Skin carcinomas in organ-transplant recipients: from early oncogenic events to therapy

Graaf, Y.G.L. de

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Leontien de Graaf

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Promotor:	Prof. dr. R. Willemze
Co-promotores:	Dr. J.N. Bouwes Bavinck
	Dr. F.R. de Gruijl
Referent:	Prof. dr. J.W. de Fijter
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CHAPTER 1

General Introduction

Skin cancer in organ-transplant recipients

In 1954, the first renal transplantation was performed in Boston.[1] With the introduction of the immunosuppressant azathioprine in the 1960s renal transplantation became a good alternative to dialysis. In the early 1980s cyclosporine was introduced and since then a handful new immunosuppressant agents were marketed. With more effective immunosuppression, long-term survival after organ transplantation has increased substantially. As a result, the number of patients with long-term complications of transplantation is also increasing. Skin cancers are the most common post-transplantation malignancies and account for substantial morbidity and mortality.[2-6] The largest group of organ-transplant recipients is formed by renal-transplant recipients. The problem of skin cancer is not limited to renal-transplant recipients, but is also eminent in recipients of other organs. This introductory chapter highlights the problem of skin cancer in organ-transplant recipients and will discuss a) risk factors and related mechanisms that are relevant to the development of skin cancer and b) the clinical management of organ-transplant recipients with skin carcinomas and multiple precursor and associated skin lesions.

The incidence of skin cancer in organ-transplant recipients increases with time after transplantation as well as with decreasing latitude (Figure 1).[7] In countries with temperate climates, such as The Netherlands, 40% of organ-transplant recipients have skin cancer 20 years after transplantation[3], compared to a percentage of 70% in subtropical countries like Australia.[7]

The most prevalent tumours in organ-transplant recipients are squamous-cell carcinomas that are predominantly located on sun-exposed areas (Figure 2).[3,8] Squamous-cell carcinomas occur 65 to 250 times more frequently than in the general population.[3,4,9] The incidence of squamous-cell carcinoma of the lip is also increased (15 to 20-fold).[9,10] The incidence of basal-cell carcinomas is increased by a factor 10 in transplant recipients.[3] This results in a reversed ratio of basal-cell to squamous-cell carcinomas in these patients compared with the general population.[2,7,11] Moreover, squamous-cell carcinomas appear to be more aggressive in organ-transplant recipients than in immunocompetent individuals. This is manifest in increases in local recurrences, regional and distant metastases and mortality.[12,13]

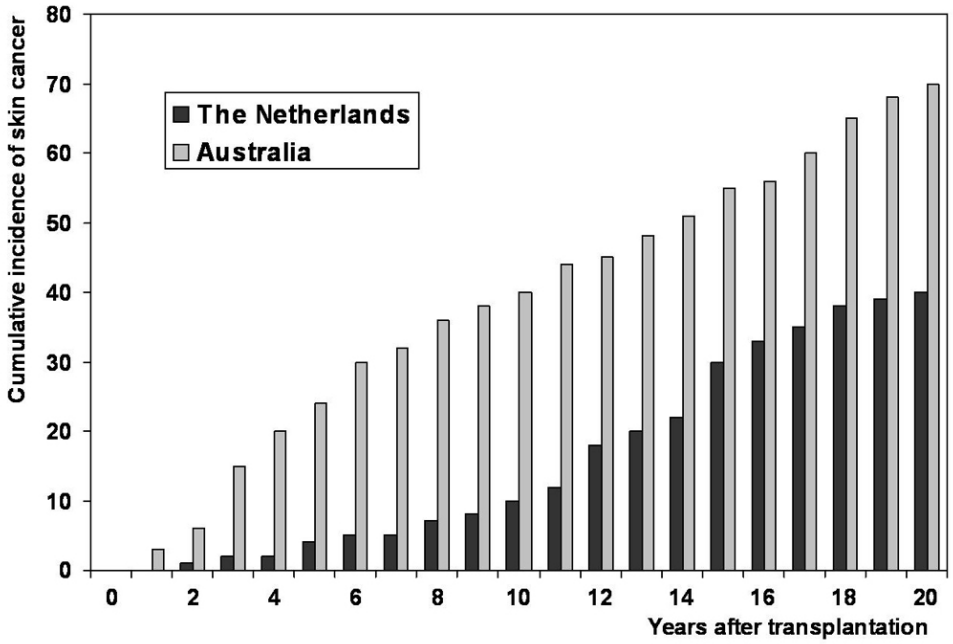

Figure 1. Cumulative incidence of skin cancer after transplantation in Queensland, Australia and Leiden, The Netherlands (modified from Bouwes Bavinck et al[7]).

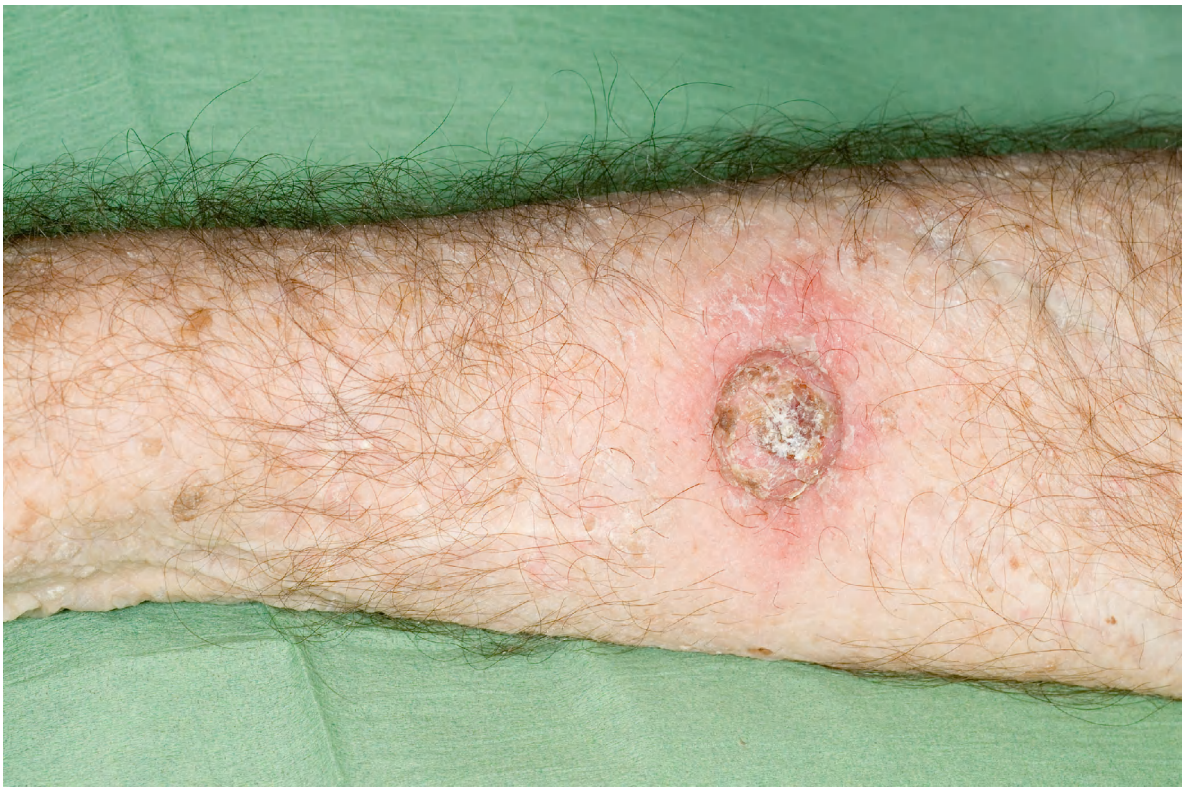

Figure 2. Squamous cell carcinoma in an organ-transplant recipient.

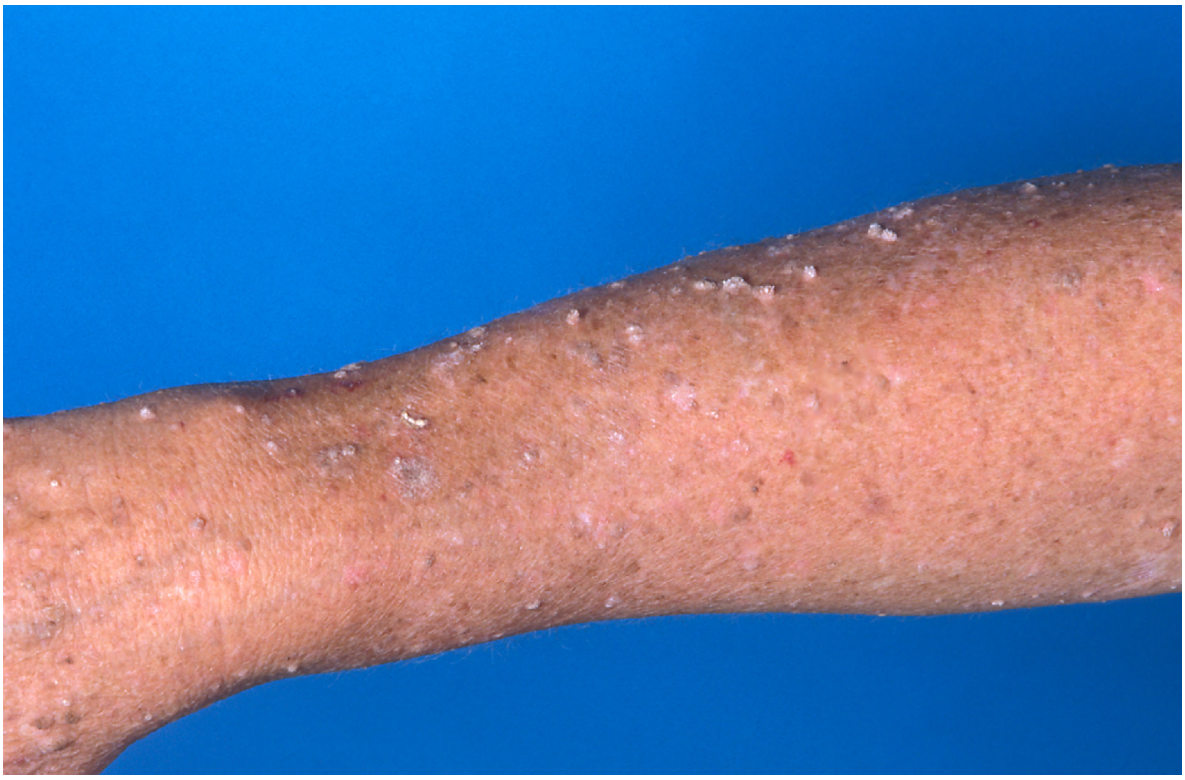

Figure 3. Hyperkeratotic lesions in an organ-transplant recipient

The risk of metastasis from squamous-cell carcinoma in these immunocompromized patients is estimated to be approximately 7%.[12] The regional lymph node has been reported to be the primary site of metastasis of squamous-cell carcinomas.[13] The presence of multiple skin cancers and localisation in the head and neck region is associated with an aggressive clinical course.[13,14] Other types of skin cancer of which the incidences are increased in organ-transplant recipients are Kaposi's sarcoma (84 to 113-fold) [6,9,15] melanoma (2 to 8-fold) [5,9,16] and Merkel cell carcinoma.[12]

In addition to the increased incidence of skin cancer, these patients develop numerous viral warts and actinic keratoses.[8,17] Compared to the general population, these lesions are more often resistant to therapy and frequently large areas are affected. In particular, the scalp and dorsal surfaces of the hands and forearms can show multiple confluent, hyperkeratotic lesions (Figure 3).[12]

Risk factors for skin carcinogenesis in organ-transplant recipients

The pathogenesis of skin cancer is multifactorial, with extrinsic and intrinsic risk factors (Figure 4). Sun exposure and prolonged immunosuppressive therapy have been recognized as the most important risk factors for skin cancer in organ-transplant recipients. In addition, human papillomaviruses might play a role in skin carcinogenesis. These topics will be discussed in more detail below.

Other risk factors for the development of skin cancer in organ-transplant recipients are gender, age, smoking, time after transplantation and the duration of pre-transplantation dialysis.[2,7,18] A fair complexion and an inability to tan are well-known genetic risk factors.[19,20]

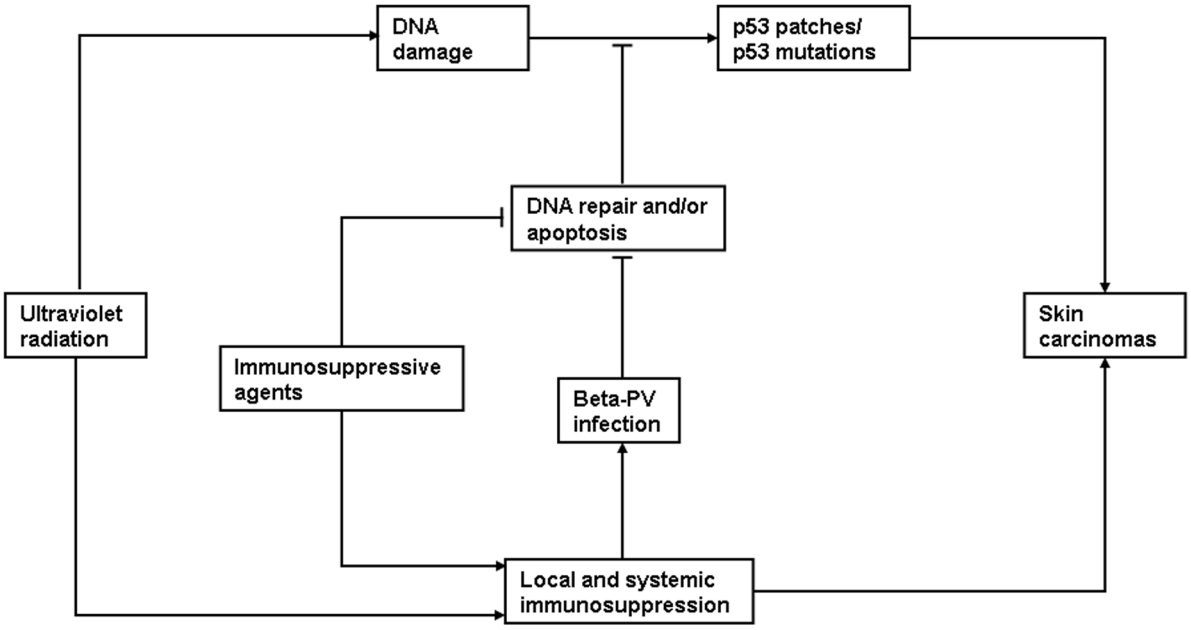

Figure 4. Hypothetical mechanisms of skin carcinogenesis in organ-transplant recipients

Ultraviolet radiation

The importance of exposure to sunlight as a risk factor is reflected by the fact that countries with high insolation have the highest incidence of skin cancer[7] and the tumours predominantly develop in sun-exposed areas.[8] It is assumed that the oncogenic properties of UV radiation are not only due to a direct mutagenic effect, but also to an immunosuppressive effect.

Mutagenic effect

Solar ultraviolet radiation at the Earth's surface, specifically the short wavelength UVB radiation, induces damage in DNA, mainly cyclobutane pyrimidine dimers and (6,4) photoproducts. If not adequately repaired, this damage gives rise to C→T and CC→TT transitions at dipyrimidine sites. These mutations are characteristic of UV radiation and are therefore called 'UV signature mutations'.[21] In the general (immunocompetent) population these mutations are found in the p53 tumour suppressor gene in the majority of squamous-cell carcinomas[22], and their precursor lesions, actinic keratoses.[23]

Upon chronic UV irradiation, clusters of epidermal cells occur that are readily immunohistochemically detectable by overexpression of the p53 protein. It has been suggested that that these clones expand at the expense of neighbouring keratinocytes owing to differential apoptotic responses under UV exposure.[24] Previous studies using microdissection showed that 30-70% of the p53 patches in human skin contained p53 gene mutations, of which the majority has the typical UV signature.[25-29]

P53 patches are found long before the appearance of skin carcinomas in the hairless mouse model.[30,31] As these p53 patches bear UV-specific mutations similar to those in the subsequent carcinomas, they appear to be early microscopic precursor lesions of the ultimate tumours.[32] This hypothesis is also supported by studies in human skin. An earlier study showed a significant dose-response relation between UV radiation and frequency of p53 patches.[26] Another study reported more p53 patches to be present adjacent to basal-cell carcinomas than adjacent to benign skin lesions[28], and again more adjacent to squamous-cell carcinomas than basal-cell carcinomas.[33]

However, it is not clear whether p53 patches are more prevalent in immunocompromized patients. Therefore we studied whether the number of p53 patches in uninvolved skin adjacent to carcinomas was increased in organ-transplant recipients when compared to immunocompetent patients. This study is described in **Chapter 2**.

Immunosuppressive effect

From classic animal experiments it is known that UV-induced skin cancers are antigenic and subject to elimination by the immune system. Subcarcinogenic doses of UV radiation can suppress the rejection and even induce specific tolerance toward the tumour.[34,35]

UV-induced immunosuppression is a highly complex process in which several different pathways are involved. UV radiation reduces the number and function of epidermal Langerhans cells, the major antigen presenting cells in the epidermis.[36] Next to DNA damage and oxidative damage[36], the formation of cis-urocanic acid by photoisomerisation of transurocanic acid can modify antigen presentation through ligation to serotonin receptors.[37] In addition, UV radiation stimulates keratinocytes, and subsequently leukocytes, to release immunosuppressive soluble mediators that affect antigen presentation, including interleukin 10, which enter the circulation and thereby also induce systemic immunosuppression.[38] Another important effect of UV radiation is the induction of regulatory T cells that appear to play a role in UV-induced tolerance.[38,39]

Immunosuppressive treatment

The lifelong immunosuppressive therapy of organ-transplant recipients usually consists of prednisone in combination with immunosuppressants such as azathioprine, cyclo-

sporine, mycophenolate mofetil, tacrolimus or more recently sirolimus (rapamycine). It is plausible that immunosuppressive drugs create a state in which immunosurveillance and eradication of malignant cells are impaired, facilitating carcinogenesis.[12] In addition, these immunosuppressive agents cause direct adverse effects on the skin cells (keratinocytes), that can increase the carcinoma risk. The classic immunosuppressives azathioprine (Imuran®) and cyclosporine (Neoral®) interfere with DNA repair.[40,41] It has been shown that azathioprine also enhances DNA damage by photosensitization; it is incorporated into the DNA as a thio-guanine pseudobase, which can sensitize the DNA to solar UVA radiation-induced damage.[42,43] In addition, it has been suggested that cyclosporine induces transforming growth factor β (TGF-β) production by tumour cells resulting in invasive growth.[44]

An experimental study from the late 1980s in which mice were treated with different immunosuppressive agents and exposed to UV radiation to induce skin tumours showed that azathioprine had the greatest effect on skin cancer development. Azathioprine increased the number of tumours per mouse and decreased the time to the first tumour, while cyclosporine decreased only the time to tumour induction to a minor extent.[45]

The clinical studies in which the role of the different treatments was studied concerning skin cancer risk are inconclusive. Some studies did not show a difference in skin cancer incidence between azathioprine and cyclosporine groups.[2,7] Other studies reported that patients receiving cyclosporine, azathioprine and prednisone had an increased risk of squamous-cell carcinoma compared with patients taking only prednisone and azathioprine.[9,46] Unfortunately, most of the clinical studies are retrospective and consist of large registry reports. Comparison of incidence rates is therefore difficult, because the patient populations (azathioprine vs. cyclosporine) are from different time periods. Moreover, the increased skin carcinoma risk in some of these studies may also be attributed to the immunosuppressive dosages, i.e., level of immune suppression, in combination with the duration of the treatment. In a randomized prospective study in which low-dose cyclosporine was compared with standard-dose cyclosporine, the low-dose regimen resulted in a significantly lower incidence of skin cancer.[47] This was consistent with another prospective study that also found an association with the overall cumulative immunosuppressive dose.[20]

More recent studies on newer drugs suggest that sirolimus, which has anti-tumour effects, confers a lower skin cancer risk compared with the classic immunosuppressive therapies.[48-50] However, this needs to be confirmed in carefully designed prospective randomized clinical trials, since skin cancers take years after transplantation to develop.

Beta-papillomaviruses

It has been shown that the development of skin cancer in organ-transplant recipients is strongly associated with the number of keratotic skin lesions, mainly viral warts and actinic keratoses.[8,17]

Numerous studies suggested that human papillomaviruses may be co-carcinogenic.[51-53] On the basis of their tropism human papillomaviruses may be classified as genital (mucosal) or cutaneous. Genital human papillomaviruses are subdivided into high- and low-risk virus types according to their association with malignancies and their in-vitro cell-transforming capacity. The cutaneous human papillomaviruses can be subdivided into the classical types associated with warts, such as verrucae vulgares and verrucae plantares, and the epidermodysplasia verruciformis types. The latter have recently been renamed as beta-papillomaviruses (beta-PV).[54]

Role of beta-PV in skin carcinogenesis

Infection with beta-PV occurs frequently and may persist for many years.[55] A wide diversity of beta-PV-types can be detected in both pre-malignant skin lesions and skin carcinomas.[17,56-59] Earlier studies provide indirect evidence that beta-PV may play a role in

skin cancer development either directly or in combination with sun exposure. The hair follicle is a possible reservoir for the beta-PV types. It has been shown that the prevalence of beta-PV-DNA in plucked eyebrow hairs is higher in immunocompetent individuals with a history of squamous-cell carcinoma than in controls.[51] Moreover, patients with a history of squamous-cell carcinoma are more likely than controls to have a sero-response against beta-PV.[52,53] The early viral protein E6 of some beta-PV types may impair the process of DNA repair or prevent apoptosis after exposure to UV radiation.[60-63] As a result, beta-PV infected, DNA-damaged cells may become genomically unstable, and survive. Such cells may ultimately give rise to actinic keratoses and squamous-cell carcinomas (Figure 5).[64] A recent study provided direct evidence for the carcinogenic potential of beta-PV by showing non-melanoma skin cancer development in HPV-8 transgenic mice without any treatment with physical or chemical carcinogens.[65]

It has been shown that beta-PV inhibit the apoptotic response to UV damage in-vitro. The aim of our study, described in **Chapter 4,** was to investigate whether apoptosis was decreased in the presence of beta-PV after an UVB challenge in human skin in-vivo.

