Intermediate-effect biomarkers in prevention of skin cancer

J.-F. Doré, R. Pedex, M. Boniol, M.-C. Chignol and P. Autier

Skin cancers, both non-melanoma and melanoma, usually progress through sequential steps towards malignant transformation, leading from an initiated cell to a mutant clone and to a precancerous lesion. Prevention of skin cancers relies on reduction of exposure to solar radiation and may be evaluated by measuring induction of intermediate-effect biomarkers such as sunburn cells or p53 mutations in the epidermis, actinic (solar) keratoses, UV-induced immunosuppression or naevi. Sunburn cells (apoptotic keratinocytes) and p53 mutations are indicators of UV-induced DNA lesions as early steps of malignant transformation of epidermal keratinocytes. Actinic keratoses are premalignant sun-induced skin lesions, characterized as keratinized patches with aberrant cell differentiation and proliferation; they represent risk factors for basal-cell carcinoma and melanoma and are precursors of squamous-cell carcinoma. Studies in humans have investigated UV-induced immunosuppression and its modulation by topical sunscreen application, focusing on contact hypersensitivity as measured by immunization or response to haptons, or on modulation of stimulation of allogeneic lymphocytes by epidermal cells, or local release of immunomodulatory molecules such as cys-urocanic acid or interleukin-10.

Naevi are focal collections of melanocytes, usually found at the junction of the epidermis and dermis or at various depths in the dermis. Common acquired naevi arise after birth both spontaneously and in response to sun exposure. Most acquired naevi are clonal, while most melanocytes in non-naevial areas are not. Although it is not yet certain whether naevi represent premalignant lesions or risk factors, many melanomas arise in acquired naevi, and the number of naevi constitutes the best predictor of individual risk of melanoma. The presence of large (i.e., >5 mm) or atypical naevi (i.e., large naevi with non-uniform colour and irregular borders) is associated with elevated melanoma risk, independently of the number of smaller naevi. Children seem particularly vulnerable to sun-induced biological events involved in the genesis of melanoma, and the greatest increase in naevus numbers per unit of skin surface occurs before adolescence. Therefore, the distribution of naevi and their development in children are relevant to understanding melanoma occurrence in adults.

Introduction
Skin cancers, both non-melanoma and melanoma, usually progress through sequential steps of malignant transformation, leading from an initiated cell to a mutant clone and to a precancerous lesion. In the absence of exogenous influences, mutant clones and precancers may remain stable or tend to regress. Only on rare occasions do these precancerous lesions finally transform into an invasive cancer (Brash & Ponten, 1998) (Table 1). Epidemiological studies, and, more recently, studies of p53 mutations in squamous-cell carcinoma (SCC) (Ziegler et al., 1994) have suggested that the main etiological agent of skin cancer in humans is exposure to solar radiation, especially early in life (Autier & Doré, 1998). Clonal expansion of an initial mutated cell may be driven by sunlight exposure either by a direct effect of UV radiation on initiated target cells or through an indirect immunosuppressive effect. Immunosuppressive drugs may exert the same effect as UV radiation in facilitating the clonal expansion of precancers. Sunlight can act twice: as tumour initiator and as tumour promoter. Predisposition to sunlight-induced precancer is a multigenic trait that involves independent factors such as skin (photo)type, eye and hair colour, individual DNA repair capacity and naevus number. However, this
latter trait results from both individual susceptibility to UV and exposure to sunlight in childhood (Autier, 1997).

Prevention of skin cancers will largely result from reduction in exposure to solar radiation and may be evaluated by measuring induction of intermediate-effect biomarkers such as induction of sunburn cells or p53 mutations in the epidermis, actinic (solar) keratoses, UV-induced immunosuppression or naevi (Tables 2 and 3).

Non-melanoma skin cancer
SCC of the skin results from chronic exposure to sunlight and progresses by stages (Table 1): sun-damaged epidermis with individually disordered keratinocytes; clones of keratinocytes harbouring a mutant p53 tumour-suppressor gene within a normal epidermis; actinic (solar) keratoses, i.e., keratinized patches with aberrant cell differentiation and proliferation (these dysplastic lesions may still spontaneously regress), carcinoma in situ; SCC and metastasis. The set of p53 mutations in tumours is more restricted than in precancers, suggesting the existence of additional events and selection in the progression from precancer to invasive cancer (Ziegler et al., 1994; Brash & Ponten, 1998).

