we determined the stage at which sunlight mutates the p53 tumour-suppressor gene and identified a function for p53 in skin. The p53 mutations induced by ultraviolet radiation and found in >90% of human SCCs[4,5] were present in AKs. Inactivating p53 in mouse skin reduced the appearance of sunburn cells[6], apoptotic keratinocytes generated by overexposure to ultraviolet. Skin thus appears to possess a p53-dependent 'guardian-of-the-tissue' response to DNA damage which aborts precancerous cells. If this response is reduced in a single cell by a prior p53 mutation, sunburn can select for clonal expansion of the p53-mutated cell into the AK. Sunlight can act twice: as tumour initiator and tumour promoter.

To identify the time of sunlight mutagenesis, we first searched for ultraviolet-induced mutations in the p53 gene of AKs. We then looked for evidence indicating whether these mutations had arisen as a prior event in sun-damaged skin or as a later event within the AK. Of 45 actinic keratoses from 24 New England patients, 60% contained a total of 35 p53 mutations (Table 1); eight carried two mutations. Loss of a p53 allele was found in 29% of informative samples. The percentage with mutations may be underestimated as most AKs are not full-thickness lesions. Of the 12 biopsies with the highest proportion of AK to flanking tissue, 75% were mutated.

The base changes found implicated sunlight as the mutagen[4,5,7]: 89% occurred at adjacent pyrimidines (Table 1) and most were C→T substitutions or CC→TT double-base changes. All coding-region mutations would alter the amino acid. Most patients had multiple AKs in the same region of sun-damaged skin. In five patients, more than one carried a p53 mutation (Table 1). These multiple AKs never shared a common mutation. Each AK's p53-mutant clone thus originated from an independent ultraviolet photon event.

If these p53 mutations arose after the AK, reflecting microscopic SCCs, no AK would contain more than a subclone of p53-mutated cells. But after microdissection, over 80% of the AKs had a mutant sequencing band with 40–100% of the signal, as expected if the mutation was present on one or both alleles in the entire AK sample (Table 1). As the p53 mutations were present throughout most lesions, sunlight evidently acted as a mutagen before or during the clonal expansion of AK, the first clinically apparent lesion in skin cancer development. It is unlikely, but not excluded, that the p53 mutations arose before the AK. A large p53-mutant clone did not precede the multiple AKs, because these AKs had different p53 mutations. If AKs arose within separate small p53-mutant clones, an AK would be surrounded by non-AK cells containing the same p53 mutation. But the frequency of the AK mutation in flanking sun-exposed skin was $\leqslant 10^{-3}$ in 7 of 8 cases, and never exceeded 10^{-2} (Table 2). It is not excluded that the p53 mutations arise within a clone of cells previously mutated at another gene but not having an AK phenotype.

To investigate the function of p53 in normal skin, wild-type mice were irradiated on the back with varying doses of ultraviolet-B. Twenty-four hours later, sunburn cells[6] were present in the epidermis (Fig. 1*a*, *b*). When stained with haematoxylin–eosin, these had the classic light-microscope appearance of apoptotic cells: pycnotic nuclei and intensely eosinophilic cytoplasm. In $p53^{-/-}$ animals[8], however, few sunburn cells were induced (Fig. 1*c*, *d*). As sunburn cells are often considered 'dyskeratotic', with the eosinophilic cytoplasm presumed to provide evidence of altered keratinization, we confirmed the apoptotic nature of sunburn cells by assaying for DNA strand breaks in keratinocyte nuclei using fluorescent *in situ* end-labelling (FISEL). Figure 1*e*, *f* shows that, in wild-type animals, keratinocytes with strand breaks in the nuclear DNA were present 24 h after ultraviolet-B irradiation. In some cells, breaks were localized at the nuclear periphery, resembling the marginated chromatin of apoptotic cells seen by electron microscopy. Ultraviolet-B itself did not induce breaks. In wild-type mice, cells with DNA strand breaks appeared with the same time course as apoptotic cells visualized by haematoxylin–eosin (not shown).