Prevention and treatment options for skin cancer in organ-transplant recipients

The most important element of preventive management in organ-transplant recipients is patient education. All patients should receive information, before and after their transplantation, about the increased risk of skin cancer and the harmful effects of excessive sunlight exposure.[66] Furthermore, education on photoprotection, self-examination and the recognition of (pre)-malignant lesions is required. Monthly self-examination of skin as well as regular examination by physicians should be encouraged. Patients with pre-malignant skin lesions should be referred to a dermatologist in an early stage for intensive surveillance and active treatment of premalignant lesions and cancers.[12,67]

Prevention of skin cancer

Available studies have suggested a beneficial effect of systemic retinoids in chemoprevention of transplant-related skin cancers. Retinoids are structural and functional analogues of vitamin A that display a wide range of biological activity. Possible mechanisms by which they prevent or reduce skin cancer development include induction of apoptosis, normal differentiation of keratinocytes, and immunomodulation.[68,69] Organ-transplant recipients who may benefit from retinoid chemoprevention are those who are developing large numbers of skin cancers.[70,71] **Chapter 5** provides a review on the role of topical and systemic retinoids in the chemoprevention of skin cancer in organ-transplant recipients.

Another possible modality in the prevention of skin cancer is photodynamic therapy, which involves the use of a photosensitizing agent and a light system. Photodynamic therapy can be used to treat superficial skin carcinomas or precancerous lesions that are accessible to light.[72] It has been shown that photodynamic therapy is a safe and effective treatment for actinic keratoses in organ-transplant recipients.[73,74] In addition, earlier experimental studies showed that photodynamic therapy can delay the development of UV-induced skin carcinomas.[75,76] We studied this hypothesis in organ-transplant recipients in a randomized-controlled trial. This study is described in **Chapter 6**.

Obviously, aggressive treatment of pre-malignant lesions, such as actinic keratoses, is essential to minimize the progression to squamous-cell carcinoma. For this purpose, treatments such as cryotherapy, topical retinoids, 5-fluorouracil or the immune-response modifier imiquimod can be used. The same is true for actinic cheilitis because of the increased risk of high-risk squamous-cell carcinoma of the lip.[12]

Finally, reduction of immunosuppression is considered a reasonable adjuvant management strategy for organ-transplant recipients who develop numerous or life-threatening skin cancers.[77]

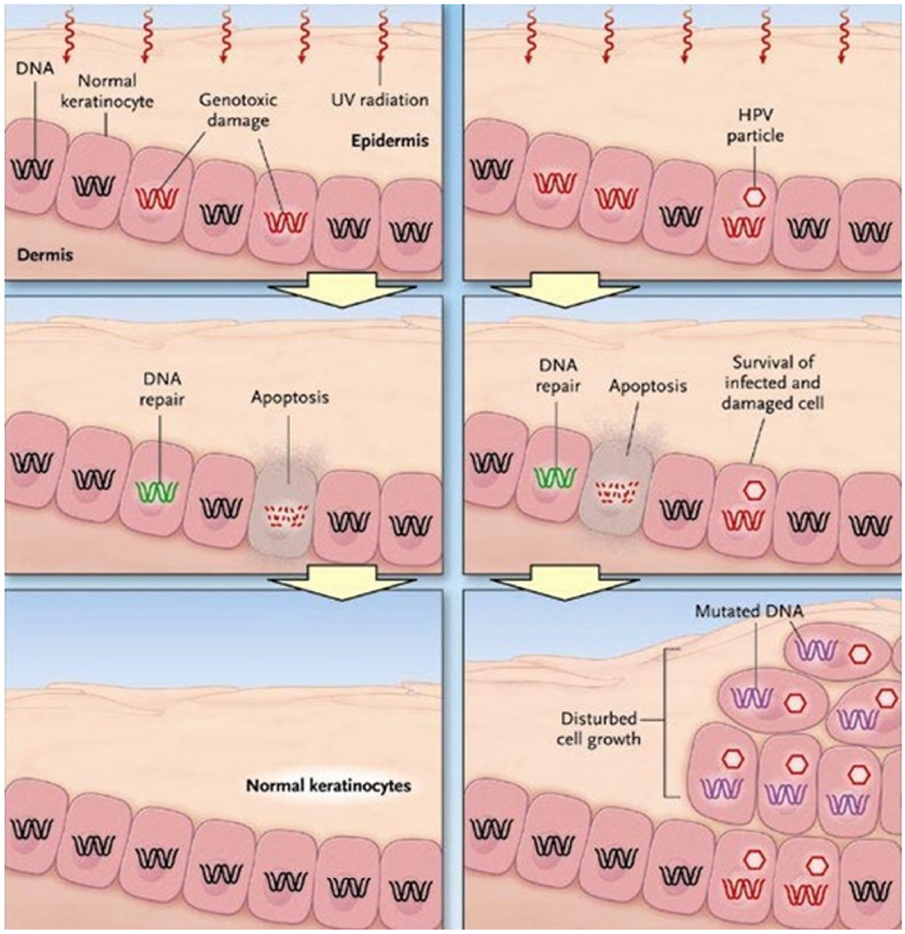

Figure 5. A proposed scheme for the development of actinic keratoses and cutaneous squamous-cell carcinoma (adapted from Bouwes Bavinck et al[64]).

Management of skin cancer

For squamous-cell carcinomas in organ-transplant recipients the treatment of choice is surgical excision with histological examination.[78] Resurfacing the dorsum of the hand can be a useful in selected patients. With this surgical procedure the tumour(s) and the actinically damaged skin are resected.[79] On high-risk tumours, Mohs' micrographic surgery can be performed.[80]

In selected tumours, curettage and electrodessication, a destructive modality, is an option, but there is not much evidence of its efficacy as a treatment of squamous-cell carcinomas in organ-transplant recipients in the literature. Only one case is described in which multiple squamous-cell carcinomas were successfully treated by curettage.[81] Nevertheless, curettage and electrodessication appears to be widely used in organ-transplant recipients, usually for superficial or early skin cancers.[82] Therefore, we evaluated the recurrence risk of squamous-cell carcinomas after treatment with curettage and electrodessication in organ-transplant recipients and compared the recurrence rates at different skin locations. This retrospective follow-up study is described in **Chapter 7**.

Aim and structure of this thesis

The aim of the studies presented in this thesis is broadly two fold:

i) identify early oncogenic events (such as high numbers of p53 patches and reduced apoptosis) that could explain

the high risk of skin carcinoma in organ-transplant recipients, and

ii) contribute to improved prevention and therapy of skin carcinomas in organ-transplant recipients.

Clearly, advancements under point i) can contribute to prevention and improved clinical intervention, mentioned under point ii).
Chapters 2 through 4 are related to point i), whereas the Chapters 5 through 7 are related to point ii).

Chapter 2 investigates whether the enhanced risk of skin carcinomas in organ-transplant recipients is reflected in increased p53 patches in their skin compared with immunocompetent patients. In addition, two possible mechanisms by which azathioprine might increase p53 patches were investigated: immunosuppression and impaired DNA repair.
Chapter 3 describes the prevalence of p53-specific serum antibodies in both renal-transplant recipients and immunocompetent individuals with and without a history of squamous-cell carcinoma.
Chapter 4 investigates whether beta-PV affect UV-induced apoptosis in unexposed skin of organ-transplant recipients and immunocompetent individuals and studies the effect of UVB exposure on beta-PV presence.
Chapter 5 provides a review on the efficacy of topical and systemic retinoids in the prevention of skin cancer in organ-transplant recipients.
Chapter 6 describes a randomized-controlled trial with paired observations in 40 organ-transplant recipients in which the effect of photodynamic therapy on the occurrence of new squamous-cell carcinomas on sun-exposed skin was assessed.
In **Chapter 7** a series of squamous-cell carcinomas from organ-transplant recipients that were treated with curettage and coagulation was studied, in order to assess the recurrence rate after this treatment and to compare the recurrence rates at different skin locations.
Chapter 8 summarizes and discusses the findings described in the preceding chapters. The results are compared with other, more recent studies. Furthermore, possibilities for future research are suggested.

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CHAPTER 2

More epidermal p53 patches adjacent to skin carcinomas in renal-transplant recipients than in immunocompetent patients: the role of azathioprine

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Original Article

More epidermal p53 patches adjacent to skin carcinomas in renal transplant recipients than in immunocompetent patients: the role of azathioprine

Ymke G. L. de Graaf[1], Heggert Rebel[1], Abdoel Elghalbzouri[1], Patricia Cramers[2], Ruud G. L. Nellen[1]*, Rein Willemze[1], Jan Nico Bouwes Bavinck[1] and Frank R. de Gruijl[1]

Departments of [1]Dermatology and [2]Toxicogenetics, Leiden University Medical Center, Leiden, The Netherlands
Correspondence: Y. G. L. de Graaf, MD, Department of Dermatology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands, Tel.: +31 71 5262638, Fax: +31 71 5248106, e-mail: y.g.l.de_graaf@lumc.nl
**Current address: Department of Dermatology, Academic Hospital Maastricht, Maastricht, The Netherlands.*

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Abstract: Immunosuppressive medication in renal transplant recipients (RTR) strongly increases the risk of cancers on sun-exposed skin. This increased risk was considered an inevitable collateral effect of immunosuppression, because UV-induced carcinomas in mice were found to be highly antigenic. Here, we posed the question whether immunosuppression also increases the frequency of p53-mutant foci ('p53 patches'), putative microscopic precursors of squamous cell carcinomas. As the majority of RTR was kept on azathioprine for most of the time, we investigated whether this drug could increase UV-induced p53 patches by immunosuppression. As azathioprine can impair UV-damaged DNA repair under certain conditions, we also investigated whether DNA repair was affected. Archive material of RTR and immunocompetent patients (ICP), as well as azathioprine-administered hairless mice were examined for p53 patches. DNA repair was investigated by ascertaining the effect of azathioprine on unscheduled DNA synthesis (UDS) in UV-irradiated human keratinocytes. P53 patches were more prevalent in RTR than in ICP in normal skin adjacent to carcinomas ($P = 0.02$), in spite of a lower mean age in the RTR (52 vs 63 years, $P = 0.001$), but we found no increase in UV-induced p53 patches in mice that were immunosuppressed by azathioprine. We found a significant reduction in DNA repair activity in keratinocytes treated with azathioprine ($P = 0.011$). UV-induced UDS in humans is dominated by repair of cyclobutane pyrimidine dimers, and these DNA lesions can lead to 'UV-signature' mutations in the *P53* gene, giving rise to p53 patches.

Key words: azathioprine – DNA repair – p53 patches – renal transplant recipients

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Introduction

Renal transplant recipients (RTR) are at an increased risk of developing skin cancer, of which squamous cell carcinomas (SCCs) are the most prevalent. These tumors develop primarily in areas exposed to the sun (1,2). The incidence of skin carcinomas in these patients increases with time after transplantation, reaching 40% in 20 years in the Netherlands (3) and in 10 years in Australia (4).

The pathogenesis of skin carcinoma is multifactorial. Solar ultraviolet radiation is recognized as a dominant etiological factor (5,6). Especially, the short wavelength (280–315 nm) UVB radiation induces DNA lesions, broad aspecific detection which by XL-PCR shows efficient repair at sub-lethal dosages in human keratinocytes, i.e. about 90% of the lesions are removed in 24 h (7). During chronic UV irradiation, clusters of epidermal cells develop that over-express the p53 protein in mutant conformation. These p53 foci or 'p53 patches' (p53-mutant clones) are detectable long before the appearance of skin carcinomas in the hairless mouse model (8), and are also found in human skin (9,10). P53 patches and SCCs show parallel UV dose-time dependencies in mice (11). As the p53 patches bear UV-specific mutations similar to those in the subsequent SCCs, they appear to be early microscopic precursor lesions of the ultimate tumors (12). Hence, the frequency of these patches can serve as a good marker of SCC risk (11,12).

Another important risk factor for skin cancer is immunosuppression. Classic animal experiments (13,14) have shown UV-induced skin tumors to be immunogenic, i.e. the tumors will be rejected upon transplantation into syngenic host, unless the host is immunosuppressed (UV radiation itself was found to induce a immunosuppressed and tumor-tolerant state). Suppression of tumor immunity

facilitates UV-induced skin carcinogenesis (15). Thus, the increased risk of skin carcinomas in RTR would appear to be an inevitable consequence of the immunosuppressive medication.

Most of the early RTR started off on the immunosuppressant azathioprine (supplemented with prednisone), and cyclosporine made its entry later on as an alternative immunosuppressant. Experiments showed that these immunosuppressants accelerated UV carcinogenesis in the hairless mouse model (16). Besides causing immunosuppression, these drugs were reported to impair DNA repair in the hairless mice (17). The repair in the epidermis was measured by unscheduled DNA synthesis (UDS). This raises the question of whether RTR could suffer from a medicinally induced DNA repair syndrome, which would make it a far more common syndrome than any hereditary syndrome of DNA instability/mutation. This in turn would make the RTR an especially interesting group of patients for basic studies to further our understanding of (skin) cancerogenesis (18).

Here, we first of all posed the question whether the enhanced risk of SCCs in RTR is reflected in increases in p53 patches in their skin. To this end, we investigated in archive material whether p53 patches were more prevalent in normal skin adjacent to skin carcinomas excised from RTR when compared with immunocompetent patients (ICP). After finding confirmative data, we pursued to identify the underlying mechanism.

As the archive material stemmed from patients who were predominantly and for the longest period of time kept on azathioprine, we focused on this immunosuppressant. We investigated two potential mechanism by which azathioprine could cause an increase in p53 patches: (i) immunosuppression, (ii) impaired DNA repair. We resorted to the hairless mouse model to assess experimentally the effect of an azathioprine-immunosuppressive regimen on the UV induction of p53 patches. In supplementation of earlier experiments in mice, we investigated the impact of azathioprine on the repair of UV-induced DNA damage in human primary keratinocytes. The results suggest that the local adverse effects of azathioprine on DNA repair in human skin increase the induction of p53 patches, and may thus – independently of immunosuppression – add to the risk of developing SCC in RTR.

Methods

Patients; selection of skin samples

Archived paraffin blocks from surgical excisions of skin carcinomas, of which the majority consisted of SCC, and the adjacent excision margins were obtained from both RTR and ICP. The adjacent skin margins had a minimal distance of 2 mm from the tumor mass. All skin samples were obtained from chronically sun-exposed sites (head, neck, dorsal surface of hands). Nineteen RTR and 13 ICP were randomly selected and included, matched for location of the tumor, and season of excision. Most skin samples were taken in autumn/winter.

Immunohistochemistry and scoring of p53 patches in human skin

P53 immune staining with DO-7 monoclonal antibody (M7001; Dakocytomation, Copenhagen, Denmark) was performed using standard procedures as described previously (10). Sections of skin tumors known to have strong p53 immunoreactivity with DO-7 were included as positive controls. Omission of the first antibody always yielded a negative result.

For description of the p53 immunoreactivity, criteria of Ren et al. were used (10). A p53 patch was defined as an uninterrupted cluster of at least 10 strongly and uniformly immunopositive nuclei in a sharply demarcated area of normal epidermis. Only these 'compact patterns' in the excision margins were scored, as this staining pattern was strongly associated with p53 mutations (19). The number of p53 patches per cm in the normal skin margins adjacent to carcinomas was determined in archive material from RTR and ICP. P53 patches were counted if there was no sign of connection to tumor in 10 successive sections. We also scored the size of the p53 patches in both groups.

Mice: UV irradiation and azathioprine treatment

Three groups of five hairless SKH-1 mice (Charles River, Maastricht, The Netherlands) entered the experiment at 9 weeks of age, under conditions as described earlier (11). Mice in the first group were both irradiated with UV and administered azathioprine, the second group was also irradiated, but received a placebo. Mice of the third group were not irradiated but did receive azathioprine. The procedure for UV irradiation and azathioprine administration was comparable to that described earlier (16) for the experiments on azathioprine-enhanced UV carcinogenesis. The mice were irradiated on working days: the first 2 weeks with 0.75 of the minimal erythemal dose (MED, 375 J/m^2 UV) per day, and in the third and fourth week with 1 MED (500 J/m^2 UV) per day from TL-12 lamps (Philips, Eindhoven, The Netherlands). Azathioprine (Pharmachemie, Haarlem, The Netherlands) was diluted in phosphate-buffered saline (PBS) at a concentration of 4 mg/ml. On Mondays, Wednesdays and Fridays, during the 4 weeks of irradiation, the mice were injected intra-peritoneally with an individual weight-corrected volume of the azathioprine solution resulting in 15 μg/g body weight. PBS injections served as placebo treatment. At 24 h after the final UV irradiation, all mice were killed by CO_2 asphyxiation. From

each mouse a defined rectangular dorsal part of the skin (2.9 × 1.9 cm) was dissected for preparation of epidermal sheets. Immunosuppression in the azathioprine-treated groups was confirmed by lymphocyte transformation tests on isolated splenocytes ($P = 0.034$ and 0.008 for the UV-exposed and unexposed groups, respectively, when compared with the control group that was not treated with azathioprine).

Immunohistochemistry and scoring of p53 patches in mouse skin

Preparation of epidermal sheets and immunostaining with the mutant-p53-specific PAb-240 antibody were described earlier (11). For scoring the p53 patches a grid, placed on top of each epidermal sheet preparation, was used to count p53 patches in 20 squares (total area 29.0 × 18.5 mm), using a light microscope equipped with a PI ×25/0.5 objective. A p53 patch was defined as a cluster of at least 10 Pab240-positive epidermal cells.

Unscheduled DNA synthesis in human keratinocytes

Primary cultures of normal human keratinocytes (PHKs) were established from skin derived from breast reduction according to earlier described procedures (20,21). PHKs were seeded in 10 cm diameter culture dishes at a density of $0.09 \times 10^6/cm^2$. PHKs from two different donors were used for two independent UDS tests.

The UDS test was performed according to van Zeeland et al. (22). At the first and third day of culture, ^{32}P was added to the medium to label the PHK DNA overall. At the sixth day the medium was replaced with fresh medium supplemented with a series of azathioprine concentrations. Two independent experiments were performed, with azathioprine concentrations of 0, 5, 25 and 100 μM in the first and 0, 10 and 50 μM in the second experiment. Per concentration two or three dishes of keratinocytes were tested.

At the seventh day the PHK cells were rinsed with PBS and irradiated with 300 J/m^2 from TL-12 lamps. Subsequently the media with or without azathioprine were returned on the cells and cultured for 6 h with ^3H-thymidine, after which the cells were harvested. ^3H uptake and ^{32}P were measured by differentiated scintillation counting of alkaline gradient fractions as described (22), and used for ratio calculations of UDS and total DNA, respectively.

Parallel cultures of human keratinocytes on glass cover slips were used for assessment of the vitality of the cells that were subjected to 0, 5, 25 and 100 μM azathioprine (two cover slips per concentration). After 7 days of culturing, slides were rinsed with PBS and stained with 0.15% trypan blue. Vital and non-vital cells were counted *in duplo* by light microscopy.

Statistical analyses

Because of high percentages of individuals without p53 patches and some RTR with exceptionally high numbers of p53 patches, the difference in the distributions of p53 patches among RTR and ICP (Fig. 2) was tested by chi-squared statistics (as differences in Kaplan–Meier curves, calculated by Graphpath Prism 3.0 software). For further statistical analyses, we used SPSS version 12.0.1 for Windows. The Student's *t*-test was used to ascertain significances of the differences in age and in UDS measurements. A log *t*-test was used to test the difference in number of p53 patches in the mice. To calculate the difference in sizes of p53 patches between RTR and ICP, the Mann–Whitney *U*-test was performed. The density of p53 patches in humans was related to other factors such as age and the period of time after transplantation and tested on significance in linear regression analyses.

Results

The baseline characteristics of the RTR and ICP are listed in Tables 1 and 2. At the time of excision, the RTR were significantly younger than the ICP, with a mean age of 52 and 63 years old, respectively ($P = 0.001$); difference with [95% CI]: 11 [5–18].

P53 patches in human skin

Figure 1 shows an example of a p53 patch in a part of the epidermis. The number of p53 patches per cm epidermis adjacent to skin carcinomas was significantly higher in RTR; median of 1.4 vs 0.3 patches/cm in RTR and ICP, respectively ($P = 0.02$). Figure 2 shows a clear difference between the groups in the distributions of p53 patches, with 20% ($n = 4$) of the RTR and none of the ICP with more than 3 patches/cm. The sizes of the patches did not differ between the RTR and ICP: in both groups predominantly small patches (10–50 cells) were found (data not shown).

The number of patches was not associated with age, gender or season in either group or both groups combined. Additionally, no association was found between the number of p53 patches and the time since transplantation.

The majority of the RTR (16/19) used azathioprine, but exclusion of the three patients that used cyclosporine and/or mycophenolate mofetil did not alter the results.

UV-induced p53 patches in azathioprine-immunosuppressed mice

A slight erythema was found in some mice after increasing the daily UV dose after 2 weeks, but most of the mice showed no apparent sunburn skin reaction, both in the azathioprine-treated and non-treated groups.