Basal-cell carcinoma (BCC) of the skin seems to arise without a precancer, probably from stem cells in the bulge region of the hair follicle, and contains mutations of the Ptch gene and, less frequently, of p53. Its relationship to sunlight exposure is less clear but it may, like malignant melanoma, be associated with intermittent recreational exposure rather than chronic exposure.

Sunburn cells and p53 mutations
Sunburn cells are identified in conventionally stained epidermal biopsies as keratinocytes with a dense, pyknotic nucleus and a homogeneously eosinophilic cytoplasm. They are keratinocytes that have sustained a lethal dose of UV radiation (apoptotic keratinocytes). It has long been observed that sunburn cells may be produced, in the absence of erythema, by doses of UV radiation below the minimum erythemal dose (Grove & Kaidbey, 1980). Hence, formation of sunburn cells in the epidermis provides a quantifiable end-point of acute damage by UV radiation.

A study conducted in healthy volunteers of skin phototypes I to III, exposed to a dose of UV radiation equivalent to the individual sun protection factor (SPF) of an SPF-15-labelled sunscreen, over areas of the middle of the back protected by
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<td>Keratinized patches with aberrant cell differentiation and proliferation induced by chronic sun exposure in a dose-dependent manner. May spontaneously regress in the absence of UV exposure</td>
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SPF: sun protection factor
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<td>Case-control</td>
<td>418 melanoma 438 controls</td>
<td>Never/ever use of sunscreens</td>
<td>Naevus count on both arms in controls increased from no use to ever use of sunscreens. Rate ratio (RR) 1.31 (95% CI, 1.19–1.43) adjusted for age, sex, hair colour, sun exposure</td>
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<td>Cohort</td>
<td>357 children (170 boys, 187 girls) 7–11 yrs (median, 9 yrs)</td>
<td>Regular, seldom or never use of sunscreen</td>
<td>Whole body naevus counts at five-year interval. RR of increase in naevi ≥1 mm: 1, 0.81 (0.65–1.01), and 0.41 (0.23–0.75) for regular, seldom or never use of sunscreen.</td>
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<td>Total and average sunscreen use, wearing of clothes, sun exposure</td>
<td>Whole-body naevus count. Median number of naevi increased with total and average sunscreen use, and decreased with average wearing clothes. Adjusted RR of high naevus count on the trunk 1.68 (95% CI, 1.09–2.59) for the highest level of sunscreen use and 0.59 (95% CI, 0.36–0.97) for the highest level of wearing clothes.</td>
<td>SPF of sunscreen has no effect. Autier et al. (1998) on naevus count. Highest risk of naevi on the trunk in children using sunscreen and who never suffered from sunburns (RR, 2.21; 95% CI 1.33–3.67)</td>
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application of an SPF-15 or SPF-30 sunscreen, showed that the SPF-30 sunscreen more efficiently prevented the formation of sunburn cells (Kaidbey, 1990).

The p53 protein plays an important role in cellular response to DNA damage. Following exposure to genotoxic agents such as ionizing or UV radiation, wild-type p53 accumulates and becomes immunohistochemically detectable. In human skin, UV irradiation induces accumulation of p53 in the epidermis; this response is rapid and transient, being detectable as early as two hours after irradiation, peaking at 24 hours and persisting for several days (Hall et al., 1993). Following UV irradiation of normally unexposed skin of healthy subjects, the pattern of p53 expression in the epidermis differs according to the UV wavelength: while UVA induces p53 expression predominantly in the basal layer, UVB induces p53 expression diffusely throughout the whole epidermis (Campbell et al., 1993). In contrast, mutation of p53 leads to persistent and strong nuclear accumulation of the protein, and in human sun-exposed skin samples from the face or the dorsal surface of the hands, epidermal areas of homogeneously stained cells sharply demarcated from their surroundings and strongly reactive with antibody to p53 (compact pattern) can be detected (Ponten et al., 1995), that have been shown by microdissection and DNA sequencing to reflect clonal proliferation of keratinocytes with mutated p53 (Ren et al., 1996). Mutation of p53 plays an important role in the onset of SCC of the skin. More than 90% of human SCCs of the skin contain UV-induced p53 mutations that are already present in actinic keratoses (Ziegler et al., 1994), but are genetically unrelated to mutations present in benign clonal keratinocyte patches with p53 mutations in undamaged epidermis (Ren et al., 1997).