The number of sunburn cells in wild-type mice increased with the dose of ultraviolet-B (Fig. 1*g*). The slope of induction was much less for $p53^{-/-}$ animals. The frequency of apoptosis was intermediate in +/− heterozygous mice (Fig. 1*g*), as was also observed for γ-irradiated thymocytes[8,9]. Sunburn cell formation was not just shifted to an earlier time, because at 12 h post-irradiation the frequency in −/− mice was still less than half that in +/+ mice (not shown). FISEL-positive cells were also reduced in the $p53^{-/-}$ strain and were intermediate in the heterozygote (not shown); the decrease with decreasing copy number of the *p53* gene was statistically significant ($P = 0.03$). The doses used in these experiments are ~1 and 2 times the dose required to cause a detectable erythema in mouse or humans[10]. For humans, a minimal erythemal dose is roughly equivalent to 20 minutes' exposure of untanned skin at noon at middle latitudes. In contrast to the ultraviolet-B response, the apoptotic features of keratinocyte differentiation and the hair cycle appear to be undisturbed in $p53^{-/-}$ mice (K. Stenn, personal communication).

The phenotype of the mutation induced by sunlight should reflect its early occurrence. p53, a transcription factor whose targets include genes involved in cell-cycle regulation[11], has been

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proposed to participate in a DNA surveillance pathway that monitors the genome for DNA damage (reviewed in ref. 12). In some cells, this appears to be a 'guardian-of-the-genome' pathway in which DNA damage by ultraviolet light induces p53 protein[13], leading to transient cell-cycle arrest at a G1 checkpoint[12,14]. In other cases, however, the endpoint of the DNA surveillance pathway is p53-dependent apoptotic death of the damaged cell[8,9]. This pathway, which might be termed a 'guardian of the tissue', aborts the aberrant cell rather than restoring its genome. The sunburn cell appears to be the result of this effector step in skin. Whereas the hallmark of sunburn is erythema, these individual dying cells are microscopic features of sunburned skin[6] and are distinct from the confluent swollen cells of necrosis. They are seen after irradiation of mammalian skin with ultraviolet-A, -B or -C[6,10] and can be initiated by DNA damage because their frequency is reduced by reversing cyclobutane pyrimidine dimers in DNA[15]. Cells that become sunburn cells seem to be those that were replicating DNA at the time of irradiation[6]. Sunburn cells now appear to be important for preventing skin cancer: they occur in the cell-type of origin for skin tumours, keratinocytes; they result from DNA damage caused by the principal skin carcinogen, ultraviolet radiation;

TABLE 1 Sunlight-induced p53 mutations in actinic keratoses

Sample	Site	LOH	Codon	Sequence	Base change	Amino-acid change	Mutation signal Intensity (%)
YK 101A	scalp	n.i.	278	tcCt	C → T	Pro → Leu	40
			282	gCcg	C → A	Arg → Leu	30
YK 104A	hand	n.i.	245	gcC*g	C → T	Gly → Ser	35
YK 106A	hand	−	264	atcTa	−T	Leu → . . . stop	50
YK 107A	scalp	+	84	gCcc	C → T	Ala → Val	100
YK 108A	scalp	−	205	aTa	T → C	Tyr → Cys	40
YK 109A	shoulder	−	198	tCca	C → A	Glu → stop	50
YK 110A	hand	n.i.	331	cttCa	C → T	Glu → stop	40
YK 112C	neck	−	92	ccCcc	C → T	Pro → Leu	50
			159	gCca	C → T	Ala → Val	50
YK 113A	forehead	−	200 or 201	atTtg	+T	Leu → . . . stop	50
			intron 4	aCtg	C → T	effect unknown	30
YK 114A	scalp	n.i.	178	cccCa	C → T	His → Tyr	60
			342	ttcC*g	C → T	Arg → stop	60
YK 115A	hand	+					
YK 115C	forearm	+	238	aCa	C → A	Cys → Phe	40
			241	ttCct	C → T	Ser → Phe	60
YK 116A	arm	n.i.	258	ttCca	C → T	Glu → Lys	50
YK 116B	arm	n.i.	192	ctCa	C → T	Gln → stop	50
YK 116C	arm	n.i.	201–202	tGC*g	GC → TT	Leu-Arg → Phe-Cys	40
			202	gTg	−T	Arg → . . . stop	40
			282	acC*g	C → T	Arg → Trp	50
YK 117A	forearm	−	286	tCct	C → A	Glu → stop	60
YK 117B	forearm	−	91	gCca	C → T	Trp → stop	30
			179	acCa	C → T	His → Tyr	50
YK 118C	forearm	n.i.	279	tcCca	C → T	Gly → Glu	30
YK 119B		n.i.	intron 2	tcCa	C → T	effect unknown	40
YK 120A		n.i.	128	ccCc	C → T	Pro → Ser	40
YK 120B		+	247–248	aCC*g	CC → TT	Asn-Arg → Asn-Trp	90
YK 121A		+	248	acC*g	C → T	Arg → Trp	50
YK 121B		−	245	gcC*g	C → T	Gly → Ser	30
YK 122A	cheek	+	135–136	gCCa	CC → AT	Cys-Gln → stop-stop	100
YK 123A	cheek	−	245	gCc*g	C → T	Gly → Asp	40
YK 124A	hand	−	222	gcC*g	C → T	Pro → Leu	50
			223	gCct	−C	Pro → . . . stop	70
YK 124B	knuckle	+	331	ttCa	C → T	Gln → stop	60