Table 1. Characteristics of renal transplant recipients ranked according to p53 patches/cm epidermis

Patient no.	Age (years)	Time after TX (years)	Sex	Type of medication	Type of skin cancer	Location of skin cancer	Season	P53 patches/cm epidermis
1	53	10	M	P+A	SCC	Neck	Spring	9.0
2	54	17	F	P+A	BCC	Dorsum of forearm	Autumn	6.7
3	60	21	M	P+A	SCC	Nose	Winter	4.6
4	55	11	F	P+C	SCC	Dorsum of hand	Autumn	4.2
5	48	19	F	P+A	SCC	Dorsum of finger	Autumn	2.5
6	54	18	F	P+A	SCC	Cheek	Winter	2.1
7	41	9	M	P+A	SCC	Shoulder	Autumn	2.0
8	43	16	F	P+A	SCC	Dorsum of forearm	Summer	1.9
9	46	24	F	P+A	SCC	Dorsum of forearm	Spring	1.7
10	51	22	F	P+A	SCC	Dorsum of hand	Winter	1.4
11	55	20	F	P+A	SCC	Dorsum of finger	Winter	0.9
12	49	7	M	P+C+M	SCC	Forehead	Autumn	0.8
13	55	23	M	P+A	SCC	Dorsum of hand	Summer	0.7
14	37	9	F	P+C	SCC	Dorsum of hand	Winter	0.1
15	69	25	M	P+A	SCC	Forehead	Summer	0.0
16	45	25	M	P+A	SCC	Dorsum of hand	Winter	0.0
17	64	17	M	P+A	SCC	Forehead	Winter	0.0
18	61	27	F	P+A	SCC	Cheek	Winter	0.0
19	49	18	F	P+A	SCC	Dorsum of forearm	Autumn	0.0

M, male; F, female; P, prednisone; A, azathioprine; C, cyclosporine; M, mycophenolate mofetil; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; TX, transplantation.

Table 2. Characteristics of immunocompetent patients ranked according to p53 patches/cm epidermis

Patient no.	Age (years)	Sex	Type of skin cancer	Location of skin cancer	Season	P53 patches/cm epidermis
1	77	F	SCC	Dorsum of hand	Autumn	2.2
2	56	M	SCC	Dorsum of forearm	Winter	1.6
3	49	M	SCC	Scalp	Winter	1.1
4	66	M	SCC	Forehead	Spring	0.9
5	69	F	SCC	Dorsum of hand	Summer	0.8
6	72	F	SCC	Nose	Autumn	0.7
7	64	M	SCC	Cheek	Spring	0.3
8	59	M	SCC	Forehead	Winter	0.0
9	69	F	SCC	Upper arm	Spring	0.0
10	69	M	SCC	Forehead	Winter	0.0
11	72	M	SCC	Face	Summer	0.0
12	34	M	SCC	Neck	Summer	0.0
13	68	F	SCC	Cheek	Winter	0.0

M, male; F, female; BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

No p53 patches were detected in the epidermal sheets from the azathioprine-treated control mice that were not UV irradiated. Clusters of epidermal cells with p53-positive nuclei (example in Fig. 3) were found in the two groups that were UV exposed for 4 weeks. The median numbers of the p53 patches per epidermal sheet were not significantly different between the two groups of UVB-irradiated mice. The median numbers of p53 patches [95% CI] in the azathioprine-treated mice and non-treated mice were 11 [7–17] and 24 [13–46], respectively ($P = 0.11$).

UDS in human keratinocytes

To investigate the effect of azathioprine on primary human keratinocytes (PHKs), we assessed the DNA repair by UDS assay, 6 h after UV irradiation. At the day of radiation the cultures were 90–100% confluent. Overall, UDS was

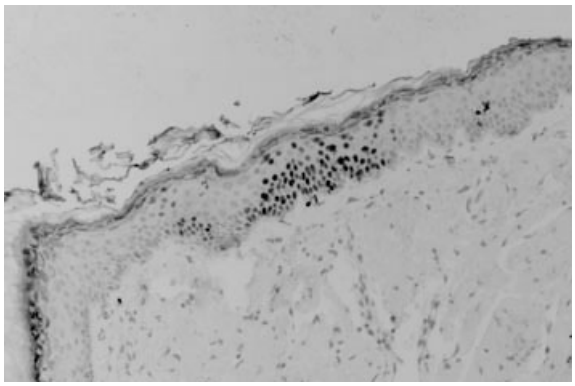

Figure 1. p53 patch in 5 μm section of human epidermis from a renal transplant recipient.

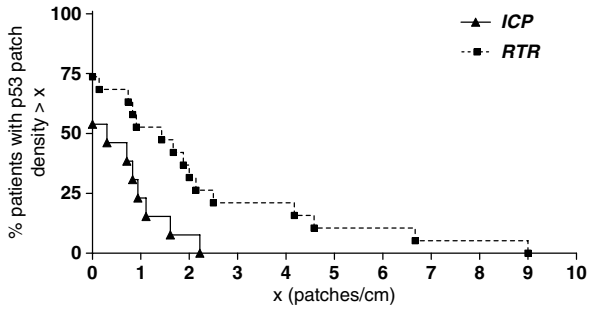

Figure 2. Plot of the percentages of patients (on ordinate) with densities of p53 patches greater than a density *X* (on abscissa); a significant difference ($P = 0.02$, chi-squared test) between the distributions from a renal transplant recipient (RTR; dashed line) and a immunocompetent patient (ICP; solid line).

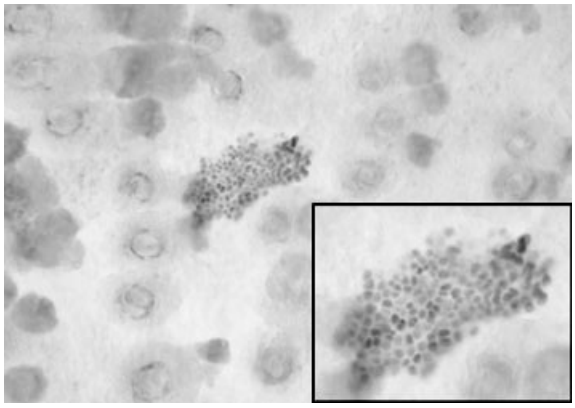

Figure 3. p53 patch in murine epidermal sheet; bottom view (circular structures are hair follicles), insert with detail of compact cluster of cells with p53-positive nuclei.

significantly inhibited when azathioprine (5–100 μM) was present in the medium in comparison with controls (0 μM, $P = 0.011$), with an apparent maximum inhibition around

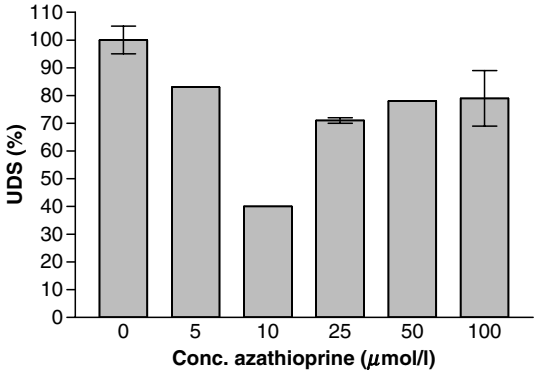

Figure 4. Effect of azathioprine on unscheduled DNA synthesis (UDS) in human keratinocytes (whiskers represent SE).

10 μM (Fig. 4). The trypan blue test revealed >95% vitality at several tested azathioprine concentrations (5, 25 and 100 μM).

Discussion

In the present study we found significantly more p53 patches in uninvolved skin neighboring carcinomas of RTR than of ICP. In contrast to this observation in humans, we found no increase in p53 patches in chronically UV-exposed mice that were treated with azathioprine. As we confirmed the immunosuppression of azathioprine in these mice, we conclude that p53 clones do not appear to be immunoreactive. This finding is in line with the results of Remenyik et al. (23) who found no differences in the induction and regression of p53 clones between immunosuppressed RAG-1 knockout and wild-type mice. Although azathioprine did not increase the number of p53 patches in our mice, it did increase the number of UV-induced skin tumors in mice in earlier experiments (16). Hence, azathioprine-induced immunosuppression did appear to affect the ultimate development of SCC. Thus the p53 patch increase in RTR and not in mice on azathioprine is not likely to be related to immunosuppression, but by another action of azathioprine.

Impairment of DNA repair is another effect of azathioprine and we found a diminished repair of UV-induced DNA damage in PHKs that were treated with azathioprine. This is in line with earlier published results of UV-induced DNA repair inhibition by azathioprine in peripheral blood mononuclear cells (24). We did not find a simple monotonous increase in inhibition with increasing azathioprine concentration, but a maximum inhibition around 10 μM. The inhibition is most likely specific inhibition, as no toxicity was measured up to 100 μM. Based on dosages per kg body weight and by the metabolites (6-mercaptopurin and 6-thiouric acid) in circulation, we estimated about 7.0 μM

azathioprine present in a patient (25), i.e. within the range that was tested in our experiments.

Unscheduled DNA synthesis is impaired by azathioprine in both mice and men (17,24); thus one would expect an increase in p53 mutations and patch formation as observed in men. However, this increase is not clear in the well-controlled experiment in mice. A plausible explanation is the difference in DNA repair between mouse and man: the cyclobutane pyrimidine dimer is the dominant carcinogenic DNA damage (26), which can cause the typical 'UV-signature' mutations in the *P53* gene (27), and this damage is poorly repaired in mice and very well repaired in men (28–30). Consequently, DNA repair impairment is likely to have more of an impact in men than in mice. The presence of p53 patches in human skin may, therefore, be attributable to a local effect on DNA repair in human keratinocytes rather than to a systemic impairment of immune surveillance and elimination.

Next to an impairment of DNA repair, azathioprine can introduce a UVA phototoxicity from thio-guanines incorporated in DNA (31,32). However, the mutation spectrum of *P53* in carcinomas from early cohorts of RTR showed no apparent deviation from the expected UVB-related point mutations normally found in ICP (33). This result provides evidence for an enhanced UVB-related mutation rate from lowered DNA repair in RTR rather than for additional mutations from UVA sensitization. The number of mutations (12) may however been too small to pick up an added effect from UVA sensitization.

In general, p53 patches can serve as a marker of skin carcinoma risk in humans. Next to the evidence from animal experiments, a significant dose–response relationship between UV exposure and frequency of p53 clones in human skin was also reported (9). Additionally, Backvall et al. found significantly more p53 clones adjacent to SCC than to basal cell carcinomas and melanocytic nevi (34). Another study reported significantly more p53 patches adjacent to basal cell carcinomas compared with benign skin lesions (35).

Earlier experimental and some clinical studies have shown that the frequency of p53 patches increases with age (11,36,37). In contrast, Jonason et al. did not find such an association (9). In the present study we did not find an association between age and number of patches either. This may, however, be due to the relatively small number of patients that was included.

Female patients appeared somewhat overrepresented among the RTR when compared with the ICP, but overall, the number of patches showed no dependence on gender. On close scrutiny, there appears to be a difference between both groups regarding location of the tumors. In the RTR the dorsum of the fingers and hands, or forearms occurs more frequent than in the ICP. Although there probably is no difference in the level of sun exposure, it cannot be entirely excluded that this site difference introduced a bias in p53 patches; a larger study with substratification to tumor site would be required.

In conclusion, in our archive material we found a higher density of p53 patches in RTR than in ICP. P53 patches in mice were not subject to immune detection and elimination (23). However, we found that azathioprine lowered DNA repair in human keratinocytes, and may thus increase mutagenesis in the *P53* gene, and consequently the development of p53 patches in human. Whether, aside from the systemic immunosuppression, this impact of azathioprine on the keratinocytes in the skin can ultimately increase the risk of skin carcinomas in RTR requires further (*in vivo*) experiments.

Because p53 patches represent an early step in skin carcinogenesis, the impact of novel immunosuppressive agents, such as sirolimus, on DNA repair and the formation of p53 patches in human should also be studied to ascertain whether these novel agents lack this additional risk.

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CHAPTER 3

P53-specific serum antibodies are not associated with a history of skin carcinoma in renal-transplant recipients and immunocompetent individuals

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LETTER TO THE EDITOR

p53-Specific serum antibodies are not associated with a history of skin carcinoma in renal transplant recipients and immunocompetent individuals

KEYWORDS
p53-Specific antibodies;
Tumor marker;
Skin cancer

Alteration of the p53 tumor suppressor gene is the most frequent genetic event found in human cancers [1]. A recent review reported a correlation between the presence of a p53-specific antibody response and p53 mutations in the tumors [1]. Titers of p53-specific antibodies have shown to be prognostic markers of lowered survival in patients with breast and colorectal cancers and may serve to detect lung cancer in early stages [1].

A high rate of p53 mutations is observed in skin carcinomas: in more than 90% of the squamous cell carcinomas (SCC) and in 75—80% of the precursor lesions, solar keratoses (SK) [2]. Also, in renal transplant recipients a large portion (48%) of the skin carcinomas contains p53 mutations [3].

The relevance of p53-specific antibodies to skin cancer is unclear [4]. To our knowledge, only one study reported on p53-specific antibodies in patients with skin carcinomas. Moch et al. [5] found a low prevalence of p53-specific serum antibodies in 105 immunocompetent patients with nonmelanoma skin cancer. Eight per cent (2/25) of the SCC patients and 1.5% (1/68) of the basal cell carcinoma (BCC) patients showed a p53-specific antibody response [5].

Renal transplant recipients run an increased risk of developing cutaneous SCCs compared to immunocompetent patients [6]. In the general population the ratio BCC over SCC is approximately 5:1, this ratio is reversed in renal transplant recipients [6]. Furthermore, SCCs in renal transplant recipients tend to be more aggressive. Therefore, it is important to detect skin cancer in an early stage in this specific population [6].

To determine whether p53-specific serum antibodies could be a relevant marker for the development of skin carcinomas in renal transplant recipients, we resorted to assess p53-specific antibodies in available archived sera (stored at −80 °C) from a well-documented bank collected from renal transplant recipients and immunocompetent subjects with and without a history of skin carcinoma. Data about age, sex and tumors after transplantation were collected. All renal transplant recipients were treated with prednisone and azathioprine [7]. The medical ethical committee of the LUMC approved the study.

In total, sera from 157 patients of the Leiden University Medical Center (LUMC) were studied: thirty-four renal transplant recipients with a history of one or more skin carcinomas, and 43 without skin carcinoma (collected in 1988) [7]. Among the patients who had a carcinoma, 17 individuals had a history of SCC, 7 of BCC, and 10 patients had a history of both tumors. As controls, sera of 80 immunocompetent individuals were randomly selected from the Leiden Skin Cancer Study [8] (collected in 1998). Among them, 39 individuals had a history of SCC and 41 had no skin cancer. None of the individuals displayed lymph node metastases or were known to have a history of other types of cancer (Table 1).

p53-Specific serum antibodies were detected using a quantitated enzyme-linked immunoabsorbent assay kit (anti-p53 ELISA, PharmaCell, France). The threshold value for presence of antibodies was set at 1.15 U/ml following the instructions of the manufacturer.

The mean age of the immunocompetent individuals was 61 years, while the mean age of renal transplant recipients was 47 years ($p < 0.01$; two-tailed Student's t-test). The mean period after transplantation was 13 years in the individuals with a history of skin carcinoma and 12 years in the skin cancer free group ($p = 0.32$).

Fig. 1 depicts levels of p53-specific antibodies in the renal transplant recipients and immunocompe-

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Table 1 Description of the patient groups according to the presence of p53-specific antibodies

	SCC only	BCC only	Both SCC and BCC	No skin cancer
No. of renal transplant recipients	17	7	10	43
p53 pos (no.)	1	2	0	2
p53 neg (no.)	16	5	10	41
No. of immunocompetent patients	28	0	11	41
p53 pos (no.)	1	—	1	2
p53 neg (no.)	27	—	10	39

SCC = squamous cell carcinoma; BCC = basal cell carcinoma.

tent patients. The distributions in these two groups were not discernibly different for SCC only, BCC only, or the combination of SCC and BCC (Table 1). The skin cancer groups were, therefore, combined in the statistical analyses.

In 6.5% (5/77) of the renal transplant recipients and 5.0% (4/80) of the immunocompetent patients p53-specific antibodies were present ($p = 0.69$). Altogether, 8.8% (3/34) of the renal transplant recipients with a history of skin carcinoma and 5.1% (2/39) of the immunocompetent patients with a history of skin carcinoma showed p53-specific antibodies ($p = 0.53$). One renal transplant recipient and one immunocompetent individual without skin cancer showed highly elevated levels of p53-specific antibodies: 16.4 and 18.7 U/ml, respectively. No obvious clinical reasons were found for these elevated serum levels. In the analyses the outliers were excluded, but inclusion of these outliers did not alter the outcome. Statistical analyses of the p53-specific serum antibody levels revealed that there were no significant differences in the mean p53-specific antibody levels between the groups. There were no significant age or sex differences between patients with and without p53-specific antibodies either.

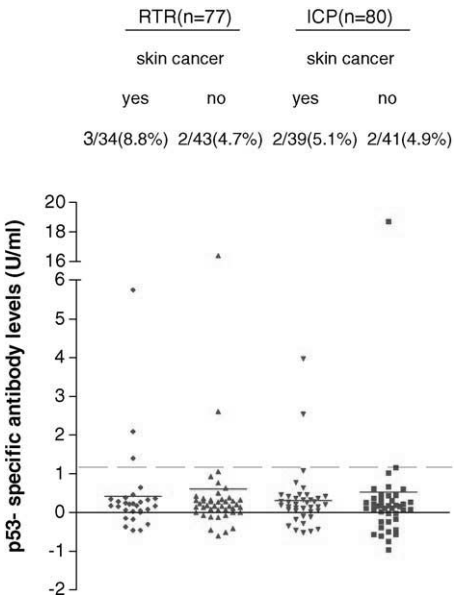

Fig. 1 Presence of p53-specific antibodies in the different patient groups. *RTR = renal transplant recipients; ICP = immunocompetent patients; the dashed line represents the cut-off value for the presence of antibodies (=1.15 U/ml); the small horizontal lines represent the mean value after exclusion of the two outliers.

Our results show that renal transplant recipients with a history of skin carcinoma and commonly carrying SK rarely display circulating p53-specific IgG antibodies. Despite the limitations of our study on archival sera sampled at various times after removal of skin carcinomas, we infer that there is no indication that circulating p53 antibodies are a useful prognostic marker of skin carcinoma risk in renal transplant recipients (nor in immunocompetent individuals).

Strikingly, 15% (10/66) of a cohort of Japanese renal transplant recipients not bearing any skin carcinomas were reported sero-positive for p53 [9]. Although we used sera of 157 patients, we did not find any indication of elevated titers of p53 antibodies [9]. Because skin carcinomas are extremely rare in Japanese renal transplant recipients [6], these patients cannot simply be compared with their Caucasian counterparts. Interestingly, the sero-positivity in this group of Japanese patients was only found among those who were treated with cyclosporine ($n = 56$), not in those taking only prednisone ($n = 10$) [9]. Cyclosporine treatment can result in p53 accumulation [10], which may well explain the Japanese results. None of the transplant recipients tested in our study was treated with cyclosporine, because they were transplanted before the cyclosporine era.

Likely explanations of the low humoral immunization against p53 in our study could be the low skin tumor mass and lack of necrosis and inflammation, in contrast to what is usually found in colon cancers.

In sum, we conclude that there is no indication of an elevated humoral response against p53 in people treated for skin carcinoma, irrespective of whether these people were on immunosuppressive medication or not.

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Ymke G.L. de Graaf*
Bert Jan Vermeer
Jan Nico Bouwes Bavinck
Rein Willemze
Frank R. de Gruijl
Department of Dermatology, Leiden University Medical Center, Albinusdreef 2, 2300 RC, Leiden The Netherlands

Daniel Schiefer
Anke Redeker
Sjoerd H. Van der Burg
Department of Immunohematology and Blood Transfusion, Leiden University Medical Center Albinusdreef 2, 2300 RC, Leiden, The Netherlands

*Corresponding author. Tel.: +31 71 5262638
fax: +31 71 5248106
E-mail address: y.g.l.de_graaf@lumc.nl

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CHAPTER 4

UV-induced apoptosis is not diminished in the presence of beta-papillomaviruses in habitually unexposed skin, but does decrease with age

Submitted

UV-induced apoptosis is not diminished in the presence of beta-papillomaviruses in habitually unexposed skin, but does decrease with age.

Y.G.L. de Graaf, M.N.C. de Koning,*† W.H. Zoutman, S.J. Weissenborn,‡ H. Pfister,‡ L. Struijk,* M.C.W. Feltkamp,* J. ter Schegget,*† R. Willemze, J. N. Bouwes Bavinck and F.R. de Gruijl.

Departments of Dermatology and *Medical Microbiology, Leiden University Medical Center, Leiden, and †DDL Diagnostic Laboratory, Voorburg, The Netherlands; ‡Institute of Virology, University of Cologne, Cologne, Germany.

Summary

Background Beta-papillomaviruses (beta-PV) may play a role in early skin cancer development either alone or in combination with UV exposure. Earlier in-vitro studies showed that beta-PVs decreased UV-induced apoptosis.

Objectives To investigate whether beta-PVs decrease UV-induced apoptosis in-vivo and whether UV exposure affects the presence and quantity of beta-PVs.

Methods Thirty organ-transplant recipients (OTR) and 30 immunocompetent individuals (ICI) were exposed to 3 minimal erythema doses (MED) UVB irradiation on unexposed buttock skin. Biopsies were taken from both irradiated and non-irradiated skin. Beta-PV types were detected and identified by PCR and reverse hybridization and quantified by q-PCR. Apoptotic cells were stained with anti-active caspase 3.

Results Beta-PV DNA was detected in 47% of non-irradiated and 53% of UVB-irradiated skin samples from OTR, and in 27% of both non-irradiated and UVB-irradiated skin samples from ICI. The quantity of beta-PV was generally below 1 copy per 1000 cells. There was no effect of a single UV exposure on the detected presence of beta-PV. We did not detect an association between beta-PV presence and UV-induced apoptosis in both groups studied. We did, however, find in both OTR and ICI a decrease in UV-induced apoptosis with age, which was not linked to any significant increase in beta-PV with age.

Conclusions In line with low copy numbers of beta-PV, we did not detect any overall decrease in UV-induced apoptosis in beta-PV positive skin samples, nor any immediate effect of UV irradiation on the beta-PV types detected. In both OTR and ICI, the number of UV-induced apoptotic cells decreased with age.