Two studies have shown that topical application of a broad-spectrum sunscreen with a sun protection factor of 15 either decreases the overexpression of wild-type p53 (a physiological response to UV-induced DNA damage) in epidermal keratinocytes following UV exposure of normal previously non-sun-exposed skin (Ponten et al., 1995) or significantly reduces the number of p53-positive keratinocytes in sun-protected skin after application of sunscreen to chronically sun-exposed skin during a one-summer period (Berne et al., 1998).

**Actinic keratoses**

Actinic, or solar, keratoses are premalignant, sun-induced skin lesions, characterized as keratinized patches with aberrant cell differentiation and proliferation. They represent risk factors for BCC and melanoma and are precursors to SCC, although the rate of transformation is low (Green & O'Rourke, 1985; Marks et al., 1988a, b). Strong clinical and experimental evidence supports their being precursors of SCC of the skin. Approximately 60% of SCCs are thought to arise in actinic keratoses (Marks et al., 1988a). Similarly to SCC, they have a strong link to sun exposure and share many features with their malignant counterpart, including a dose-response relationship with cumulative sun exposure (Vitsas et al., 1990) and an increased incidence in immunocompromised individuals such as transplant patients (Bloehme & Larko, 1984).

Two randomized, placebo-controlled studies have investigated the prevention of new actinic keratoses and the reduction of pre-existing keratoses by sunscreens in high-risk populations with actinic keratoses or even non-melanoma skin cancer. The Australian study (Thompson et al., 1993), conducted over a period of six months (one summer) in 431 subjects, as well as the North American study (Naylor et al., 1995), conducted on a smaller number of subjects (50) over a two-year period, both showed that regular application of a high sun protection factor broad-spectrum sunscreen significantly reduced the total number of actinic keratoses. The Australian study further showed that this reduction in total number of actinic keratoses stemmed from a reduction in the development of new solar keratoses and an increased remission rate of keratoses present at baseline.

**Immunosuppression**

Experimental studies have shown that UV irradiation, and more especially UVB, induces local and systemic suppression of cell-mediated immunity that plays an important role in the control of growth of UV-induced malignant tumours (Krlpke, 1994). Although there is no direct evidence that UVB-induced immunosuppression plays a role in the growth of sunlight-associated skin malignancies in humans, there is abundant circumstantial evidence; for instance, immunosuppressed
patients such as transplant recipients have greatly elevated rates of non-melanoma skin cancer incidence (Granstein, 1995).

Recent studies have investigated UV-induced immunosuppression and its modulation by topical sunscreen application in humans, focusing on contact hypersensitivity as measured by immunization or response to haptens, on modulation of stimulation of allogeneic lymphocytes by epidermal cells, or on release of immunomodulatory molecules such as cis-urolcanic acid or interleukin-10.

Dinitrochlorobenzene (DNCB) is a potent contact sensitizer to which spontaneous sensitization is rarely encountered in human populations and has been widely used for evaluation of immune capacities of patients. Typically, sensitization to DNCB is induced by applying to the skin in a Finn chamber a small patch of filter paper containing 30-50 μg DNCB in acetone solution; the patch is removed after 48 hours. The sensitization induced is tested two weeks after the first contact with DNCB by applying of challenge patches containing a range of concentrations of DNCB (usually from 3.125 to 12.5 μg). The challenge patches are removed after 48 hours and the contact hypersensitivity reactions are assessed 24 hours later.

Several studies have shown that in healthy volunteers an erythemal UV exposure significantly impairs the afferent arm of the contact hypersensitivity reaction, and that the application of a high sun protection factor sunscreen can at least partially prevent the UV-induced suppression of contact hypersensitivity, without itself interfering with the contact sensitization, probably by preventing the decrease in epidermal Langerhans cells at the irradiated site that usually follows exposure to UVB (Whitmore & Morison, 1995; Hayag et al., 1997; Serre et al., 1997). However, conflicting reports have shown that the ability of sunscreens to interfere with UV-induced modulation of cell-mediated immune responses critically depends on the UV irradiation protocol and assay end-points that may involve different mechanisms of UV-induced immunomodulation (Van Praag et al., 1991; Hurks et al., 1997).