Bold, sample with more than one p53 mutation; boxed, patient with p53 mutation in more than one actinic keratosis; n.i., non-informative at codon 72; *, potential 5-methylcytosine; −T indicates T deleted; mutation signal intensity represents sequencing-gel band intensity of mutant divided by intensity of (wild type + mutant). AKs were removed by Mohs surgery. For samples YK101–118, tissue was also removed from adjacent sun-exposed skin more than 1 cm away from the AK, and from sun-shielded skin on the inner aspect of the arm. Half of each specimen was fixed in neutral-buffered formalin, embedded in paraffin and stained with haematoxylin–eosin. The remainder was frozen and DNA isolated using proteinase K. Some paraffin sections were microdissected before DNA isolation to reduce contamination with normal tissue[4]. The absence of associated SCC in these patients excluded the possibility that the lesion studied was a morphological variant of SCC. Exons 2 to 11 of the *p53* gene were amplified by PCR and directly sequenced using the buffers, primers, cycling conditions and controls described previously[4,5]. To determine allelic loss, the region of polymorphic codon 72 was amplified from microdissected paraffin sections and PCR fragments were restricted. p53 protein was detected in unbaked paraffin sections using rabbit polyclonal antibody CM-1 (NovoCastra, Newcastle, UK) at a dilution of 1:4,000 (ref. 5). Staining was restricted to the epidermis and was absent when CM-1 antibody was omitted.