Introduction

The majority of organ-transplant recipients (OTR) develop multiple skin cancers, especially squamous-cell carcinomas, on sun-exposed skin.[1] In transplant recipients the development of skin cancer is strongly associated with the number of keratotic skin lesions, consisting of viral warts and actinic keratoses.[2;3]

Recently, epidermodysplasia verruci-formis (EV)-related human papillomaviruses were re-classified into beta-papillomaviruses (beta-PV). Infection with beta-PV occurs frequently and may persist for many years.[4] A wide diversity of beta-PV-types can be detected in both pre-malignant skin lesions and skin carcinomas.[2;5-7] Beta-PV-DNA has been detected in up to 90% of squamous-cell carcinomas of OTR.[8] In immunocompetent individuals (ICI) detection rate of beta-PV-DNA in skin carcinomas is lower, namely between 30 and 50%.[8;9] The presence of beta-PV-DNA in plucked eyebrow hairs of ICI is associated with a history of squamous-cell carcinoma.[10] Sero-epidemiological studies also showed an association between sero-response against beta-PV and skin cancer risk.[11;12] The presence of antibodies against HPV8 was particularly associated with the development of squamous-cell carcinomas[11;13-15] and actinic keratoses.[13] All

these findings together provide indirect evidence that beta-PV may play a role in skin cancer development in organ-transplant recipients and immunocompetent individuals either directly or in combination with ultraviolet exposure.[16;17]

Ultraviolet B (UVB) exposure has been recognized as the most important etiological factor in the development of skin cancer. UVB irradiation causes the induction of somatic mutations through the formation of pyrimidine dimers and 6-4 photoproducts. The failure to repair this DNA damage or to remove severely damaged cells by apoptosis may ultimately lead to skin cancer.[18-20]

In-vitro, beta-PV have been reported to hamper DNA repair.[21;22] Recently, it also has been shown that beta-PV E6 proteins inhibit apoptosis in-vitro in response to UV damage.[23;24] The impact on apoptosis has not been studied in-vivo. The aim of the present study was to investigate whether apoptosis was decreased in the presence of beta-PV after an UVB challenge in human skin in-vivo. Furthermore, we investigated whether the presence and diversity of beta-PV in previously non-sunexposed skin were modified by a single UVB exposure. For this purpose we used a novel, highly sensitive beta-PV detection technique.[25]

Material and methods

Subjects OTR who were regularly seen at the Department of Dermatology from the Leiden University Medical Center (LUMC) in the period between January 2003 and January 2005 were eligible for the study. To study the impact of beta-PV separately in immunocompetent individuals, volunteers were recruited from the LUMC and the University of Leiden through advertisements. Consequentially, the groups were not matched, but studied independently for the effects of beta-PV. Inclusion criteria among OTR were a functioning graft of 5 years or longer; for both patients and controls inclusion criteria were an age of 18 years or older; skin type I through III and sun-unexposed buttock skin, without pre-existent solar damage. ICI with systemic diseases were excluded. The medical ethical committee of the LUMC approved the study and all participants provided written informed consent. In total, 30 organ-transplant recipients and 30 immuno-competent individuals were included in the study.

MED testing and UVB irradiation For the determination of the minimal erythema dose (MED), which is defined as the smallest dose of UV radiation to result in just detectable erythema, a phototesting device designed by Diffey *et al*. was used.[26;27] The device is made of two flexible metal foils, containing 10 ports with mesh attenuators giving 10 irradiance levels with an 8 : 1 ratio from the highest to the lowest exposure. The device was slightly modified to maintain a constant distance of 3 mm to the skin.

At the day-1 visit, the MED test was performed on the previously non-sunexposed buttock skin of each individual. The phototesting device was placed on the skin and irradiated with broadband UVB TL-12 tubes (Philips, Eindhoven, The Netherlands) for 4 minutes, giving an irradiance ranging from 30 to 240 mJ cm^{-2} UV. The MED was assessed visually 24 hours later. No difference between the MED values of the OTR and the ICI was observed. The average MED values [95% CI] were 125 [117-133] and 135 [120-150] mJ cm^{-2}, respectively. Subsequently, an area of 1.5 cm^2 of the opposite unexposed buttock skin was exposed to 3 MED UVB irradiation. On the third day, 24 hours after irradiation, one 4-mm punch biopsy was taken from the 3-MED area, together with a control biopsy from adjacent (a maximum distance of 1 cm) non-irradiated buttock skin of OTR and ICI. All biopsies were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), snap frozen in liquid nitrogen and stored at −80 °C.

To investigate chronically sunexposed skin, we also took paired biopsies of UV-irradiated and non-irradiated dorsal forearm skin. MED determination was, however,

very difficult in this heavily sun-damaged skin with high numbers of solar keratoses and other hyperkeratotic skin lesions. The MED readings were considered very unreliable. Therefore, these forearm specimens were not suitable for comparative measurements of apoptosis. After testing 9 OTR on the forearm, this part of the experiment was discontinued, and only the presence of beta-PV types was ascertained in the acquired forearm skin biopsies.

Processing of the samples For all samples, first 5 µm cryosections for immuno-histochemistry were cut. Six serial cryosections were used for DNA isolation. DNA was extracted for both the PM-PCR RHA method (see below) and the quantitative PCR using the Genomic Tip kit (Qiagen, Hilden, Germany).
To prevent contamination, after each sample strict cleaning of the blade was performed. Furthermore, a control (blank) sample, consisting solely of Tissue-Tec, was cut after each pair of biopsies of one individual. All control samples were processed identically to the skin samples. During analysis seven out of 60 blank control samples were beta-PV-positive, each for a different type of beta-PV. Since these types showed no correspondence with the ones found in samples cut directly preceding or following the control sample, they were considered false positives from minor random contamination. An airborn infection cannot be excluded and appears to be the most likely explanation. This false positive rate constituted a low background noise, which was substantially and significantly lower than the positive rates in the skin samples of both OTR and ICI ($p<0.001$).

The PM-PCR RHA method and quantitative PCR Beta-papillomavirus detection and genotyping was carried out with the PM PCR Reverse Hybridization Assay (PM-PCR RHA) method (Skin (beta) HPV prototype research assay; Diassay BV, The Netherlands). We have applied strict guidelines adapted from Kwok et al.[28] to prevent contamination. The method was designed for the identification of 25 established beta-PV types, namely beta-PV genotypes 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, cand92, 93 and cand96. This method was described in detail earlier.[25] In short, a broad-spectrum consensus PCR primer set was used for amplification of a 117 bp fragment from the beta-PV E1 gene. Generated amplimers were subsequently analysed in the RHA on nitrocellulose membrane strips containing oligo nucleotide probes for 25 beta-PV types. The RHA includes several hybridisation and (stringent) washing steps followed by an enzymatic colouring reaction that visualises the presence of biotinylated amplimer bound to the probes. The analytical sensitivity of the PM-PCR RHA method was reported to be 10 to 100 viral genomes. Intra- and interlaboratory variability experiments showed that the reproducibility of the assay was very high. Furthermore, no aspecific results were reported as assessed by theoretical alignments, challenging the genus specificity of the PCR and analysis of high concentrations of amplimers derived from the 25 beta-PV types.[25]
For a subset of positive buttock skin samples a quantitative PCR for beta-PV types 5, 8, 15, 20, 23, 24, 36, 38 was performed, as described previously.[29] The quantity of beta-PV was expressed as 1 beta-PV copy per n cells.

Activated caspase 3 immunohistochemistry and scoring apoptosis Frozen skin sections (5 µm) on 3-amino-propyltriaethoxysilane-coated glass slides were fixed for 10 min. in acetone at room temperature. Before and between incubation steps, the slides were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 for 5 min. After blocking with 2% normal human serum diluted in PBS /1% BSA for 20 min, the slides were incubated overnight with the primary antibody; polyclonal rabbit anti-active caspase 3 (Cat. No. 557035, BD Pharmingen) diluted 1:30 in PBS/1% BSA. After washing, skin sections were incubated

with the second antibody for 60 min; biotinylated goat anti-rabbit (IgG) (1:200; Vector, Burlingame, CA, USA). Subsequently, the slides were incubated with a horseradish peroxidase (HRP)-labelled avidin-biotin complex (DAKO, diluted 1:100 in PBS/1% BSA) for 60 min. Antibody binding was visualized by incubating the sections in 0.1 M acetate buffer (pH 5) containing 20 mg of 3-amino-ethyl-carbazole (Sigma, St Louis, MO, USA) and 100 µl of 30% hydrogen peroxide per 100 ml during 7 min. The skin sections were counterstained with haematoxylin and rinsed with running tap water for 10 min. All slides were mounted with Kaiser's glycerin and kept at room temperature. Anti-active caspase 3 positive cells in the epidermis were quantified per high-power field (original magnification x 400). The number of positive cells in all fields (mean 17; range 4-36) was assessed. Subsequently, the average number of positive epidermal cells per high-power field was calculated.

Statistical analyses The sample size of this study was calculated according to the criterion that a difference of 2 apoptotic cells/high-power field between beta-PV-positive and -negative skin still should be detectable. Beta-PVs have been detected more frequently in normal skin samples of organ-transplant recipients (17-87% positivity) than in immunocompetent individuals (16-35% positivity).[4;6;30;31] It was expected that in 80% of the OTR and 30% of immunocompetent individuals beta-PV would be present in healthy skin. With a power of 80% and a significance level (alpha) of 0.05, we calculated that 30 OTR would be sufficient to distinguish significantly between beta-PV-positive and -negative skin in level of apoptosis. We also included 30 ICI and studied both groups separately.

For the statistical analyses, we used SPSS version 12.0.1 for Windows. To compare the mean levels of apoptosis between beta-PV-infected and non-infected skin in and between OTR and ICI the Student's T-Test was used. The association between age and apoptosis was tested by linear regression analysis. All analyses were performed for the OTR and ICI, separately.

Results

Baseline characteristics

The baseline characteristics of the organ-transplant recipients and immunocompetent individuals are listed in Table 1.

Beta-PV DNA was frequently detected in low quantities and not affected by UV exposure

Tables 2 and 3 show the results of beta-PV DNA typing in skin biopsies of 30 OTR and 30 ICI, respectively. In the OTR beta-PV DNA was detected in 14 (47%) non-irradiated and 16 (53%) irradiated buttock skin biopsies (Table 2). In the 30 ICI beta-PV DNA was detected in 8 (27%) of both non-irradiated and irradiated buttock skin biopsies (Table 3). In the forearm skin of 9 OTR the beta-PV positive percentages were 89% and 63%, respectively (Table 2). In both groups, the most prevalent beta-PV types in buttock skin were beta-PV 8, 23, and 38. In OTR vs. ICI beta-PV 8 was present in 12% (7/60) and 7% (4/60) of the samples respectively, beta-PV 23 was found in 17% (10/60) and 7% (4/60), and beta-PV 38 in 15% (9/60) and 3% (2/60) of the samples, respectively (Tables 2 and 3).

Age was not associated with the presence of beta-PV (Figure 1). The mean age (SD) of the OTR with 1 or more beta-PV types in any biopsy was 55 (11) compared with an age of 54 (14) years in OTR without beta-PV. In ICI with and without beta-PV present the mean age was 33 (12) and 32 (12) respectively. Time after transplantation did not influence beta-PV positivity either. The beta-PV positive patients were tested at a mean of 19 years after transplantation, while the beta-PV negative patients were 16 years post transplantation. Also, gender was not significantly associated with presence of beta-PV DNA in the OTR and ICI. There was a large inter- and intraindividual variation in the number and types of beta-PV

Table 1. Characteristics

	Organ-transplant recipients	Immunocompetent individuals
No. of individuals	30	30
Sex		
Male	15	15
Female	15	15
Age (years)		
Mean ± SD	55 ± 12	32 ± 12
Range	32 – 77	19 – 57
Time after transplantation (years)		
Mean ± SD	18 ± 9	
Range	5 – 36	
History of skin cancer		
No. of patients with skin cancer (%)	19 (63%)	0 (0%)
Mean no. of skin cancers ± SD	6 ± 9	
Range	1-38	
Medication type (No. of patients; %)		
P + A	14 (47%)	
P + C	8 (27%)	
P + C + A	2 (7%)	
P + M	2 (7%)	
P + C + M	2 (7%)	
P	1 (3%)	
Unknown (clinical trial)	1 (3%)	

Abbreviations: SD=standard deviation; P=prednisone; A=azathioprine; C=cyclosporine; M=mycophenolate mofetil

Table 2. Beta-PV types in buttocks and forearms and apoptosis in buttock skin of organ-transplant recipients, presented with increasing levels of apoptosis.

No.	Gender and age (yrs)	Buttocks		Forearms		Apoptosis after UV in buttock skin (no. of pos. cells/ field)
		Control biopsies (not irradiated)	Biopsies irradiated skin	Control biopsies (not-irradiated)	Biopsies irradiated skin	
1	M 56	-	-	76	-	0.1
2	F 53	19	23,37	-	-	0.4
3	M 53	20,23	8,17,38	22,23,80,92	22,23,80,92,93	0.6
4	M 65	22,24,76	24,36	N.d.	N.d.	0.9
5	F 52	-	5,23,80	17,24	-	1.0
6	M 44	-	-	20,37	93	1.2
7	F 42	20,23	9	8,12,20,23,75	23,24	1.4
8	F 69	8,25	25,38	17,23,25,37,38,96	5,15,17,23,36,38	1.4
9	M 57	-	-	N.d.	N.d.	1.6
10	F 45	96	-	N.d.	N.d.	1.6
11	F 50	-	38,76	12,22,24,80	12,22,37	1.6
12	F 63	8,36,38,92	8,36	N.d.	N.d.	2.0
13	M 77	-	-	N.d.	N.d.	2.9
14	M 74	8,23,38	-	N.d.	N.d.	2.9
15	F 57	-	15,92	N.d.	N.d.	3.0
16	M 70	-	-	N.d.	N.d.	3.2
17	M 61	-	-	N.d.	N.d.	3.4
18	F 65	75	-	N.d.	N.d.	3.5
19	F 64	38	38	N.d.	N.d.	3.6
20	M 72	19,23,93	23,37,80,93	N.d.	N.d.	4.5
21	F 36	17	-	N.d.	N.d.	4.9
22	F 60	-	22	38	-	6.0
23	M 39	-	8,23	N.d.	N.d.	6.7
24	M 68	-	-	N.d.	N.d.	7.0
25	M 44	-	-	N.d.	N.d.	7.3
26	M 44	22,38,92	23	N.d.	N.d.	7.4
27	M 32	-	-	N.d.	N.d.	8.0
28	F 42	-	38	N.d.	N.d.	12.4
29	F 57	8,23,80,92,93	24,92	N.d.	N.d.	12.9
30	F 40	-	-	N.d.	N.d.	24.5

Abbreviations: N.d.=not done; M=male; F=female; - =no beta-PV present

Table 3. Beta-PV-types and apoptosis in buttock skin of immunocompetent individuals, presented with increasing levels of apoptosis.

No.	Gender and age (yrs)	Buttocks		
		Control biopsies (not irradiated)	Biopsies irradiated skin	Apoptosis after UV (no. of pos. cells/ field)
1	M 56	-	-	1.0
2	F 57	-	-	2.4
3	F 30	15	-	2.6
4	M 24	-	-	3.3
5	M 57	-	8,38	3.7
6	M 25	-	-	3.7
7	M 42	-	-	3.8
8	F 51	-	24	4.3
9	F 36	-	23	4.3
10	M 32	-	-	5.5
11	F 26	-	-	6.3
12	M 46	-	-	6.4
13	M 21	-	38	6.5
14	F 21	-	-	7.9
15	M 40	8	-	8.6
16	F 39	-	-	9.3
17	F 25	80	15	9.9
18	M 44	-	-	10.1
19	F 20	-	-	10.2
20	F 25	9	-	10.6
21	F 23	-	-	10.7
22	M 27	-	25	11.6
23	M 23	8	-	12.0
24	M 19	-	-	12.0
25	M 24	-	-	12.2
26	M 23	14	-	13.9
27	F 20	-	-	15.7
28	F 32	23	23	16.9
29	F 49	8	-	20.0
30	F 22	-	5,22,23	37.0

Abbreviations: M=male; F=female; - = no beta-PV present

detected in both OTR and ICI (Tables 2 and 3). In the OTR, the number of types detected in irradiated skin varied from 1 to 4 (mean 1.9), and in the non-irradiated skin from 1 to 5 (mean 2.3). In the ICI the number of types in irradiated skin varied from 1 to 3 (mean 1.4), and in the non-irradiated biopsies all beta-PV positive samples showed only 1 beta-PV type. UV-irradiation did not affect the presence or number of beta-PV in buttock skin nor in forearm skin (Tables 2 and 3).

The quantity of beta-PV was determined in 26 beta-PV positive samples, of which only two samples yielded a positive signal in the quantitative PCR (for beta-PV 23 and 38). One copy of beta-PV DNA was found in less than around 1000 cells in both samples. In the remaining 24 samples beta-PV-DNA loads were apparently below the detection limit (< 10 copies of beta-PV per reaction) of the quantitative PCR (data not shown).[29]

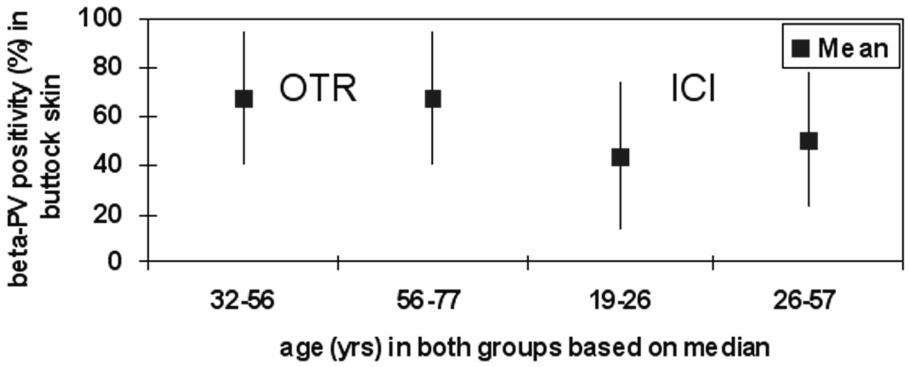

Figure 1. Beta-PV positivity (%) with 95% confidence intervals in different age groups based on median age in organ-transplant recipients and immunocompetent individuals. The findings were based on the cumulative test results obtained from two buttock skin biopsies per individual

Apoptosis was not reduced in beta-PV-infected skin but decreased with age

As expected no apoptotic cells were detected in non-UVB irradiated skin. In UVB-exposed buttock skin of organ-transplant recipients the number of apoptotic cells/high-power field varied from 0.1 to 24.5 (median 3.1). In the immunocompetent individuals the number of apoptotic cells varied from 1.0 to 37.0 (median 9.0) (Figure 2) (Tables 2 and 3).

Interestingly, in the UVB-irradiated biopsies of both the OTR and ICI, the number of apoptotic cells decreased with age (p=0.05 and 0.06 respectively) (Figure 3). Gender was not associated with apoptosis.

In both groups there was no difference in apoptosis between beta-PV positive and negative buttock skin (Figure 4). Also, when examining the effect of the most prevalent beta-PV-types, beta-PV 8, 23, 38, separately, no effect on apoptosis could be detected. Additionally, there was no difference in apoptosis levels between organ-transplant recipients with and without a history of skin cancer. The type of medication (cyclosporine or azathioprine) was not associated with the level of apoptosis either (data not shown). Adjusting our analyses for age and sex of the OTR and ICI did not change these findings.

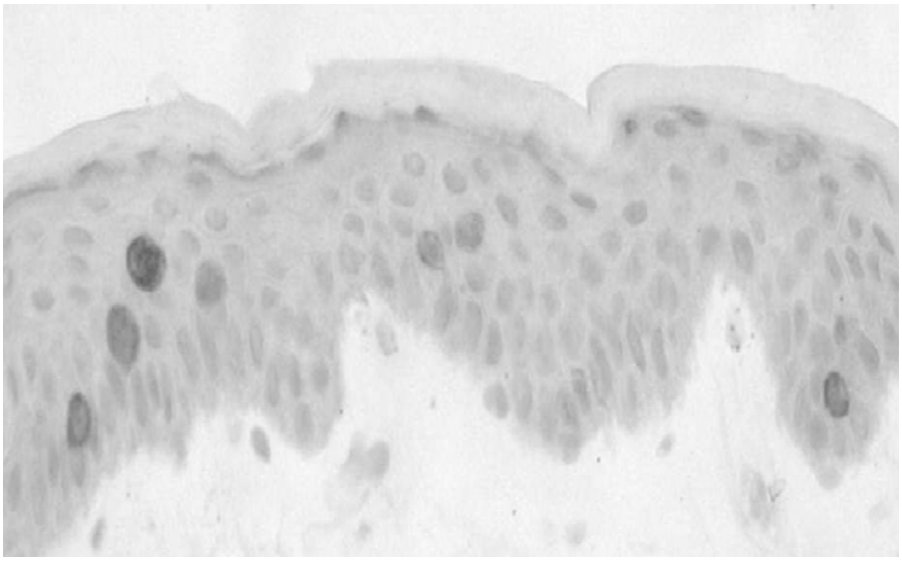

Figure 2. Anti-active caspase 3 positive cells in the epidermis (original magnification x 400).