Numerous mediators are released by keratinocytes upon UV irradiation. cis-Urocanic acid, formed by photoisomerization in the epidermis of trans-urocanic acid, accumulates in the stratum corneum and is considered an important mediator of local immunosuppression resulting from exposure to UV radiation. Formation of cis-urocanic acid can be measured in stratum corneum strippings and has been used to evaluate photoprotection by sunscreens (Krien & Moyal, 1994; de Fine Olivarius et al., 1999). More recently, interleukin-10 (IL-10) mRNA expression has been studied by reverse transcription polymerase chain reaction in epidermal cells obtained from suction blisters (Hochberg & Enk, 1999).

**Melanoma**

Cutaneous melanoma represents the end of a continuum starting from sun-damaged melanocytes in...
the epidermis and progressing through a common acquired naevus which may show cellular atypia, an atypical (dysplastic) naevus with architectural atypia, melanoma in situ, early (radial growth phase) primary melanoma with no competence for metastasis, late (vertical growth phase) primary melanoma, and finally to metastatic melanoma (Table 1).

Although the pathogenesis of melanoma is far from fully understood (Gilchrest et al., 1999), the main exogenous etiological factor of melanoma appears to be intermittent (recreational) sun exposure, especially in childhood (Autier & Doré, 1998). It has been proposed that melanomas arising before the age of 50 years occur electively in body areas intermittently exposed to sunlight, while melanomas arising at a later age occur in body areas more likely to be chronically sun-exposed (Elwood & Gallagher, 1998).

**Naevi**

Naevi are focal collections of non-dendritic melanocytes, usually found at the junction of the epidermis and dermis (junctional naevi) or at various depths in the dermis (compound or dermal naevi). Common acquired naevi arise after birth, both spontaneously and in response to various factors, particularly exposure to the sun (Harrison et al., 1994).

Many, if not most, cutaneous melanomas arise in acquired naevi, and it has recently been shown that most acquired naevi are clonal, while most melanocytes in non-naeival areas are not (Robinson et al., 1998). Although it is not clear whether acquired naevi represent actual premalignant lesions or risk factors, the number of common naevi in any given individual, which is determined by genetic factors and sun exposure, constitutes the best predictor of individual risk of melanoma (Boyle et al., 1995; Berwick & Halpern, 1997). The presence of large naevi (e.g., those with one dimension >5 mm) or of atypical naevi (e.g., large naevi with non-uniform colour and irregular borders) is also associated with higher melanoma risk, independently of the number of smaller naevi (Bataille et al., 1996; Tucker et al., 1997). Large naevi are not uncommon in children, but atypical naevi are rare in North American or European children before onset of puberty (Greene et al., 1985).

Children seem particularly vulnerable to sun-induced biological events possibly involved in the genesis of melanoma (Holman & Armstrong, 1984; Autier & Doré, 1998). Also, the greatest increase in naevus numbers per unit of skin surface takes place before adolescence (English & Armstrong, 1994). Therefore, the distribution of naevi and their development in children is relevant to understanding melanoma occurrence in adults.

A recent study by our group (Autier et al., 2000) assessed the body-distribution of naevi, with particular reference to differences in sun exposure by body-site, in 649 European children aged 6–7 years from Brussels (Belgium), Bochum (Germany), Lyon (France) and Rome (Italy). Counts of naevi of size 2–4.9 mm and ≥5 mm were performed using a standard method. The numbers of naevi 2–4.9 mm and of naevi ≥5 mm were strongly correlated, especially on the trunk. For naevi 2–4.9 mm, the highest relative densities were found on the face, back, shoulders and the external surface of the arms. Lowest relative densities were found on the hands, legs, feet and abdomen (Figure 1). The relative density of naevi ≥5 mm was higher on the trunk than on any other body site (Figure 2). Similar body distributions were observed in both sexes and at each centre. The body-site distribution of naevi 2–4.9 mm seemed to parallel the usual sun exposure patterns of young European children. It is suggested that the development of naevi ≥5 mm might be a marker of the vulnerability of melanocytes to the harmful effects of solar radiation. Such vulnerability would be maximal on the trunk, and would decrease distally, with melanocytes of the hands and feet having the lowest vulnerability. The number of naevi acquired on a specific area of skin would result from the combined effects of local vulnerability to solar radiation and local sun exposure history. The origin of acquired body-site differences in the susceptibility of melanocytes to UV radiation is unknown, although it seems to parallel the body-site density of sensory innervation.