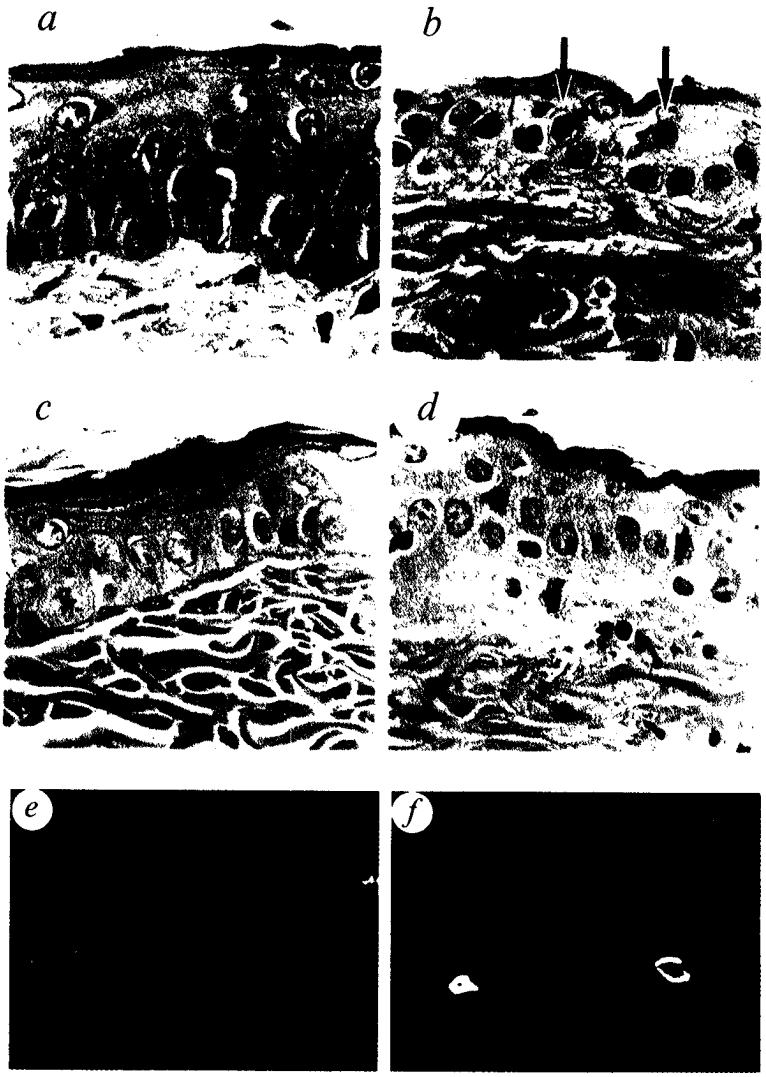
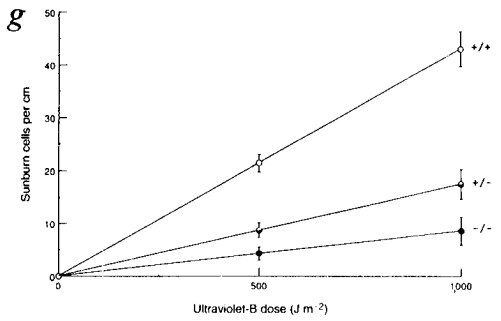


FIG. 1 Sunburn cell formation in different *p53* genotypes. *a*, *b*, Haematoxylin–eosin staining of epidermis of $p53^{+/+}$ mice, 0 and 500 J m^{-2} UV-B; *c*, *d*, $p53^{-/-}$, 0 and 500 J m^{-2}. *e*, *f*, FISEL detection of nuclear DNA strand breaks in $p53^{+/+}$ mice after 0 and 500 J m^{-2}. *g*, Dose response of sunburn-cell formation in different p53 genotypes.
METHODS. The germline *p53* gene was disrupted between exons 2 and 6, preventing production of p53 protein[8]; mice were C57BL6 × 129/sv hybrids, age 7–8 weeks. Hair was removed by waxing a 2-cm^2 region of the back; because removal of the stratum corneum stimulates cell proliferation, this procedure also enhances the number of sunburn cells seen. 12 h later mice were irradiated 8 cm beneath three UV-B lights (FS20T12-UVB; National Biological) at a dose rate of 11.3 J m^{-2} s^{-1}; vertical movement was restrained by a wire screen with 1.2-cm grid. Mice were killed 24 h after UV-B, skin flaps were fixed and embedded, and 5-μm sections stained with haematoxylin–eosin. To detect DNA strand breaks, the TUNEL method[30] was modified so that terminal transferase directly incorporated fluorochrome-labelled dCTP (Cy-5; Biological Detection Systems, Pittsburgh, USA). Cells were visualized with a BioRad MRC-600 laser confocal microscope, with RHS filter set. Criteria for scoring sunburn cells were intensely eosinophilic cytoplasm, pycnotic nuclei, and separation from adjacent cells. For each of 2–5 mice per point, about 6 haematoxylin–eosin sections, spaced three sections apart, were counted for sunburn cells per linear cm of epidermis. Data for each genotype were analysed using weighted linear regression of sunburn cell frequency versus UV dose, with weights proportional to the mean at each dose. Bars represent standard errors; slope differences were statistically significant by *t*-test ($P<10^{-6}$ for +/+ versus −/−; $P=8\times10^{-6}$ for +/+ versus +/−; $P=0.03$ for +/− versus −/−). Sunburn cells: 400 ×, tungsten bulb, LBD-2 standard daylight blue filter, Kodak Ektar 25 film; FISEL: 504 ×, Kodak Elite 100 film, 1 s.

and now they are shown to depend largely on a gene, *p53*, that prevents tumours in mouse skin[16] and is mutated in most human skin cancers and precancers.