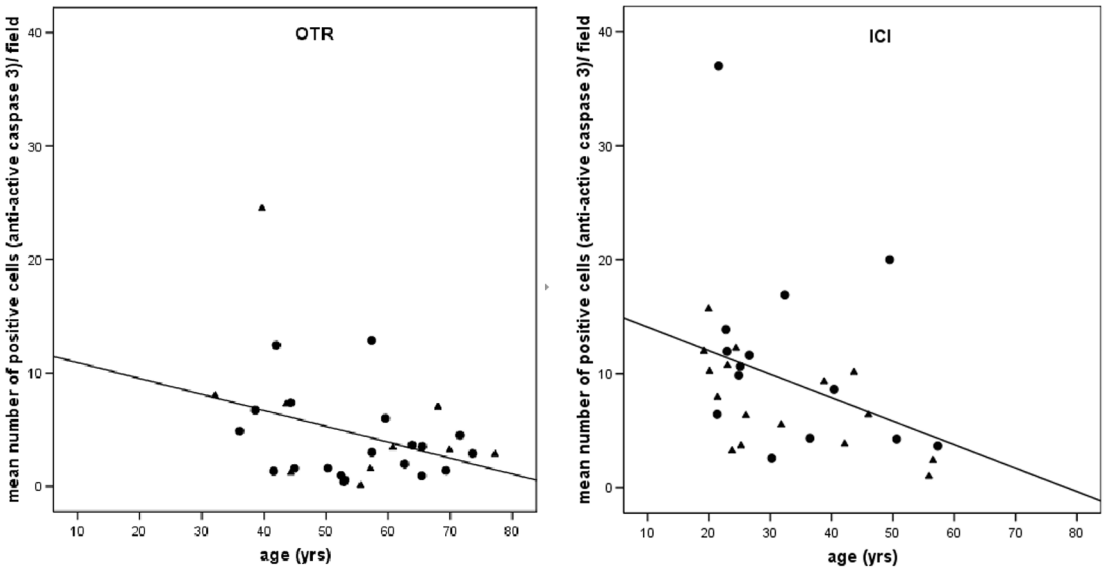

Figure 3. Relation between mean number of anti-active caspase 3 cells per high-power field and age in organ-transplant recipients and immunocompetent individuals.●: beta-PV positive, ▲: beta-PV negative

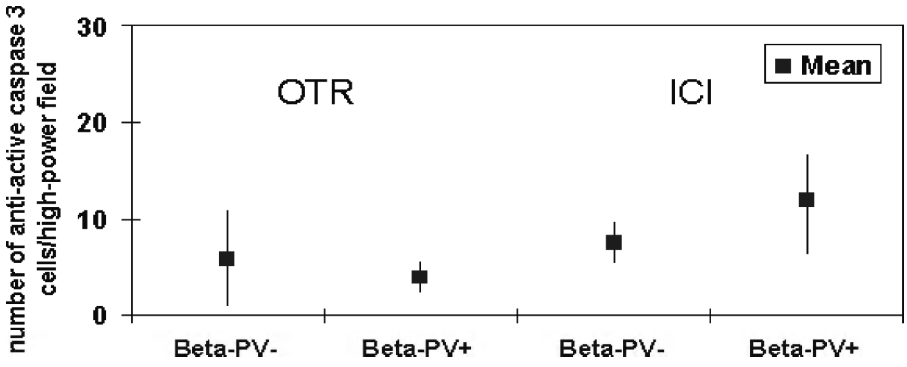

Figure 4. Mean number of anti-active caspase 3 cells per high-power field with 95% confidence intervals in beta-PV-positive and -negative skin samples in organ-transplant recipients and immunocompetent individuals

Discussion

In our study, we could not confirm in-vivo the hypothesis that beta-PVs may decrease UV-induced apoptosis. Based on our study, however, we cannot immediately conclude, that beta-PVs do not decrease UV-induced apoptosis. The low quantity of beta-PV DNA in the skin biopsies, with generally less than one copy of beta-PV per 1000 cells, would explain that we did not find any appreciable decrease in the number of apoptotic cells in beta-PV positive biopsies. In-situ hybridization with beta-PV types of individual apoptotic and non-apoptotic cells is a more precise method to study the effect of beta-PV infection on UV-induced apoptosis in individual cells. Unfortunately, this technique is not available yet and would be not conclusive in our biopsies because of the low viral DNA loads.

It cannot be entirely excluded that part of the beta-PV DNA is present on the surface of the skin. But this DNA probably originated from local, viral replication as can be inferred from the recent findings that beta-PV DNA is persistently present in plucked hairs[32] and, importantly, also in swaps of skin surface.[33] These data strongly suggest that the presence of this viral DNA is due to persistent beta-PV infections and not to contamination.

It is likely that not all beta-PV types exert similar effects on UV-induced apoptosis,

which may be an alternative explanation of the non-detectable effect of beta-PV infection on UV-induced apoptosis. To study the effect of all 25 known individual beta-PV types, separately, much larger series of skin biopsies would be needed. To discriminate better between the effects of the different beta-PV genotypes and to overcome the problem of low viral loads, the impact on UV-induced apoptosis could be studied in cultures of beta-PV transfected keratinocytes.

The dynamics of beta-PV infection in reaction to UV-irradiation is also not known yet. We observed no appreciable effect, but a single UV exposure may not be sufficient to measure an effect of beta-PV infection on UV-induced apoptosis in-vivo. Multiple exposures may lead to a more pronounced effect. We did find a tendency of a higher prevalence of beta-PV in the forearms than in the buttocks of OTR, which supports the notion that beta-PV is more prevalent in chronically sun-exposed, lesional skin. A recent study by Harwood et al. did not show such a difference between chronically sun-exposed and unexposed skin.[31] An earlier study by de Jong-Tieben et al. showed a higher prevalence of beta-PV-DNA in benign skin lesions from chronically sun-exposed sites compared with unexposed skin.[2] This latter result is in line with our supposition that chronic sun-exposure may induce beta-PV replication. It is known that at least some beta-PVs have UV responsive elements [34;35]

In our samples numerous beta-PV genotypes were detected and the mean number of beta-PV types in buttock skin was clearly higher in skin from OTR than from

ICI. However, the OTR and ICI were not age and sex-matched, and therefore not directly comparable. But in the overlapping age range from around 30 to 57 years the beta-

PV positive rate was higher in OTR than ICI (75 vs. 50 %, Figure 1), although not significantly.

Beta-PV effects were meant to be assessed in each group separately. The viral loads were indeed found to be low, and most frequently below the detection limits of the quantitative PCR, but in the similar range as reported before.[29] Corresponding to a sparseness of the viruses in the biopsies, there was a large inter- and intraindividual variation in the number and types of beta-PV.

Interestingly, our study showed that the level of UV-induced apoptosis decreased with increasing age in both OTR and ICI without a link to any appreciable age dependence of beta-PV or of MED values (the overall difference in apoptosis between OTR and ICI is fully attributable to age differences).

A relation between decreased apoptosis and skin cancer development has been established in animal experiments.[36] A study in which UV-induced apoptosis was measured in peripheral blood lymphocytes, showed a significant decrease in apoptosis with increasing age in melanoma patients.[37]

In conclusion, our data show that normal unexposed skin frequently contained viral DNA of various beta-PV types, but in very low quantities. We observed a decreased level of apoptosis with increasing age, not linked to any increase in beta-PV. However, we did not find a relation between beta-PV presence and overall UV-induced apoptosis. To ascertain the effect of beta-PV infection on UV-induced apoptosis in-vivo studies need to focus specifically on beta-PV-carrying cells.

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CHAPTER 5

Systemic and topical retinoids in the management of skin cancer in organ-transplant recipients

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Systemic and Topical Retinoids in the Management of Skin Cancer in Organ Transplant Recipients

Y. G. L. DE GRAAF, MD,* S. EUVRARD, MD,† AND J. N. BOUWES BAVINCK, MD, PHD*

*Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands, and †Department of Dermatology, Hôpital Edouard Herriot, Lyon, France

BACKGROUND. **Nonmelanoma skin cancers are the most frequent malignancies in organ transplant patients. Patients who develop multiple new skin cancers may benefit from retinoid chemoprevention.**

OBJECTIVE. **The objective of this study was to advise on the use of retinoids in organ transplant recipients.**

METHODS. **A summary was performed of the existing literature regarding experience with retinoid chemoprevention for skin cancer in organ transplant patients.**

RESULTS. **Systemic retinoids, specifically acitretin, are effective in inhibiting tumor development in organ transplant patients. This effect is only present during therapy, however. Topical retinoids have some effect in the treatment of actinic keratoses.**

CONCLUSIONS AND RECOMMENDATIONS. **Systemic retinoids can be used for chemoprevention of skin cancer. For a good result, long-term treatment with acitretin is necessary. Side effects, however, limit the use of retinoid chemoprevention. It is advised that treatment be started at a low dose, and patients should be monitored regularly for triglyceride and cholesterol levels and transaminases.**

Y. G. L. DE GRAAF, MD, S. EUVRARD, MD, AND J. N. BOUWES BAVINCK, MD, PHD HAVE INDICATED NO SIGNIFICANT INTEREST WITH COMMERCIAL SUPPORTERS.

Skin Cancer in Organ Transplant Recipients

NONMELANOMA SKIN cancers, especially squamous cell carcinomas, are the most frequent malignancies in organ transplant recipients.[1,2] The frequency of skin cancers increases progressively with time after transplantation. In temperate climates such as in the Netherlands, 40% of the renal transplant recipients will develop skin cancer 20 years after transplantation.[3] In sunny climates like Australia these percentages are even higher.[4]

Furthermore, in this particular population squamous cell carcinomas tend to be multiple and may have a more aggressive course with frequent recurrences compared to the immunocompetent host. Often there is an association with warts, premalignant keratoses, Bowen's disease, and keratoacanthomas.[2]

Prevention and Management of Skin Cancer

In organ transplant recipients, intensive surveillance and treatment of precancers and cancers in an early stage is necessary.[5] This consists mainly of multiple sessions of cryotherapy, curettage and coagulation, and repeated surgical excisions of tumors. Resurfacing the dorsum of the hand may also be useful in selected patients.[6]

Organ transplant recipients who may benefit from retinoid chemoprevention are those who are actively developing large numbers of skin cancers.[7,8] In this overview we will summarize the most important literature regarding the experience with systemic and topical retinoids in the chemoprevention of skin cancer in organ transplant recipients.

Mechanism of Action

Retinoids are structural and functional analogs of vitamin A that display a wide range of biologic activity.[9] Results of basic investigations suggest that retinoids act chemopreventively by inducing growth arrest or apoptosis of tumor cells, by modulating the immune response or differentiation of keratinocytes, or a combination of these events.[10–12]

Intracellularly, retinoids interact with cytosolic proteins and two families of nuclear receptors, the retinoic acid receptors and retinoid X receptors. As a result, specific genes are expressed.[12] It has also been shown that retinoic acid is more effective in inhibiting the growth of human papillomavirus (HPV)-infected human keratinocytes than that of normal

Address correspondence and reprint requests to: J. N. Bouwes Bavinck, Leiden University Medical Center, Department of Dermatology, Albinusdreef 2, PO Box 9600, 2300 RC Leiden, the Netherlands, or e-mail: J.N.Bouwes_Bavinck@lumc.nl.

keratinocytes, probably by suppressing the early transcription of HPV genes.[13,14]

Rook et al.[15] reported a marked increase of Langerhans cells within the epidermis in three patients after treatment with etretinate and topically applied tretinoin. The increase of Langerhans cells was correlated with duration of therapy. This may indicate a possible immunologic mechanism. In addition, a (statistically not significant) trend was reported for an increase in Langerhans cell density in squamous cell carcinomas during etretinate therapy.[16]

Warts in renal transplant recipients showed pronounced K13 expression in contrast to warts in the immunocompetent host. This may reflect an important molecular event inherent in the malignant degeneration of warts in renal transplant recipients. Retinoid treatment significantly correlated with a specific pattern of K13 expression in skin lesions of renal transplant recipients. It has been suggested that by keeping keratinocytes in this esophageal-type differentiation, retinoids might act chemopreventively.[17] Six months of treatment with topical tretinoin resulted clinically in an increased skin thickness. Histologic and ultrastructural examination of retinoic acid treated "normal skin" of renal transplant recipients revealed epidermal and dermal changes evoking increased cellular metabolism in the treated sites.[18]

Use of Retinoids in Nonimmunosuppressed Individuals

Systemic Retinoids

Systemic retinoids can be administered to treat precancers and cancers, but more often are used as chemopreventive agents. Treatment of actinic keratoses and Bowen's disease in immunocompetent patients was effective with the use of various retinoids.[12] Treatment of basal cell carcinomas and squamous cell carcinomas, however, showed no clear beneficial effects.[12]

In most trials, retinoids have been administered to prevent skin cancer. These trials were performed in immunocompetent individuals and in patients with xeroderma pigmentosum.[19]

Xeroderma pigmentosum is a rare recessive disease of defective DNA repair, in which patients have a 1000-fold increased risk of developing skin cancers.[20] In a 3-year controlled prospective study of oral isotretinoin at a dose of 2 mg/kg/day in five xeroderma pigmentosum patients, a reduction of 63% of tumors ($p = 0.02$) was reported in the treatment period compared to the pretreatment interval.[20] With these high doses, however, severe adverse effects were reported. As a result two of the seven patients were unable to complete the protocol.[20]

In a double-blind, randomized controlled trial of retinol versus placebo in 2297 immunocompetent individuals with actinic keratoses, daily retinol was associated with a statistically significant reduction in the risk of developing squamous cell carcinomas.[21] In contrast, low doses of isotretinoin were not effective in reducing basal cell carcinomas.[22]

The adverse effect profile of synthetic retinoids resembles the symptoms associated with hypervitaminosis A and may hamper the use of these agents.[12] The most frequent systemic side effect is an increase in plasma triglycerides. Increases of total and LDL cholesterol levels are also frequently observed.[23] Other adverse effects are abnormalities of liver function tests, arthralgias, mucocutaneous xerosis, and alopecia.[12] In addition, treatment at high doses or for a long term has been associated with various skeletal abnormalities such as calcifications of tendons and ligaments around joints, hyperostosis of the spine, and osteoporosis.[7,12] Retinoids are highly teratogenic if given orally during embryogenesis.[12] Isotretinoin's link to psychiatric disorders such as depression is still controversial.[24]

Topical Retinoids

The advantage of topical retinoids is the avoidance of systemic toxicity. Good results were obtained in the treatment and chemoprevention of actinic keratoses in immunocompetent patients using topical retinoids, in particular isotretinoin and tretinoin.[12] Topical retinoids have not been systematically tested in the treatment or prevention of nonmelanoma skin cancer in immunocompetent individuals.[12]

Retinoid Chemoprevention in Organ Transplant Recipients

Systemic Retinoids

The tables provide a summary of the clinical studies with retinoids in organ transplant recipients that are discussed in this overview.

Initially, studies were performed with etretinate. Later etretinate was replaced by its active metabolite, acitretin.

The four studies summarized with etretinate were all without an explicit control group and showed a reduction of new skin cancers, compared to the pretreatment period (Table 1). The well-known side effects of retinoids were observed but were well manageable (Table 1). Specifically no deterioration of renal function was noted.[15,25–27]

Table 1. Clinical Studies With Etretinate in Organ Transplant Recipients

Study	*Technical Details of Study**	*Treatment Result*	*Side Effects*
Shuttleworth et al.[25]	NCG, $n = 6$, 1 mg/kg/day, 6 months	5 patients, no new skin cancers; 1 patient, 2 new SCCs after 6 months.	Cheilitis, increased triglycerides ($n = 3$), increased cholesterol ($n = 2$); no deterioration in renal function.
Kelly et al.[26]	NCG, $n = 4$, 50 mg/day, 8–13 months	Considerable reduction new SCCs.	Mild mucocutaneous side effects, thrombocytopenia ($n = 1$); renal function unchanged.
Rook et al.[15]	NCG, $n = 11$, 10 mg/day in combination with topically applied 0.025% tretinoin ($n = 7$) or tretinoin alone ($n = 4$), 9 months	3 of 4 patients, no new SCCs after 9 months of etretinate + topical tretinoin (2 of 3 patients, no new SCCs after 9 months of topical tretinoin alone); 4 patients, nonevaluable.	Mild mucocutaneous side effects.
Gibson et al.[27]	NCG, $n = 11$, 0.3 mg/kg/day, 17 months	10 patients, significant reduction of new skin cancer after 3 + 6 months compared to pretreatment period; 1 patient, nonevaluable.	Mild mucocutanous side effects, increased triglycerides ($n = 3$), increased cholesterol ($n = 1$).

*Respectively, the type of trial (NCG, study without any explicit control group), the study size, the dose of etretinate, and the duration of the study are depicted.
Abbreviation: SCC, squamous cell carcinoma.

Table 2. Clinical Studies With Acitretin in Organ Transplant Recipients

Study	*Technical Details of Study**	*Treatment Result*	*Side Effects*
Vandeghinste et al.[39]	CR, $n = 1$, 0.5 mg/kg/day, 15 months	No new dysplastic skin lesions observed.	Mild mucocutaneous side effects.
Bouwes Bavinck et al.[28]	RCT (compared with placebo), $n = 38$, 30 mg/day, 6 months	Statistically significantly fewer patients with new skin cancers compared to placebo reduction of number of actinic keratoses.	Side effects: mild mucocutaneous, hair loss, increased cholesterol + triglycerides ($n = 3$); renal function unchanged.
Yuan et al.[30]	NCG, $n = 15$, 10–50 mg/day, <6 to >12 months	Variable effects on skin cancer.	Mild mucocutaneous side effects.
McKenna et al.[31]	NCG, $n = 16$, 0.3 kg/mg/day, 5 years	Statistically significant reduction of new skin cancers after 4 years compared to pretreatment period.	Mild mucocutanous side effects, increased triglycerides ($n = 1$), increased cholesterol ($n = 1$).
McNamara et al.[32]	NCG, $n = 5$, 10–25 mg/day, 10–24 months	3 patients, significant decrease in new tumors compared to pretreatment period; 2 patients, moderate decrease in new tumors.	Mild mucocutaneous side effects.
George et al.[33]	RCT (compared to no therapy), $n = 23$, 25 mg/day, 2 year	Number of SCCs significantly lower on acitretin compared to drug-free period.	Cheilitis, headache, increase of musculoskeletal symptoms, gastritis, increased triglycerides.
de Sévaux et al.[29]	RCT (0.4 mg/kg/day compared to 0.2 mg/kg/day), $n = 26$, 0.4 or 0.2 mg/kg/day, 1 year	Decrease of actinic keratoses by 50% in both groups; no effect on development of skin cancers in both groups compared to pretreatment period.	Mild mucocutaneous side effects, mild hair loss.

*Respectively, the type of trial (CR, case report of single cases; RCT, randomized controlled trial; NCG, study without any explicit control group), the study size, the dose of acitretin, and the duration of the study are depicted.
Abbreviation: SCC, squamous cell carcinoma.

With acitretin, one placebo-controlled trial and two other randomized controlled trials were performed (Table 2). In addition, three studies without an explicit control group and one case report were published (Table 2). The randomized, double-blind, placebo-controlled trial showed a significant effect on the prevention of skin cancer at a acitretin dose of 30 mg/day. During a 6-month period, 2 of 19 patients (11%) in the acitretin group reported a total of two new squamous cell carcinomas, compared with 9 of 19

patients (47%) in the placebo group who developed a total of 18 new carcinomas ($p = 0.01$).[28] Furthermore, the relative decrease of keratotic skin lesions was 13% in the acitretin group, compared to a relative increase of 28% in the placebo group ($p < 0.01$). After the end of treatment, however, skin cancers and keratotic skin lesions relapsed.[28] The other studies generally confirmed the favorable effect of acitretin on the reduction of development of new skin cancers and actinic keratoses.[29–33] In the Australian open randomized crossover trial, 25 mg acitretin/day was prescribed to 14 patients, and 9 patients received no retinoid treatment. They crossed over after 1 year. A significantly lower number of squamous cell carcinomas was shown in patients while on acitretin compared to the drug-free period ($p = 0.002$).[33] A recent randomized study with low-dose acitretin was performed by de Sévaux et al.[29] in 26 renal transplant patients of whom 13 were treated with 0.4 mg/kg/day acitretrin and 13 with 0.2 mg/kg/day for 1 year. A decrease in actinic keratoses of 50% was reported in both groups. Nevertheless, no effect on the development of nonmelanoma skin cancer was reported in the two low-dose groups of patients when compared to the pretreatment period.[29]

As far as we know, there are a few reports about the use of isotretinoin in transplant patients with skin cancer.[8,34] In one study with one patient a 50% reduction of new lesions compared to the pretreatment period was observed.[34]

Side effects of retinoids in organ transplant recipients are identical to those reported in the immunocompetent host. In organ transplant recipients there is a theoretical concern of immunostimulation caused by the systemic retinoids, which theoretically could lead to rejection of the transplanted organ.[8] Osteoporosis is also a particular problem for the posttransplant recipient, who is already at risk for osteoporosis from glucocorticoid exposure.[5] Furthermore, elevation of serum lipids is more common in organ transplant recipients also without systemic retinoids. This problem is usually manageable by the use of antihyperlipemics.[35]

Topical Retinoids

Two studies with topically applied tretinoin and one with topically applied adapalene are summarized in Table 3. A statistically significant reduction of actinic keratoses was observed in two studies.[36,37] Side effects were generally mild.[36–38] No significant effect was shown in the study of Smit et al.,[38] possibly because of the smaller sample size, the lower concentration of tretinoin, the shorter study duration, the complexity of the study, the focus on immunohistochemical and histologic parameters, or a combination of these factors. As far as we know, no studies with topically applied retinoids were performed to study the effect on the development of new skin cancers.

Personal Experience With Acitretin in Organ Transplant Recipients

Acitretin in a dose between 10 and 30 mg per day has, in our experience, a clear effect on the number and thickness of the hyperkeratotic skin lesions and the number of new skin cancers. Most of the approximately 75 patients we have treated in Leiden (The Netherlands) and Lyon (France) together could tolerate this regimen for a 4- to 6-month period. Approximately one-third of these patients were able to continue this regimen for at least 5 years without significant side effects. About half of the patients, however, have preferred to discontinue this treatment after 4 to 6 months because of dry skin, dry lips, significant hair loss, pruritus, or arthralgias. With

Table 3. Clinical Studies With Topical Retinoids in Organ Transplant Recipients

Study	*Technical Details of Study**	*Treatment Result*	*Side Effects*
Euvrard et al.[36]	RCT (vs. placebo), $n = 22$, 0.05% tretinoin once daily, 3 months	Statistically significant reduction of keratotic lesions after 3 months compared to placebo (45% vs. 23%).	Local tolerance generally good.
Euvrard et al.[37]	RCT (vs. placebo), $n = 40$, 0.3% vs. 0.1% adapalene, 6 months	Significant decrease in actinic keratoses in 0.3% group compared to placebo (32% vs. 21%).	Skin irritation ($n = 1$).
Smit et al.[38]	CT (four treatment modalities), $n = 13$, 0.02% tretinoin vs. calcipotriol vs. both vs. emollient twice daily, 6 weeks	Treatment of actinic keratoses, alone or in combination with calcipotriol, showed no effect on clinical, immunohistochemical, and histologic measures.	Pruritus in all test areas (number unknown).