Several studies have investigated the development of naevi as a function of sun protection or sunscreen use (Table 3). In a case-control study conducted in Europe in 418 melanoma cases and 438 controls (Autier et al., 1995), it was noted that the use of sunscreen was associated with a higher density of pigmented lesions of the skin in controls. The naevus count on both arms in
control subjects significantly increased from those reporting no sunscreen use to those who ever used sunscreen.

A study of naevus development was conducted in Germany among schoolchildren (Luther et al., 1996). Naevus counts were performed in 866 children between the ages of 1 and 6 years (median age, 4 years), and a second time after a five-year period in 424 children of whom the parents had agreed to participate. Univariate analysis showed a significant relationship between the regular use of sunscreen and a high increase in numbers of melanocytic naevi. This association was interpreted by the authors as resulting from a tendency of children who use sunscreen regularly to have more cumulative sun exposure.

A retrospective cohort study examined the number of naevi in 631 6–7-year-old European children in elementary schools in Brussels, Bochum, Lyon and Rome, according to their sun exposure history, physical protection, sunscreen use and sunburn history from birth to the moment of skin examination (Autier et al., 1998). In all study locations, the median numbers of naevi tended to increase with total or average sunscreen use during holidays, whereas the reverse was true for average wearing of clothes in the sun. Median naevus counts increased with both increasing sun exposure and average sunscreen use. After adjustment for sun exposure and host characteristics, the relative risk for a high naevus count on the trunk was 1.68 (95% confidence interval, 1.09–2.59) for the highest level of sunscreen use and 0.59 (95% confidence interval, 0.36–0.97) for the highest level of wearing clothes while in the sun. The average sun protection factor of the sunscreen used had no demonstrable effect on naevus counts. The highest risk of naevi on the trunk associated with sunscreen use was seen in children who never suffered from sunburn (relative risk, 2.21; 95% confidence interval, 1.33–3.67).

Conclusion
Few intermediate end-points related to the biology of tumour progression can be reliably used as biomarkers to assess chemoprevention of skin cancers. Assessment of sunburn cell formation or of p53 mutation induction in the epidermis provides information on the early steps of non-melanoma skin cancer. However, due to their invasive measurement, these biomarkers would be difficult to use in large-scale prevention trials. Actinic keratoses, precursors to SCC, share a number of
features with their malignant counterpart, including a dose-dependent link to cumulative sun exposure, and can be easily measured clinically. They have therefore been measured in large-scale trials in humans that have shown that a reduction of actinic keratoses can be obtained by use of sunscreens. The use of such an intermediate-effect biomarker in chemoprevention studies may be regarded as validated by a recent Australian trial showing a reduction in risk of SCC, but not of BCC, in a prospective controlled trial of sunscreen use in the prevention of non-melanoma skin cancer (Green et al., 1999).

Naevi and melanomas share a number of common features and it is thought that long-term prospective studies on melanoma occurrence could be replaced by prospective studies of naevus development. In this respect, the maintenance of a low number of naevi should be considered in the evaluation of the efficacy of protection against long-term harmful effects of sunlight exposure. However, the prevalence of common acquired naevi exceeds by far that of melanoma, and future studies should focus on more precise identification of subjects at risk, for example by considering markers of DNA damage susceptibility or of DNA repair proficiency such as the inherited susceptibility to induction of chromatic breaks by an UV-mimetic mutagen, 4-nitroquinoline-1-oxide (Wei et al., 1996; Wu et al., 1996). In public health, it is increasingly evident that a reduction in the incidence of melanoma will be preceded by a reduction in naevus numbers. The evolution of naevus number at different ages could serve as an indicator of the likely trend of melanoma incidence in the coming years.

References


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