If the p53 amino-acid substitutions found in AK are as detrimental to sunburn-cell formation as the null mutations studied here, chronic sunlight exposure would lead to a sequence of events resembling the early stages of human skin cancer (Fig. 2). In particular, sunburn subsequent to a p53 mutation will act as a selection pressure favouring the cell containing that mutation. Ultraviolet-damaged normal cells will die as sunburn cells, but about half of damaged $p53^{+/-}$ cells will be resistant to apoptosis (Fig. 1*g*). These mutated cells can then clonally expand into an actinic keratosis[17]. Because the differential selection must occur after the p53 mutation, this second effect of sunlight fits the definition of tumour promotion[18]. Ultraviolet light can act as a tumour promoter in mouse skin, as can other DNA-damaging agents such as oxygen radicals[19,20]. The influence of selection can be profound owing to the exponential nature of cell proliferation: growth of mouse skin papillomas can be sustained by only a 5% excess of cell production over cell loss[21]. The expanding cell-death-defective clone: (1) presents a larger target for future ultraviolet exposure (refs 17, 18 and refs therein), and (2) is a target in which a greater fraction of cells can survive irradiation with ultraviolet to carry an additional mutation in *p53* or another gene[17]. These effects can increase the number of mutant cells without requiring a mutator phenotype. The delicate balance between cell death and cell proliferation rates can allow



even a two-gene carcinogenesis mechanism to proceed slowly enough to appear multigenic[22]. The biological effects of p53 mutations on keratinocytes will be quantitative rather than all-or-none.

The mutagenicity of sunlight in the *p53* gene and the subsequent selection pressure exerted by sunburn cell formation, acting over the course of years, can account for the salient features of human skin carcinogenesis: (1) skin cancer and the precancerous actinic keratosis are most frequent in individuals who sunburn rather than tan, particularly those of Celtic descent[23,24]; (2) an actinic keratosis usually regresses in the absence of exposure to sun but progressively enlarges if exposure