*Respectively, the type of trial (RCT, randomized controlled trial; CT, controlled trial), the study size, the dose of the topical retinoids, and the duration of the study are depicted.

regard to objective adverse effects, we have observed liver toxicity in two kidney transplant recipients necessitating treatment discontinuation, although we have not observed significant renal dysfunction or allograft rejection. Elevated serum triglycerides and cholesterol levels have been easily manageable. We have utilized intermittent therapy in some patients with alternating 1 to 3 months, with and without acitretin. This regimen seems to be effective, but randomized-controlled trials should be performed to prove the efficacy of this treatment regimen.

Recommendations and Conclusion

Systemic retinoids, specifically etretinate and acitretin, have shown to be effective in inhibiting the development of new dysplastic skin lesions and skin cancers in organ transplant recipients. Because etretinate is not marketed anymore, acitretin is the medication most often utilized and the recommendations in this overview therefore only pertain to acitretin. Experience with isotretinoin in organ transplant patients is limited. Nevertheless, it is probably the most widely studied retinoid in the chemoprevention of skin cancers in nonimmunosuppressed patients. The results in transplant patients are expected to be similar as compared to nonimmunosuppressed patients. We have no data to compare the antitumoral effect of either drug in transplant or in nontransplant patients. Isotretinoin could be useful in female patients because of the shorter time required to avoid pregnancy (1 month after the discontinuation of the treatment compared to acitretin (2 years)).

Systemic retinoids are variably tolerated by organ transplant patients. Because the benefit of acitretin is present only during treatment, long-term treatment usually is necessary. Long-term use of systemic retinoids, however, may be limited by the occurrence of side effects in individual patients. Specifically, subjective side effects, such as dry skin, dry lips, and hair loss, may be reason to discontinue therapy with acitretin, despite treatment with topical moisturizers.

Although no patients were reported in whom retinoid therapy affected the transplanted organ, the possible immune-stimulating effect of retinoids causes some theoretical concern for rejection of the transplanted organ. Use of acitretin therefore should in our opinion be limited to patients with dozens of hyperkeratotic skin lesions and/or at least one squamous cell carcinoma in the medical history, but other levels of involvement of the skin have also been suggested.

Because the minimal effective dose varies greatly between patients, it may be best to start at a low dose (between 10 and 30 mg/day), which can be increased as tolerated if insufficient benefit is observed.[5] In our experience a dose of 30 mg/day acitretin or less was sufficiently effective. When treatment is limited by subjective side effects, intermittent acitretin treatment could be considered with periods on acitretin therapy intermingled with treatment-free periods. In addition to the intermittent acitretin regimen, lowering the dose of acitretin is an alternative method of decreasing the side effects.

Patients should be monitored for the usual measures when using oral retinoids. Triglyceride levels, cholesterol levels, and transaminases should be tested before the start of the treatment and at regular intervals during treatment, initially monthly and later at 3-month intervals. In renal transplant recipients, renal function should be monitored. Radiologic examination of the spine and long bones could be performed in patients with long-term use of retinoids but, in our opinion, is not useful in organ transplant recipients, because osseous side effects of treatment with corticosteroids can be expected.

Although topical retinoids seem effective in the treatment of actinic keratoses and warts in organ transplant patients, the effect is clearly less than with treatment with systemic retinoids. Topical retinoids may be warranted as first-line therapy in high-risk patients once actinic keratoses begin to develop, but before a critical threshold necessitating oral retinoids is reached.

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CHAPTER 6

Photodynamic therapy does not prevent cutaneous Squamous-cell carcinoma in organ-transplant recipients: results of a randomized-controlled trial

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Photodynamic Therapy does not Prevent Cutaneous Squamous-Cell Carcinoma in Organ-Transplant Recipients: Results of a Randomized-Controlled Trial

Ymke G.L. de Graaf[1], Cornelis Kennedy[1], Ron Wolterbeek[2], Annemie F.S. Collen[1], Rein Willemze[1] and Jan N. Bouwes Bavinck[1]

A randomized-controlled trial with paired observations was performed with 40 organ-transplant recipients to assess the preventive effect of photodynamic therapy (PDT) on the development of new squamous-cell carcinomas and to evaluate the effect of PDT on the number of keratotic skin lesions. The treatment area consisted of a randomly assigned forearm and the corresponding hand, whereas the other forearm and hand served as the control area. After the initial visit, follow-up visits were scheduled at 3-monthly intervals during 2 years. No statistically significant difference was found in the occurrence of new squamous-cell carcinomas between the treated and untreated arms: after 2 years of follow-up, we observed 15 squamous-cell carcinomas in nine out of 40 PDT-treated arms and 10 squamous-cell carcinomas in nine out of 40 control arms. The number of keratotic skin lesions increased in both arms, but was less pronounced in the PDT-treated arm. After 1 year of follow-up, a trend in favor of the PDT-treated arm was observed, but statistical significance was not reached. Nearly 80% of the patients reported mild to severe adverse effects consisting of pain and a burning sensation, immediately after the treatment. No long-term adverse events were noted. In conclusion, PDT does not appear to prevent the occurrence of new squamous-cell carcinomas in organ-transplant recipients, but to some degree, reduces the increase of keratotic skin lesions.

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INTRODUCTION

Skin carcinomas, especially squamous-cell carcinomas, that develop on sun-exposed skin, are a serious hazard to organ-transplant recipients (London *et al.*, 1995; Euvrard *et al.*, 2003). The frequency of skin carcinomas increases progressively with time after transplantation. In the Netherlands, the risk to develop squamous-cell carcinoma increases up to 40%, 20 years after transplantation (Hartevelt *et al.*, 1990).

The majority of organ-transplant recipients develop multiple squamous-cell carcinomas, which appear to be more aggressive in these patients in comparison with immunocompetent individuals (Euvrard *et al.*, 2003).

An association exists between squamous-cell carcinoma and multiple keratotic skin lesions, and most lesions are localized on sun-exposed skin such as the forearms and dorsum of the hands (Bouwes Bavinck *et al.*, 1993; Euvrard *et al.*, 2003).

[1]*Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands and* [2]*Department of Biostatistics, Leiden University Medical Center, Leiden, The Netherlands*

Correspondence: Dr Jan N. Bouwes Bavinck, Department of Dermatology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands. E-mail: J.N.Bouwes_Bavinck@lumc.nl

Abbreviations: ALA, aminolevulinic acid; PDT, photodynamic therapy

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Currently available therapies for skin carcinomas such as excision and for solar keratoses such as topical application of liquid nitrogen are less satisfactory for the treatment of large affected areas, which is often the case in organ-transplant recipients.

Prevention of squamous-cell carcinomas and reduction of keratotic skin lesions with topical photodynamic therapy (PDT) would, therefore, substantially improve the quality of life of organ-transplant recipients.

PDT is a relatively safe procedure where a photosensitizer is applied to the affected area and subsequently irradiated with a light system. This treatment can be used to treat superficial skin carcinomas or precancerous lesions that are accessible to light (Morton *et al.*, 2002). PDT involves the activation of intracellular photosensitizers by visible light in order to generate cytotoxic singlet oxygen and other free radicals, which selectively destroy rapidly proliferating cells (Hopper, 2000; Ormrod and Jarvis, 2000). As photosensitizers, aminolevulinic acid (ALA) or methylaminolevulinic acid are used (Morton *et al.*, 2002).

PDT with topical ALA is a safe and effective treatment for solar keratoses (Ormrod and Jarvis, 2000; Brown *et al.*, 2004). The efficacy has been demonstrated for non-hyperkeratotic solar keratoses on the face or scalp of immunocompetent individuals. Response rates were comparable with cryotherapy and 5-fluorouracil, but a better cosmetic result was

reached (Morton *et al.*, 2002). Furthermore, Bowen's disease and superficial basal-cell carcinomas can also be effectively treated with PDT (Varma *et al.*, 2001; Kormeili *et al.*, 2004). PDT has also been shown to be effective in treating recalcitrant viral warts (Stender *et al.*, 2000; Ibbotson, 2002).

Only two studies concerning the efficacy of PDT in immunosuppressed patients are available. Although lower cure rates are reported in organ-transplant recipients compared to the immunocompetent controls (Dragieva *et al.*, 2004a), both studies report a good efficacy of PDT for solar keratoses in organ-transplant recipients (Dragieva *et al.*, 2004a, b). In addition, experimental studies in hairless mice have shown that PDT can delay the development of UV-induced skin carcinomas (Stender *et al.*, 1997; Sharfaei *et al.*, 2002).

The primary objective of this trial was to assess the occurrence of new squamous-cell carcinomas in organ-transplant recipients in the treated arm compared with the control arm following PDT. A second objective was to evaluate the difference in (re)occurrence of keratotic skin lesions in the arm treated with PDT compared to the control arm. Finally, we studied the difference in efficacy between a regimen with one PDT treatment or two PDT treatments with a 6-month period in between the treatments.

RESULTS

A total of 45 patients gave informed consent. Forty of them were randomized two-fold: 21 to receive PDT to the left forearm and hand, 19 to receive PDT to the right forearm and hand and 23 patients were randomized to one PDT treatment (at T0) and 17 to two PDT treatments (at T0 and T6). The other five patients had been included previously in the non-randomized pilot part of the study and they received only one PDT treatment (at T0).

The characteristics of the patients are listed in Table 1. During the 2-year follow-up period, seven patients were lost to follow-up. Five patients died from another cause then skin cancer after 3 months (two patients), and after 9, 12, and 21 months, respectively. Two patients were lost to follow-up after 18 and 24 months, respectively, without any apparent reason. The characteristics of the treated and control arm at the start of the trial are depicted in Table 2.

During the 2-year follow-up period, a total of 25 squamous-cell carcinomas and no other types of skin cancer were detected on the forearms and hands of the 40 patients in the randomized part of the trial. Figure 1b shows the distribution of the clinical outcome among these patients. In six patients one squamous-cell carcinoma developed in the control arm and none in the PDT-treated arm, and in one patient two squamous-cell carcinomas developed in the control arm and none in the PDT-treated arm. By contrast, in six patients one squamous-cell carcinoma developed in the PDT-treated arm and none in the control arm, in one patient three squamous-cell carcinomas developed in the PDT-treated arm and none in the control arm, and in one patient five squamous-cell carcinomas developed in the PDT-treated arm and one in the control arm. In one patient one squamous-cell carcinoma developed in the PDT-treated

Table 1. Characteristics of the patients

	Randomized trial	Pilot trial
No. of patients	40	5
Sex		
Male	21	2
Female	19	3
Age (years)		
Mean ± SD	55.0 ± 8.8	58.0 ± 6.9
Range	39–71	50–68
Immunosuppressive treatment		
Prednisone and azathioprine	37	5
Prednisone and cyclosporine	1	0
Prednisone, azathioprine, and cyclosporine	2	0
Time after transplantation (years)		
Mean ± SD	22.0 ± 6.3	17.9 ± 3.3
Range	7–34	12–21
History of SCC anywhere on the body		
No. of patients with SCC (%)	31 (78)	4 (80)
Mean no. of SCC ± SD	8.2 ± 9.9	6.8 ± 6.5
Range	0–42	0–17

SCC: squamous-cell carcinoma; SD: standard deviation.

Table 2. Characteristics of the PDT-treated and the control arm and hand at the start of the trial

	PDT-treated arm	Control arm
Randomized part of the trial		
No. of arms	40	40
Side of the arm		
Left	19	21
Right	21	19
History of SCC per arm		
No. of arms with SCC (%)	18 (45)	19 (48)
Mean no. of SCC ± SD	1.0 ± 1.3	1.1 ± 1.7
Range	0–4	0–8
No. of keratotic lesions per arm		
Mean ± SD	31 ± 22	27 ± 19
Range	9–96	6–103
Pilot part of the trial		
No. of arms	5	5
Side of the arm		
Left	2	3
Right	3	2
History of SCC per arm		
No. of arms with SCC (%)	2 (40)	2 (40)
Mean no. of SCC ± SD	0.6 ± 0.9	0.4 ± 0.5
Range	0–2	0–1

SCC: squamous-cell carcinoma; SD: standard deviation.

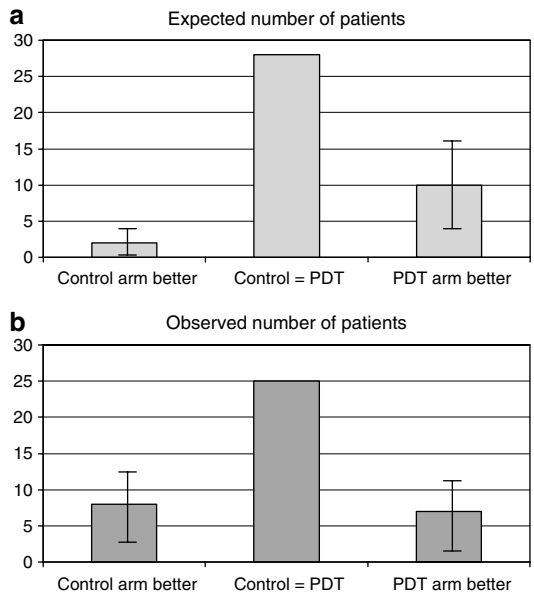

Figure 1. Expected and observed number of patients after 2 years of follow-up. (**a**) Expected number of patients with 95% confidence intervals based on the assumption that after a 2-year follow-up period, 5% of the PDT-treated arms and 25% of the control arms would develop one or more squamous-cell carcinomas. The numbers are provided for a total of 40 patients (80 arms). (**b**) Observed number of patients with squamous-cell carcinoma with 95% confidence intervals after a 2-year follow-up period, showing that the control arm was better than the PDT-treated arm in eight (20%) of the 40 patients and the PDT-treated arm was better in seven (17.5%) of the 40 patients.

arm and one in the control arm and in 24 patients no squamous-cell carcinomas developed in any arm. Altogether, 15 squamous-cell carcinomas developed in nine out of 40 PDT-treated arms and 10 squamous-cell carcinomas in nine out of 40 control arms.

In the pilot study, four squamous-cell carcinomas developed in two out of five patients: one in the PDT-treated arm and three in the control arm. One patient developed one squamous-cell carcinoma in the control arm and none in the PDT-treated arm and one patient developed two squamous-cell carcinomas in the control arm and one in the PDT-treated arm.

The occurrence of new squamous-cell carcinomas was not statistically significantly different between the PDT-treated arm and the control arm within the 2-year follow-up period neither in the randomized part of the study ($P=0.80$) nor in the pilot study ($P=0.16$) and also not when the outcomes of both studies were combined ($P=0.81$). One or two PDT treatments did not influence the outcome of squamous-cell carcinoma significantly (data not shown).

Figure 2 depicts the distribution and mean number of keratotic skin lesions in the control arms (panel a) and in the PDT-treated arms (panels b–d). Panel b shows the distribution and mean number of keratotic skin lesions of all PDT-treated arms, irrespective of whether they were treated one time or two times, panel c shows the arms that received one PDT treatment only, and panel d shows the arms that received two PDT treatments only.

Unexpectedly, at the start of the study, we found a relevant difference between the number of keratotic skin lesions in the arms randomized to be treated with PDT and the control arm with a mean of 4.5 more keratotic skin lesions in the arms that were randomly selected to be treated with PDT (Figure 2e). For this reason, all time points were compared with baseline (T0) as depicted in Figure 2f.

Planned pairwise comparison on the basis of a linear mixed model approach focused on the comparison between time points T3, T6, T9, and T12 versus T0 resulted in a statistically significant difference at time points T9 and T12 (Figure 2f). The overall fixed effect of time, however, was not statistically significant ($P=0.06$).

Two PDT treatments appeared to reduce the increase of keratotic skin lesions at time points T9 and T12 slightly more than one PDT treatment (Figure 2c and d), but using a summary measure approach based on Wilcoxon's rank-sum test on regression slopes from simple linear regression of individual patients, the difference in the median values of the regression slopes was not statistically significant ($P=0.39$).

In total, 79% of patients reported adverse effects during PDT treatment or in the first week after treatment. Most prevalent were a burning sensation (38%) and pain (31%). A minority of patients reported itch (9%) and blisters (2%). No long-term adverse effects were reported. There were no withdrawals of the patients because of adverse effects.

DISCUSSION

PDT, using topical δ-ALA and violet light (400–450 nm) applied to the forearm and corresponding dorsum of the hand, did not significantly prevent the development of new squamous-cell carcinomas in organ-transplant recipients within a 2-year follow-up period. The PDT procedure, however, to some extent, diminished the increase of keratotic skin lesions in the PDT-treated arm and hand compared to the nontreated control area, but at the expense of pain and irritation during and shortly after the procedure.

Generally, the forearms and dorsum of the hands of organ-transplant recipients have undergone a field change, meaning that the skin in this area shows histological atypia (Berg and Otley, 2002). This may be clinically visible by the presence of numerous premalignant actinic keratoses and other keratotic skin lesions and results in an increased risk to develop squamous-cell carcinomas in this area of the skin. Hypothetically, treating the whole area with PDT and thus selectively destroying the premalignant cells should result in a reduced risk of squamous-cell carcinoma. Unfortunately, such an effect was not observed in this randomized-controlled trial.

A possible explanation that we did not observe a preventive effect of the PDT procedure on the development of new squamous-cell carcinomas may be that the follow-up period of 2 years was too short. However, after 2 years of follow-up, nine out of 40 patients (22.5%) developed new squamous-cell carcinomas in the control arm, which is close to the approximately 25% of patients who were expected to develop new squamous-cell carcinomas in the control arm during the 2-year follow-up period. In addition, 23 out of 40

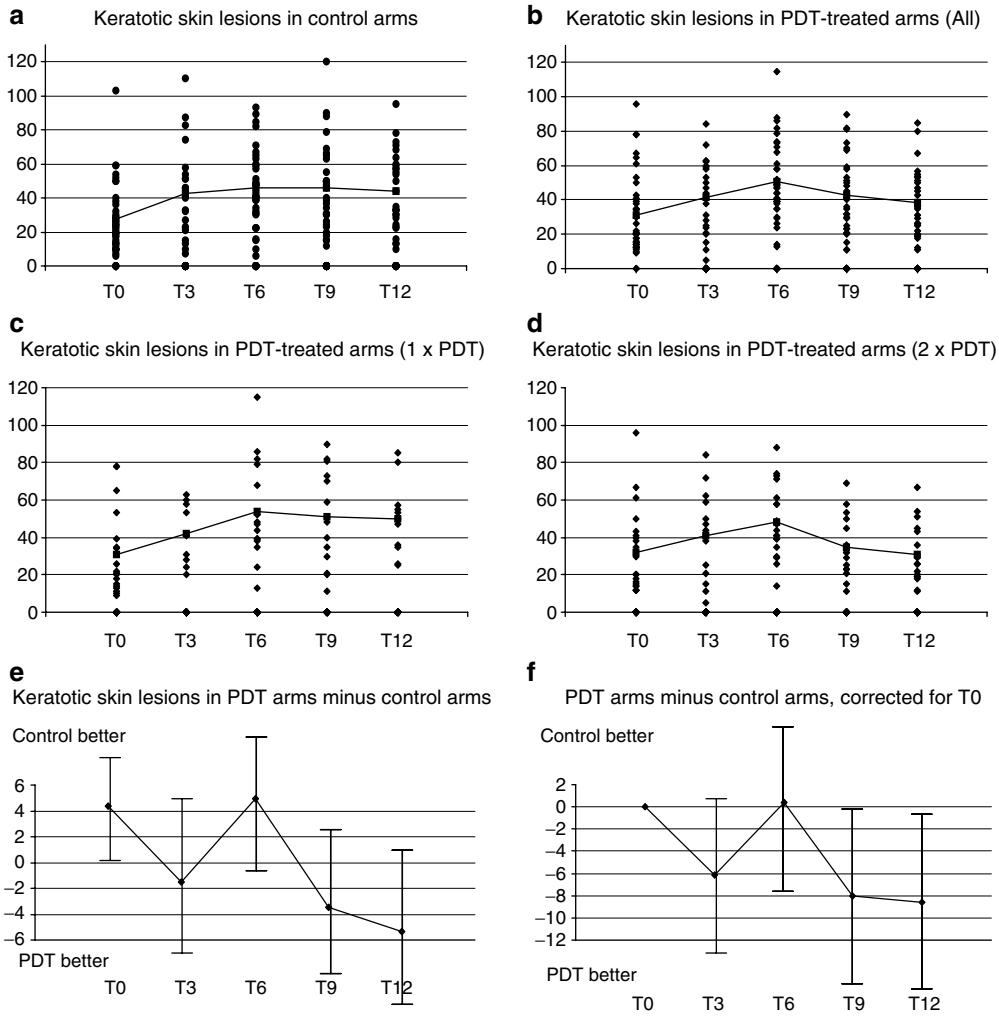

Figure 2. Distribution and mean number of keratotic skin lesions at the start of the trial and at 3-monthly intervals (a) in the control arm, (b) in the PDT-treated arm, irrespective of one or two treatments, (c) with one treatment only, and (d) with two treatments only. (**e**) Uncorrected difference between the number of keratotic skin lesions in the PDT-treated arm and the control arm and (**f**) difference corrected for the difference at the start of the trial are shown. The bars indicate 95% confidence intervals.

patients (57.5%) had developed a total of 84 squamous-cell carcinomas on the rest of the body during the 2-year follow-up period (data not shown), indicating that a 2-year follow-up period should be sufficient to harbor enough squamous-cell carcinoma events in the PDT-treated arm and the control arm. If the PDT procedure had been effective, a significantly lower occurrence of squamous-cell carcinoma would have been observed in the PDT-treated arm, which, obviously, was not the case.