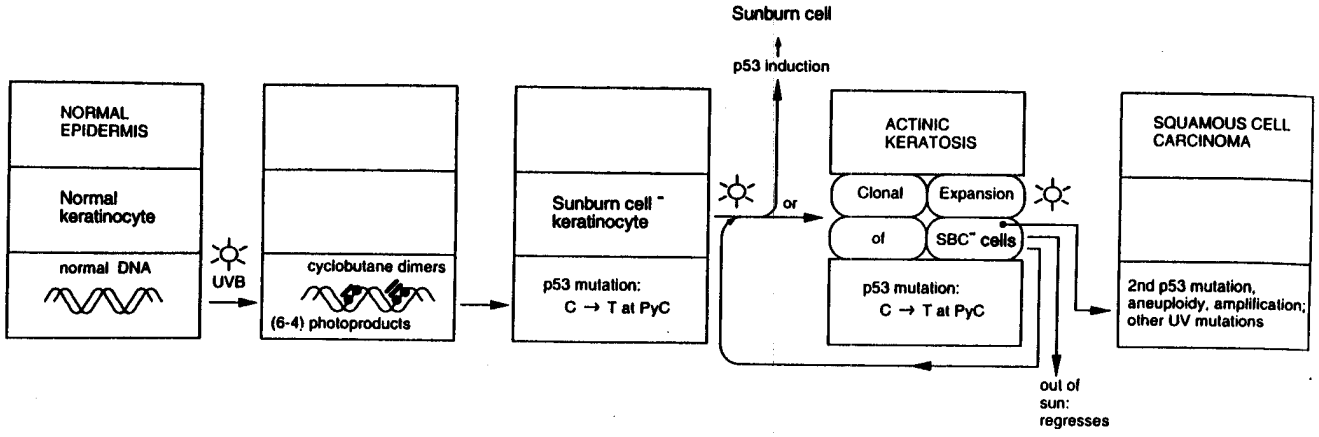


FIG. 2 Early genetic, cellular and tissue-level events in human skin cancer: selection for p53-mutated cells by repeated sunburn. Sunlight UV-B induces cyclobutane pyrimidine dimers and (6–4) photoproducts in epidermal cell DNA; most photoproducts are repaired, but upon DNA replication some cells acquire a C→T point mutation in the *p53* gene (refs 4, 5) and Table 1). At the cellular level, these $p53^{+/-}$ keratinocytes (sunburn cell$^-$ keratinocytes; SBC$^-$ cells) are partially defective for sunburn cell production (Fig. 1*g*). When the next sunlight exposure induces p53 protein[13], normal cells UV-damaged during S phase die by apoptosis, but only about half of the $p53^{+/-}$ cells die (Fig. 1*g*). A $p53^{+/-}$ cell can then clonally expand, and may become an actinic keratosis (Table 1). Thus, sunburn subsequent to a p53 mutation can act as a selection pressure for the mutated, damage-resistant, cell. These events may be frequent, because the lifetime-expectancy of AK in Australia approaches 100% (ref. 26). Other mutated genes may contribute to the AK phenotype. In the absence of further sun exposure, 25% of actinic keratoses regress each year[25]. But continued sun exposure selects for further clonal expansion of the SBC$^-$ cells in the AK, potentially allowing one cell to become $p53^{-/-}$ (ref. 5 and Table 1; YK121A and 124B may contain point-mutant subclones emerging within an allelic-loss AK). Homozygous mutation could lead to aneuploidy and gene amplification[12], to UV-induced mutations in additional genes, and to squamous cell carcinoma of the skin[4].

continues[25]; (3) skin cancers and precancers can acquire sunlight-induced p53 mutations at a rate much higher than is achievable *in vitro*[5]; (4) although sunlight exposure significant for actinic keratosis and squamous cell carcinoma occurs in childhood, skin cancer appears after forty or more years of chronic relatively low-dose sun exposure[24,26].

The clinical consequence of early mutation induction by sunlight contrasts with tissues in which p53 influences conversion of low-grade tumours to more malignant tumours[27]. SCC, already more frequent in Australia and the United States than colon, lung or breast cancer, has been increasing exponentially[28], but these rates are based on the elderly population in whom SCC arise. Because sunlight acts early, these rates should increase further as individuals with greater early-sun exposure, now in their 30s and 40s, reach the age at which tumours appear. □

TABLE 2 Analysis of sun-damaged skin flanking an actinic keratosis for the p53 mutation found in that actinic keratosis

Sample	Codon	Mutation frequency
YK 107B	84	10^{-3}
YK 110E	331	$<10^{-3}$
YK 114B	178	$<10^{-3}$
YK 114B	342	10^{-3}
YK 115D	241	$<10^{-3}$
YK 116D	282	10^{-2}
YK 117C	179	$<10^{-3}$
YK 118D	279	10^{-3}

For 8 cases in which a p53 mutation was found in an AK and a biopsy of sun-damaged skin had been taken, 1–3 cm distant to ensure that only non-AK tissue was examined, DNA was isolated from the sun-damaged skin and amplified by PCR. To detect low frequencies of the actinic keratosis mutation, 28-nucleotide primers were extended opposite the mutated base (T) with [^{32}P]dATP (Amersham) as described[29], except that the reaction included *Pfu* polymerase (Stratagene), 50 μM dideoxyGTP (New England Biolabs) to reduce nonspecific incorporation, and, depending on the sequence, either 3 μM dNTP, corresponding to the 3′ base of the primer, or a primer with phosphorothioate linkages at the 3′ two positions to reduce primer shortening by the proof-reading exonuclease of *Pfu*. A similar reaction with ^{32}P-dGTP measured the frequency of wild-type molecules to correct for variations in DNA concentration and loading. Template was 10 ng of PCR product. Mutation frequency was determined by comparing the signal strength of the skin sample to a standard curve.

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