Another possible explanation that the PDT procedure was not effective may be the selection of the patients. We selected patients who needed intervention the most, that is, patients who often had a history of one or more squamous-cell carcinomas and at least 10 hyperkeratotic skin lesions present on the forearms and dorsum of the hands. This selection is illustrated by the fact that the time period since transplantation ranged between 7 and 34 years. We cannot exclude that PDT intervention at an earlier time period after transplantation, at an earlier stage of skin cancer development, may be more effective.

Still another possible explanation that the PDT procedure was not successful in our hands may be the character of the light source and the photosensitizer that we used. Red light (570–750 nm) is often used as the light source for PDT because of the greater depth of penetration. We used violet light (400–450 nm) because violet light has a maximum overlap with the excitation spectrum of protoporphyrin IX, and, therefore less light energy is required for the same effect (Dijkstra *et al.*, 2001). As photosensitizer we used δ-ALA. Methylaminolevulinic acid, the methyl ester of ALA, has the advantage of penetrating deeper into the skin, and possibly could result in a better therapeutic result.

Additionally, we did not pretreat the keratotic skin lesions with curettage, because this was not feasible considering the large amount of keratotic skin lesions in our patients. As a result, thick keratotic skin lesions were possibly not optimally

treated with PDT, because of insufficient penetration of the photosensitizer in these lesions. Inferior response to PDT of thick keratotic skin lesions on the hands compared to the thinner lesions on the face has been demonstrated in immunocompetent patients (Morton *et al.*, 2002), and in immunocompromised patients it is probably even more difficult to treat these keratotic skin lesions with PDT (Dragieva *et al.*, 2004a).

Still another explanation that the PDT procedure may not have been successful may be that the favorable effect of PDT was counterbalanced by a harmful effect of the violet light source we used, but data to substantiate this suggestion are not available.

Whereas the occurrence of new squamous-cell carcinomas is a solid end point of this study, the number of keratotic skin lesions is a much weaker end point. Counting keratotic skin lesions is difficult to standardize; observers tend to improve their counting scales during time, and intra-observer and inter-observer variation may be significant. The advantage of paired analyses is that intra-observer variation is minimized and variation during time is reduced, because inaccurate counting should equally affect both the treated and control arms in all patients. Despite the methodological limitations, a trend to reduction of the increase of keratotic skin lesions in the PDT-treated arm and hand compared to the nontreated control area was observed. With a more accurate measurement of keratotic skin lesions, this effect is likely to be more discernible and would possibly reach statistical significance.

In summary, PDT using topical δ-ALA with a violet light source (400–450 nm) does not appear to prevent the development of new squamous-cell carcinomas in organ-transplant recipients. PDT has also no significant effect on the reduction of keratotic skin lesions although a trend in favor of the PDT-treated arm was observed. A possible positive effect of PDT, however, goes at the expense of significant short-term side effects, and, therefore, PDT performed with topical δ-ALA and violet light does not appear to be a promising preventive therapy in organ-transplant recipients.

MATERIALS AND METHODS

Eligible patients

Organ-transplant recipients who were regularly evaluated at the Department of Dermatology and/or Nephrology from the Leiden University Medical Center in the period between August 2000 and February 2003 were eligible to participate in the trial.

Inclusion criteria were a history of skin carcinoma and/or at least 10 keratotic skin lesions present on both forearms and hands at the day of inclusion; an age of 18 years or older; and a functioning graft of 5 years or longer.

Keratotic skin lesions consisted of solar keratoses, flat warts, seborheic warts, and common warts. Because it is difficult to discriminate these different keratotic skin lesions on clinical and histological grounds, they were considered together. Patients who presented with a skin carcinoma at the start of the trial were only included after excision of the skin cancer, which needed to be fully healed before topical application with the photosensitizer. Patients who were using acitretin and women with childbearing potential were excluded from the study.

The medical ethical committee of the Leiden University Medical Center approved the study, and all participants provided written informed consent. The study adheres to the Declaration of Helsinki Principles and was approved by the local medical ethics commission.

Study design

A randomized-controlled trial with a self-controlled design, consisting of a right/left comparison was performed. One forearm and the corresponding hand were randomly allocated to the PDT procedure, and the other forearm and hand served as the control area and remained untreated. In addition, patients were randomly allocated to one or two PDT procedures. The first group received only one treatment at the start of the trial (T0), whereas the second group received a second PDT procedure 6 months later (T6).

The clinical pharmacy from our hospital performed the randomization procedure. A computer program automatically generated a randomization list with a study number and the patients were randomized accordingly. Owing to the nature of the treatment, blinding of the patients was not possible. The physician, however, was blinded for the treatment arm at the follow-up visits and the patients were requested not to inform the physician which arm had been treated. Follow-up visits were scheduled every 3 months during 2 years. At the start of the trial and at each follow-up visit, the skin of both forearms and hands was checked for the presence of squamous-cell carcinomas and any other possible type of skin cancer. All newly suspected lesions were evaluated histologically. Only histologically confirmed squamous-cell carcinomas were included in the study. A follow-up time of 24 months was completed to evaluate the occurrence of new squamous-cell carcinomas. The numbers of keratotic skin lesions on both forearms and corresponding hands were counted during a follow-up time of 12 months. To minimize inter-observer variation, the same physician evaluated each patient at all visits, with few exceptions.

The randomized-controlled trial had been preceded by an open non-randomized-pilot phase with five different organ-transplant recipients to optimize the PDT procedure itself. The same inclusion and exclusion criteria had been applied to the pilot study as for the later randomized-controlled trial. In the pilot study, only new skin cancers were evaluated during follow-up.

PDT treatment

The PDT treatment was adapted from a protocol used by our colleagues in Utrecht, The Netherlands (Dijkstra *et al.*, 2001). The clinical pharmacy from our hospital freshly produced the ALA formulation for each patient visit. Patients received topical cream containing 200 mg δ-ALA HCl per 1 g of Lanette cream base on the randomly allocated forearm and hand. After application of the cream, the forearm and hand were covered with a Tegaderm dressing, which was applied for a duration of 4 hours. The dressing was removed shortly before the irradiation procedure. The light source we used produced a wavelength band of 400–450 nm (Philips HPM-10, 400 W) (Dijkstra *et al.*, 2001). The duration of the irradiation procedure was 17 minutes, resulting in a total light dose of 5.5–6 J/cm^2. The patients were instructed to cover the treated arm

and hand after the PDT procedure for the rest of the day to avoid extra irradiation.

We decided not to pretreat the keratotic skin lesions with curettage, because this was not feasible considering the large amount of keratotic skin lesions in our patients. Other ''field'' treatments, such as 5-fluorouracil or imiquimod cream, were not allowed during the 2-year post-treatment period.

Statistical analysis

The sample size was calculated based on the findings of a previous double-blind placebo-controlled study assessing the efficacy of acitretin in the prevention of new squamous-cell carcinomas (Bouwes Bavinck *et al.*, 1995). We had observed that in the placebo group within a 6-month period, nine out of 19 patients had developed altogether 15 new squamous-cell carcinomas anywhere on the body.

Based on these findings, it was expected that, within a 2-year follow-up period, at least 50% of the organ-transplant recipients would develop one or more squamous-cell carcinomas on both forearms or hands (25% per arm and hand). In order to have an effective preventive treatment, we stipulated that only 5% of the treated arms were allowed to develop new squamous-cell carcinomas compared to 25% in the control arm during the same 2-year follow-up period. With a power of 90% and a significance level of 0.05, we calculated that 45 patients would be sufficient to distinguish significantly between a skin cancer occurrence (at the 2-year follow-up) of 5% in the treated arm and 25% in the untreated arm (Figure 1a).

For the statistical analyses, we used SPSS version 12.0.1 for Windows. The difference regarding the number of new squamous-cell carcinomas between the PDT-treated and the control arm was calculated with a Wilcoxon's signed rank test.

For the effect of the PDT treatment on the number of keratotic skin lesions, we used a linear mixed model approach with fixed time effects for repeated measurements based on the differences in the number of keratotic skin lesions between the PDT-treated arm and hand and the control arm and hand at different time points. We used a summary measure approach based on Wilcoxon's rank-sum test on regression slopes from simple linear regression of individual patients to assess whether there was a difference in the time course of change of number of lesions for arms and hands receiving one or two PDT treatments after 9 (T9) and 12 months (T12) of follow-up.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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CHAPTER 7

The occurrence of residual or recurrent squamous-cell carcinomas in organ-transplant recipients after curettage and electrodessication

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The occurrence of residual or recurrent squamous cell carcinomas in organ transplant recipients after curettage and electrodesiccation

Y.G.L. de Graaf, V.R. Basdew, N. van der Zwan-Kralt, R. Willemze and J.N. Bouwes Bavinck

Department of Dermatology, Leiden University Medical Centre, Albinusdreef 2, PO Box 9600, 2300 RC Leiden, the Netherlands

Correspondence
Y.G.L. de Graaf.
E-mail: *y.g.l.de_graaf@lumc.nl*

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curettage and electrodesiccation, organ transplant recipients, recurrence rates, squamous cell carcinoma

Conflicts of interest
None declared.

Summary

Background Organ transplant recipients frequently develop multiple squamous cell carcinomas (SCCs). Surgical excision and Mohs micrographic surgery are frequently used treatments for these carcinomas; however, curettage and electrodesiccation are a useful alternative in these patients.

Objectives To evaluate the efficacy of curettage and electrodesiccation for the treatment of appropriately selected low-risk SCCs in organ transplant recipients at different sites.

Methods Between April 1989 and December 2004, 211 SCCs in 48 organ transplant recipients were treated by curettage and electrodesiccation. Only histologically confirmed SCCs were considered in this study. The charts of these patients were retrospectively reviewed and checked for the rate of residual or recurrent SCCs. The occurrence of residual or recurrent SCCs at different locations after treatment of SCCs with curettage and electrodesiccation was estimated with Kaplan–Meier survival analysis.

Results The mean follow-up time after curettage and electrodesiccation of the individual SCCs was 50 months (median 41; range 3–186). In total, 13 residual or recurrent SCCs were observed in 10 patients. The overall rate of residual or recurrent SCCs was 6%, with 7% for SCCs on the dorsum of the hands or fingers, 11% for SCCs on the head and neck, 0% for the forearms, and 5% for the remaining nonsun-exposed areas (shoulder, legs). No major clinical or cosmetic adverse events were registered after treatment.

Conclusions In organ transplant recipients with many SCCs curettage and electrodesiccation can be a safe therapy for appropriately selected low-risk SCCs, with an acceptable cure rate.

Organ transplant recipients are at an increased risk of developing nonmelanoma skin cancer, of which cutaneous squamous cell carcinomas (SCCs) are the most prevalent tumours.[1,2] In these immunocompromised patients SCCs appear to be more aggressive than SCCs in immunocompetent patients, and multiple tumours frequently develop in short periods of time.[3]

SCCs in organ transplant recipients are often treated by surgical excision with histological examination.[4] Moh's micrographic surgery can be performed in high-risk tumours.[1] Recently, two international groups of mainly dermatologists published guidelines about treatment and prevention of SCC in the transplant population and recommended that these lesions should be managed by destructive or excisional modalities.[5,6]

Curettage and electrodesiccation is a treatment option in selected tumours, for example nonulcerated SCCs with well-defined margins, smaller than 2 cm and localized on low-risk locations such as the trunk and extremities.[6] Successful outcome is associated with the physician's experience.[6,7]

Large case series in immunocompetent patients are available that report on the efficacy of curettage and electrodesiccation for the treatment of skin cancer.[8] However, there are few data published on the specific outcome after curettage and electrodesiccation of different clinical types of SCCs and larger tumours.[9] A study in which 981 SCCs were treated with curettage and electrodesiccation reported recurrence rates of 1·3–3·7%.[10] Three larger series of 947, 894 and 104 cases, respectively, of both SCCs and basal cell carcinomas that were treated with curettage and electrodesiccation, reported excellent 5-year cure rates ranging from 96% to 100%.[11–13]

As far as we know there are no studies examining curettage and electrodesiccation as a treatment of SCCs in organ

transplant recipients. In the literature, there is only one case of multiple SCCs in an organ transplant recipient that were successfully treated by curettage.[14] Although not substantiated by the literature, curettage and electrodesiccation have been widely used in organ transplant recipients, usually for superficial or early skin cancers.[15] The purpose of this retrospective follow-up study was to evaluate the cure and recurrence rate of SCCs after treatment with curettage and electrodesiccation in organ transplant recipients and to compare the cure and recurrence rates at different skin locations.

Patients and methods

Since 1966, roughly 2000 patients received a kidney or kidney–pancreas transplant at the Leiden University Medical Centre (LUMC), Leiden, the Netherlands. Approximately 200 organ transplant recipients with skin problems were regularly seen at the Department of Dermatology at the LUMC. Liver and heart transplant recipients were also seen occasionally; these had received their organs at other centres. Initially, all SCCs were treated by surgical excision. In April 1989 we started to treat some SCCs with curettage and electrodesiccation. Gradually this treatment became a more common scenario in our clinic in appropriately selected low-risk SCCs.

Low-risk SCCs were selected based on clinical grounds only: we used curettage and electrodesiccation in tumours which were less than 2 cm in size, which had developed within less than 3 months, and which did not clinically appear to infiltrate into the deeper tissues. After local anaesthesia most of the tumour mass was removed with a scalpel and this material was always sent for histological examination. The remaining tumour mass was removed with a curette and the bottom and the margins of the tumour were subjected to electrodesiccation. The procedure of curettage and coagulation was repeated several times.

Only SCCs confirmed by histological examination and treated with curettage and electrodesiccation between April 1989 and December 2004 were included in the study. All patients in the study were routinely seen in the outpatient dermatology clinic of the LUMC at 3-monthly intervals or more frequently when indicated. Their medical records were reviewed. Data were collected on localization of the tumour; possible residual or recurrent SCC, and time period to this occurrence; length of follow-up; and complications of treatment.

Using curettage and electrodesiccation, the difference between noncured or residual tumours and *de novo* or recurrent tumours cannot be made clearly based on clinical or histological grounds. An SCC was considered not to be cured or to recur if a histologically confirmed SCC occurred at the same location as the primary tumour during the follow-up period. Most of the time the locations of new SCCs were clearly different compared with the initial SCC. If there was any doubt about the location, the new SCC was considered to be a residual or recurrent SCC. Sun-exposed skin was defined as skin on the dorsum of the hands and fingers, forearms, and the head and neck region; nonsun-exposed skin was defined as all remaining locations.

Data were analysed using SPSS version 12·0 for Windows (SPSS, Chicago, IL, U.S.A). The rate of residual or recurrent SCCs after treatment of SCC by curettage and electrodesiccation was calculated with a Kaplan–Meier survival analysis. The date of the curettage and electrodesiccation was used as the opening date for this calculation. As the closing dates we used the date of the histological diagnosis of the residual or recurrent SCC, the patient's death, or the last visit of the patient in our outpatient clinic. Survival functions for the different locations of the SCCs were compared using the log rank test.

Results

Altogether, 211 SCCs occurring in 48 organ transplant recipients were treated with curettage and electrodesiccation in our Medical Centre between 1989 and the end of 2004. All the lesions with a few exceptions were treated by one experienced dermatologist (J.N.B.B.) or under the direct supervision of this dermatologist.

The main characteristics of the patients with and without residual or recurrent SCCs are depicted in Table 1. The 48 patients were followed for a mean ± SD period of 73 ± 48 months (median 70, range 3–186) as the first SCC was treated with curettage and electrodesiccation.

The mean follow-up period of the individual 211 SCCs was 50 months (median 41, range 3–186). Residual or recurrent SCCs were observed in 10 of 48 patients. Patients with residual or recurrent SCCs tended to be younger, were more often female and had more SCCs in their medical history (Table 1). These differences did not reach statistical significance with the exception of the total number of SCCs treated with curettage and electrodesiccation, which was much higher in the patients with recurrent SCCs (Table 1).

Most of the SCCs treated with curettage and electrodesiccation were located on sun-exposed skin (n = 129, 61%), and predominantly on the dorsum of the hands and fingers (n = 81, 38%). The tumour characteristics for the different skin localizations are displayed in Table 2.

Residual or recurrent SCCs were clinically and histologically documented in 13 (6%) of the 211 treated SCCs occurring in 10 of the 48 patients (Table 3). The mean ± SD time to recurrence was 10 ± 10 weeks (median 8, range 2–40). All residual or recurrent skin cancers were treated by surgical excision and we did not observe any additional recurrences after this excision during the follow-up period of between 8 and 128 months (Table 3). Four patients died from non-SCC-related causes during the follow-up period. One of these patients had a residual or recurrent SCC on the left temple, 11 years before his death.

The rate of residual or recurrent SCC after treatment with curettage and electrodesiccation of the SCCs for the different locations is shown in Figure 1. The differences were not statistically significant (P = 0·44). The majority of the residual or recurrent SCCs, 11 of 13, developed within 12 weeks of

Table 1 Characteristics of the patients

	Without residual or recurrent SCCs	With residual or recurrent SCCs
Total number of patients	38	10
Sex: M/F	22/16	3/7
Type of transplantation		
Kidney	33	9
Kidney-pancreas	3	1
Liver	1	0
Heart	1	0
Time period after transplantation at last outpatient visit (years), mean ± SD (range)	24 ± 8 (7–38)	27 ± 7 (12–37)
Age at last outpatient visit (years), mean ± SD (range)	62 ± 9 (43–79)	56 ± 10 (44–72)
Age at time of first SCC (years), mean ± SD (range)	52 ± 10 (36–77)	47 ± 8 (37–62)
No. of SCCs per patient (total), mean ± SD (range)	8 ± 8 (1–38)	18 ± 11 (6–39)
No. of SCCs per patient treated with curettage and electrodesiccation, mean ± SD (range)[a]	3 ± 3 (1–18)	10 ± 7 (3–19)
Follow-up time since first SCC treated with curettage and electrodesiccation (months), mean ± SD (range)	70 ± 50 (3–186)	84 ± 39 (26–145)

SCC, squamous cell carcinoma. [a]The differences between the two groups were not statistically significant with the exception of number of SCCs per patient treated with curettage and electrodesiccation ($P = 0{\cdot}01$).

Table 2 Tumour characteristics

Location of SCCs	No. of primary SCCs (%)	No. of residual or recurrent SCCs	Time to residual or recurrent SCCs (weeks)
Dorsum of the hand and fingers	81 (38%)	6 (7%)	2, 6, 8, 9, 16, 40
Head and neck	28 (13%)	3 (11%)	5, 8, 8
Forearms	20 (10%)	0	
Remaining locations	82 (39%)	4 (5%)	3, 6, 10, 11
Total	211 (100%)	13 (6%)	

SCC, squamous cell carcinoma.

Table 3 Characteristics of patients with residual or recurrent SCCs after curettage and electrodesiccation

Patient no.	Sex	Age (years)	Localization of SCC and histological type	Time to residual or recurrent SCCs (weeks)	FU after excision residual or recurrent SCC (months)
1	M	42	Finger web (I-II) right hand[a]	2	25
2	M	55	Dorsum left hand[a]	8	33
		57	Left shoulder[a]	3	8
3[c]	M	50	Left temple[b]	5	128
4	F	45	Dorsum left hand[a]	6	85
5	F	49	Ventral part right upper leg[a]	6	26
		50	Right shoulder[a]	11	16
6	F	55	Frontal part of the scalp[a]	8	82
		55	Anterior part of the scalp[b]	8	82
7	F	39	Dorsum left hand[a]	9	53
8	F	66	Right lower leg[a]	10	81
9	F	67	Third finger right hand[a]	16	65
10	F	39	Third finger right hand[a]	40	83

SCC, squamous cell carcinoma; FU, follow-up. [a]Well-differentiated SCC; [b]poorly differentiated SCC; [c]patient died at age 61 years from other cause.

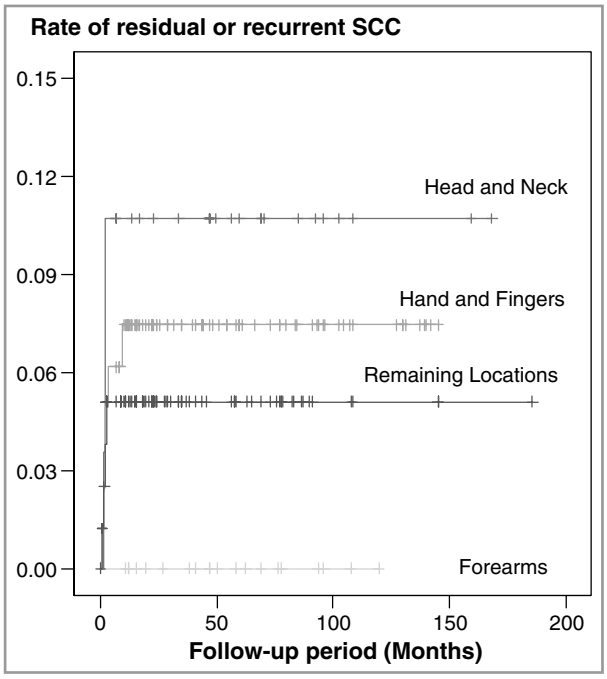

Fig 1. Kaplan–Meier survival analyses showing residual or recurrent SCCs on different locations of the body. The censored values are indicated.

follow-up; two developed within 40 weeks of follow-up. After this time period no additional residual or recurrent SCCs were observed (Fig. 1). No major clinical or cosmetic adverse events were registered after treatment.

Discussion

Our data show that curettage and electrodesiccation performed by a person with experience in this procedure is an effective treatment for SCCs in organ transplant recipients with multiple appropriately selected low-risk SCCs, with a low rate of residual or recurrent SCC of 6%. This rate is slightly higher than the rate of 1·3–3·7% in earlier case series with immunocompetent patients.[10–13]

Although we treated a limited number of SCCs in the head and neck region without encountering significant problems, these high-risk SCCs are usually not recommended for treatment with curettage and electrodesiccation, because of the more aggressive nature of these SCCs and a higher risk of metastases. Therefore, this procedure should be discouraged for the head and neck region until the safety of curettage and electrodesiccation in these locations has been proved.

Curettage and electrodesiccation have many advantages. It should be emphasized, however, that curettage and electrodesiccation should only be performed by somebody with experience in this procedure. The cosmetic result is generally good or excellent and it is a relatively easy procedure. Clinical diagnosis, biopsy and definitive treatment can be completed in one visit. Therefore, it is possible to treat more lesions at the same time. Furthermore, no sutures have to be removed afterwards. All this makes curettage and electrodesiccation convenient for the patient who usually has more than one SCC.

SCCs on the dorsum of the hands and fingers may require reconstructive surgery or the use of a skin graft. This may necessitate a short stay in the hospital and sometimes complete anaesthesia. Most of the appropriately selected low-risk SCCs on the dorsum of the hands and fingers can be treated effectively with curettage and electrodesiccation, which is a much simpler procedure. In case of a residual or recurrent SCC reconstructive surgery is still a good option.

The main disadvantage of curettage and electrodesiccation is the lack of histopathological evaluation of the tumour margins. If a patient is examined regularly, this should not form a major problem. Other disadvantages of curettage and electrodesiccation are slow healing, the possibility of impaired wound healing, the increased risk of superficial infections and the risk of hypopigmentation and more prominent scars as a result of the procedure.

Remarkably, nearly all recurrences were observed within the first 12 weeks after treatment. This suggests that most 'recurrences' can be regarded as residual tumour. We did not observe any residual or recurrent SCCs later than 10 months after treatment, suggesting that in the case of tumour cells remaining, regrowth will occur rapidly, usually within weeks or at the most within several months after the procedure. We can conclude that the most critical period for evaluation is the first year after treatment, but patients should continue to be monitored regularly.

This is the first study that reports on the efficacy of curettage and electrodesiccation for SCCs in organ transplant recipients. A substantially long follow-up time was completed. Prospective randomized controlled trials are the best way to compare two treatment modalities, but this retrospective noncomparative study also provides valuable information. A randomized controlled trial with sufficient power to distinguish between a rate of residual or recurrent SCC of 2% and 6% after surgical excision and curettage and electrodesiccation, respectively, would require 424 SCCs in each treatment arm (power calculation with $\alpha = 0{\cdot}05$ and $\beta = 0{\cdot}8$), which is practically impossible to perform in one centre. In addition, the question can be asked whether a rate of residual or recurrent SCC of 2% instead of 6% would be clinically relevant, if excision offers the patient a second chance for complete cure, anyway.

In conclusion, based on the low rate of residual or recurrent SCC and the absence of significant clinical or cosmetic adverse events, we recommend curettage and electrodesiccation for organ transplant recipients who develop multiple appropriately selected low-risk SCCs, provided that the procedure is performed by an experienced person.

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CHAPTER 8

Summary and general discussion

Introduction

Skin carcinomas develop at a high rate in organ-transplant recipients who are kept on immune suppressive drugs to prevent graft rejection. The present study dealt with a broad range of aspects of this elevated carcinoma risk, starting from the earliest oncogenic events to the ultimate therapy. Advancements on any of these aspects may be of significant benefit to the patient and his/her physician in the management of multiple and progressive skin carcinomas.

The studies presented in Chapter 2 - 4 focused on the early pathogenesis of skin cancer in organ-transplant recipients to gain a better understanding of the underlying mechanism(s) of the increased skin cancer risk in these patients. We specifically focused on the role of p53 and beta-PV in early skin carcinogenesis. The clinical studies in Chapter 5 - 7 investigated the management of skin cancer in organ-transplant recipients.
Here we summarize our most important findings and place these findings in a broader perspective.

Early oncogenic events: p53

Mutations in the p53 tumour suppressor gene, often leading to overexpression of the protein, appear to constitute an almost obligatory step in the development of squamous-cell carcinomas by chronic sun (UV) exposure. P53 patches are defined as clusters of epidermal cells that overexpress the p53 protein, and appear to be early microscopic precursor lesions of actinic keratoses and skin cancers.[1] We, therefore, investigated the early occurrence of p53 patches occurring in organ-transplant recipients (**Chapter 2**). Additionally, we studied a possible immune reactivity against the resulting overexpressed p53 protein in the same category of patients (**Chapter 3**).

P53 patches and immunosuppression

In **Chapter 2** we showed that p53 patches were more frequently present adjacent to skin cancers of renal-transplant recipients than of immunocompetent patients, indicating that p53 patches are more frequently present in immunosuppressed patients.
As the majority of the organ-transplant recipients in our study were treated with the classic immunosuppressant azathioprine, we investigated the effect of this drug on experimental p53 patch induction by chronic UV exposure in hairless mice. In earlier experiments, azathioprine had been shown to accelerate UV carcinogenesis in the hairless mouse model.[2] Since p53 patches precede the development of actinic keratoses and skin cancer one would expect an increased number of p53 patches in UV-irradiated mice immunosuppressed with azathioprine. In our study, however, we did not find an increase in p53 patches after daily UV irradiation in mice immunosuppressed with azathioprine compared with control mice. Hence, unlike the skin carcinomas, the number of p53 patches did not appear to be accelerated by immunosuppression of the mice. This is consistent with the results of another study in which the mutant p53 foci developed and regressed equally fast in immunocompromized RAG-1 mice and in wild-type mice.[3] These findings indicate that p53 patches are not subjected to immunosurveillance and elimination.

Azathioprine inhibits DNA Repair

We were able to demonstrate the inhibiting effect of azathioprine on DNA repair of UV-induced DNA damage in primary human keratinocytes, as measured by unscheduled DNA synthesis (UDS) (**Chapter 2**). In humans UDS is dominated by a repair of cyclobutane pyrimidine dimers (CPD), whereas in mice the carcinogenic CPD are hardly repaired and UDS is therefore dominated by repair of less abundant DNA lesions (6-4 photoproducts).[4,5] In humans, an azathioprine-related decreased repair of CPD is likely to explain increased amounts of p53 patches in renal-transplant recipients. In mice, on the other hand, CPD repair is already poor and consequently azathioprine will not have any apparent impact on CPD-driven mutagenesis and p53 patch formation

in mice. Thus, our results indicate that carcinoma risk in renal-transplant recipients, is, at least in part, related to an increase in p53 patches, which in turn is attributable to a direct adverse effect on DNA repair from azathioprine (and similar effects from cyclosporine and tacrolimus (Prograft®) [6-8]).

In contrast to the classic immunosuppressive agent azathioprine and the calcineurin inhibitors cyclosporine and tacrolimus, the novel immunosuppressive agents, mycophenolate mofetil (MMF, Cellcept®) and sirolimus (rapamycin, Rapamune®) and everolimus appear to impair tumour growth. However, the impact of these novel immunosuppressive agents on DNA repair and early stages of UV-induced carcinogenesis has not been evaluated. On forehand, mTOR (mammalian target of rapamycin) inhibitors such as sirolimus and everolimus, act through pathways (down-stream Akt), which are not known to affect DNA repair. Additionally, there are reports that sirolimus enhances apoptosis in p53 dysfunctional cells.[9]

We would therefore expect that sirolimus carries an importantly lower risk from local adverse effects in skin cells, when compared with the classical immunosuppressive agents. (i.e. lower mutation rate, fewer p53 patches and correspondingly fewer skin carcinomas) Moreover, the mTOR inhibitors inhibit angiogenesis and tumour growth, resulting in a further anticipated beneficial effect against tumour formation.[10] This is currently studied in animal experimental studies running in our institute and in several multicentre clinical studies.

Azathioprine and p53 mutations in skin carcinomas

Early studies showed the typical UVB-related p53 mutations in a majority of squamous-cell carcinomas[11,12], and "founding" p53 mutations were found to be commonly present throughout the tumour mass.[13,14] However, a recent study differed in its results and found disjunctive regions with independent p53 mutations in tumour masses of squamous-cell carcinomas, i.e. no indication of a founder p53 mutation, and a predominance of UVA-related p53 mutations in the basal layers of the tumour.[15] With a lowered DNA repair, owing to either azathioprine or cyclosporine, one would suspect an enhanced induction of UVB-related p53 mutations and ultimate carcinomas dominated by such founder mutations sprouted from p53 patches. With photosensitization of the DNA to UVA radiation by azathioprine, one would anticipate mutations in relation to potential thio-guanine sites, i.e. a shift away from UVB-like mutations. We therefore performed a small pilot study on 5 squamous-cell carcinomas from organ-transplant recipients. Mutation analysis was performed for exons 4b to 8 of the p53 gene from 4 different regions in each tumour mass. The results are shown in Table 1 (unpublished data). Four out of 5 squamous-cell carcinomas (80%) showed at least one p53 mutation present in at least two regions of the tumour. Two tumours showed at least one p53 mutation consistently present in all 4 regions dissected from the tumour, i.e. the apparent presence of a founder mutation.

In these 5 squamous-cell carcinomas a total of 13 p53 mutations were found of which only 2 (15%) were UVB-specific (Table 1). Hence, the p53 mutation spectrum that we found suggests a deviation from what is normally encountered in squamous-cell carcinomas from immunocompetent patients (in general 60% C $\rightarrow$ T transitions and 10% CC $\rightarrow$ TT tandem mutations).[11,12] Interestingly, 4 out of 5 patients that we studied used azathioprine. These data suggest that azathioprine might cause alterations in the p53 mutation spectrum. In an earlier report, McGregor et al.[16] found a total of 12 mutations in 23 squamous-cell carcinomas from renal-transplant patients. The majority of mutations were UVB-specific, although no characteristic CC $\rightarrow$ TT mutations were present. Unfortunately, the medication (azathioprine or otherwise) was not taken into account, and, as in our study, the number of mutations was probably too small. Future studies are clearly needed to infer the impact of immunosuppressive drugs, most specifically azathioprine, on the p53 mutation

Table 1. Distribution of P53 mutations in squamous-cell carcinomas of organ-transplant recipients.

Patientno.	medication	exon	intron	mutation	amino acid	codon	no. of sites in tumour with mutation
1	P+M	4		gtCtggg→gtGtggg	Leu→Val	111	3
		4		caGAggc →caATggc	Glu →Met	68	2
			4	ttgcCctga →ttgcActga			4
			5	acaGtac →acaAtac		*	3
2	P+A						
3	P+A	4		caGAggc →caATggc	Glu →Met	68	2
		8		tgCgtgt →tgTgtgt**	Arg →Cys	273	2
			4	ttgcCctga →ttgcActga			2
			6	tgcAact →tgcTact			1
4	P+A	4		caGAggc →caATggc	Glu →Met	68	3
		5		caCcccc →caTcccc**	Pro →Ser	151	4
			4	ttgcCctga →ttgcActga			4
5	P+A	4		tttCCgtc →tttATgtc	Phe →Tyr	109	3
					Arg →Cys	110	3
			4	ttgcCctga →ttgcActga			2

Abbreviations: P=prednisone; M=mycophenolate mofetil; A=azathioprine
*mutation at splice site
**C →T transition

spectrum and founder or later stage p53 mutations in skin carcinomas.

Immune reaction to p53

Overexpression of mutant p53 may lead to immune reactions against p53, as was found in patients with colon cancers. A p53-positive serology in these patients spelled a poor prognosis.[17] We posed the question whether abundant overexpression of p53 in skin lesions of renal-transplant recipients would lead to p53 positive serology and whether the serology would be an early indication of malignant progression.

In **Chapter 3** we showed that p53-specific serum antibodies were not associated with a history of skin carcinoma in renal-transplant recipients and immunocompetent individuals. The increased number of p53 patches and actinic keratoses in renal-transplant recipients clearly did not induce a humoral response against p53, in contrast to colon carcinomas with obviously larger tumour masses, more necrosis and inflammation.

A recent study showed increased p53-specific CD8+ T-cell responses in immunocompetent individuals with cutaneous squamous-cell carcinoma compared with controls.[18] Although the cellular immunity is suppressed in organ-transplant recipients, it would be interesting to compare p53-specific T-cell responses in organ-transplant recipients with and without skin cancer.

Early oncogenic events: beta-PV and apoptosis

Besides the epidemiologic evidence for a role of beta-PV in skin carcinogenesis, recent experimental studies provided evidence of oncogenic properties of beta-PV types: E6 proteins of some beta-PV types block the pro-apoptotic protein Bak, which impairs apoptosis in response to UV exposure.[19] In our study, described in **Chapter 4**, we could find no evidence in-vivo that beta-PV infection lowered the apoptotic response of UV-irradiated epidermis. However, the quantity of beta-papillomavirus DNA in our skin samples was very low and often below detection limit of quantitative PCR. (The loads were in the range of those reported in another study.[20]) The keratinocytes containing beta-PV DNA were sparse among an abundance of non-infected cells. Furthermore, probably not all beta-PV types carry a high carcinoma risk and exert a similar effect on UV-induced apoptosis. It has recently been reported that the E6 and E7 proteins of the beta-PV type 38 display in-vitro transforming properties[21] by selectively activating transcription of ΔNp73, an isoform of the p53-related protein p73. As a result, transcriptional functions of p53 involved in growth suppression and apoptosis are impaired.[22]

As an important side product of our study, we found clear evidence of a decrease in UV-induced apoptotic response with increasing age. Such a decline in clearance of damaged cells may enhance the cancer risk but also extend longevity of the tissue.[23]

We chose unexposed buttock skin to ascertain how immunosuppression would affect basic levels of beta-PV infection, i.e., in absence of regular solar UV exposure. We did find beta-papillomaviruses to be present at higher densities in the organ-transplant recipients than in controls, but the difference was not statistically significant and the density in organ-transplant recipients was still extremely low. Nor did we measure any effect on beta-PV infection from a single UV exposure. Possibly, a single UV exposure is simply not sufficient, and multiple exposures might have led to a more pronounced effect.

In our study we found a tendency for a higher prevalence of beta-PV in forearm skin compared to buttock skin, which is consistent with an earlier study that reported a higher prevalence of beta-PV in chronically sun-exposed lesional skin compared to non-exposed skin.[24] This is in line with our supposition that chronic sun-exposure may induce beta-PV replication. It has been shown that at least beta-PV types 5, 8 and 77 have UV responsive elements.[25,26]

To ascertain the effect of beta-PV infection on UV-induced apoptosis in-vivo, studies

need to focus specifically on beta-PV-carrying cells, preferably after repeated UV exposures. It is recommended to consider also the influence of the different immunosuppressive agents on UV-induced apoptosis in future studies.

Early oncogenic events: beta-PV and DNA repair

Beta-PV could also contribute to tumour development by interfering with the DNA damage repair process. It recently has been reported that thymine dimers repair after UV irradiation was impaired in cells expressing the E6 protein of beta-PV type 5.[27]

In addition, it has been shown that the E6 protein of beta-PV types 1 and 8 binds and inhibits the human XRCC1 protein, which is required for the repair of single-strand breaks and genetic stability.[28]

Possibly, the repair of DNA damage is impaired because of the interference of certain beta-PV types. However, the exact role of beta-PV in the etiology of skin carcinoma development is still uncertain. Additional studies are required to understand the role of beta-PV and the possible interaction with other factors in skin cancer development.

Management of skin cancer in organ-transplant recipients

Two international groups of mainly dermatologists published guidelines on prevention and treatment of squamous-cell carcinomas in the transplant population.[29,30] We contributed to improved management of skin cancer by studying both retinoids and photodynamic therapy as preventive modalities, and the use of curettage and electrodessication as a treatment of squamous-cell carcinomas in organ-transplant recipients. Tables 2 and 3 present the Leiden clinical guidelines for prevention and treatment of skin cancer in organ-transplant recipients adapted from two international guidelines.[29,30]

Prevention

To prevent the development of skin carcinomas, cryosurgery, topical 5-fluorouracil and curettage with electrodessication are recommended to treat premalignant lesions.[30] Concerning the use of imiquimod, photodynamic therapy and retinoids in the prevention of skin cancer, there is no consensus in the guidelines.[30]

In **Chapter 5** we summarized the studies on the use of retinoids in the chemoprevention of skin cancer in organ-transplant recipients. We can conclude that low-dose retinoids, in particular acitretin, are effective in inhibiting the development of skin cancer. Our conclusion was supported by a recent study in which organ-transplant recipients received retinoid treatment for up to 16 years.[31] Long-term use of systemic retinoids appears to be required. However, this may be limited by the occurrence of side effects in individual patients and a theoretical risk of graft rejection because of immunoactivation. Systemic retinoids, should therefore, in our opinion, be limited to patients with numerous hyperkeratotic skin lesions and/or at least one squamous-cell carcinoma in the medical history. The guidelines[29] are more strict and do only recommend systemic retinoids if multiple skin cancers have developed.

Although topical retinoids seem effective in the treatment of actinic keratoses and warts in organ-transplant recipients, the effect is clearly less than systemic retinoids. Following the guidelines, topical retinoids may be beneficial in patients with significant photodamage to normalize actinically damaged skin.[29]

Chapter 6 shows that photodynamic therapy with aminolevulinic acid and violet light (400-450 nm) does not appear to prevent the development of new squamous-cell carcinomas in organ-transplant recipients. We observed a small, but non-significant, effect on the keratotic lesions. An explanation for this is the location of treatment in our study: an inferior response to photodynamic therapy of thick keratotic skin lesions on the hands compared to the thinner lesions on the

Table 2. Guidelines for prevention of skin cancer in organ-transplant recipients (adapted from the Expert Group on Renal Transplantation[29] and Stasko et al[30]).

Primary prevention	• patient education on skin cancer risk and self examination • patient education on sun protection: avoidance of sun exposure prohibition of tanning beds use of protective clothing use of an effective UVB/UVA sunscreen (SPF>15) for sunexposed skin on a daily basis
Secondary prevention	• early referral to a dermatologist in case of premalignant lesions • treatment of premalignant lesions by: cryotherapy curettage & electrodessication 5-fluorouracil or possibly by imiquimod, PDT*, topical retinoids* • use of systemic retinoids, if well tolerated* • reduction of immunosuppression, if possible, by transplant physician

* Details on efficacy and indications are given in text

Table 3. Guidelines for treatment of skin cancer in organ-transplant recipients (adapted from the Expert Group on Renal Transplantation[29] and Stasko et al[30]).

Diagnosis	Treatment of 1st choice	Additional modalities
Basal-cell carcinoma	surgical excision curettage & electrodessication	
Squamous-cell carcinoma	surgical excision curettage & electrodessication*	resurfacing dorsum of hand
Recurrent squamous-cell carcinoma	surgical excision Mohs' surgery	reduction of immunosuppression systemic retinoids
Metastatic squamous-cell carcinoma	surgical excision Mohs' surgery lymphadenectomy	radiotherapy reduction of immunosuppression systemic retinoids

* Details on efficacy and indications are given in text

face has been demonstrated in immunocompetent patients.[32]
Moreover, our treatment protocol may not have been optimal, there was a 6-month period between the two treatments, which is rather long. In a recent study, photodynamic therapy with methyl aminolevulinic acid was shown to be more effective than 5-fluorouracil in treating premalignant skin lesions in 8 organ-transplant recipients.[33] However, the study design is completely different from ours and therefore the results are not comparable. Although the use of red light (570-750 nm), methyl aminolevulinic acid and pretreatment of thick hyperkeratotic lesions could have resulted in a better therapeutic outcome (by an optimal penetration of light and photosensitizer) in our study, it has to be considered that photodynamic therapy might be less effective in organ-transplant recipients compared with immunocompetent patients.[34,35] A possible explanation for this is the growing evidence that both innate and adaptive immune responses play a role in the outcome of photodynamic therapy.[36] In our opinion, photodynamic therapy with aminolevulinic acid and violet light is not a promising preventive therapy in organ-transplant recipients. Probably repeated treatments for large areas are necessary to prevent skin cancer in these patients, which is obviously expensive and painful. Obviously, future studies on the use of photodynamic therapy in immunosuppressed patients are warranted.

Treatment

In the above mentioned guidelines, it is recommended that all skin cancers in organ-transplant recipients should be completely removed with destructive or excisional modalities (Table 3).[29,30] Non-ulcerated squamous-cell carcinomas with well-defined margins that are smaller than 2 cm and localized on low-risk locations such as trunk and extremities could form an indication for curettage and electrodessication.[30] However, until now there is not much evidence for its efficacy as a treatment of skin carcinomas in organ-transplant recipients. In **Chapter 7** we demonstrated that curettage and electrodessication is an effective treatment for squamous-cell carcinomas in organ-transplant recipients with multiple tumours with a low recurrence rate of 6%. The cosmetic result was good and a substantially long follow-up period was completed (50 months per tumour). Nearly all recurrences in our study were observed within the first 12 weeks after treatment. This suggests that most recurrences can be regarded as residue tumours and that in case of presence of residual tumour cells, outgrowth will occur rapidly.
A disadvantage of this treatment is the lack of histopathological evaluation of the tumour margins, and therefore patients should be monitored regularly after treatment with curettage and electrodessication. Recurrent tumours should be treated with surgical excision. Although we did not encounter significant problems in treating squamous-cell carcinomas in the head and neck region, we do not recommend curettage and electrodessication, because of a more aggressive nature of these squamous-cell carcinomas and a higher risk of metastases.[30] In conclusion, we can recommend curettage and electrodessication as a treatment of squamous-cell carcinomas at trunk or extremities in organ-transplant recipients.

Follow-up strategy

Recommended follow-up intervals for dermatologic examinations should be communicated to both patients and transplant physicians.[37] These intervals have to be determined by the individual's risk for skin cancer development.[30] In general, it is recommended to examine patients without skin cancer or actinic keratoses every year, patients with actinic keratoses and/or one skin cancer every 6 months and patients with multiple or high-risk skin cancers should be examined every 2 to 4 months. Patients without skin cancer and actinic keratoses can be examined annually by their transplant physician until lesions arise.[30,37]

Conclusion and perspectives

In the first part of this thesis it was demonstrated that the number of p53 patches is increased in renal-transplant recipients compared to immunocompetent individuals. However, an increased number of p53 patches was not found in immunosuppressed mice treated with azathioprine. Unlike the skin carcinomas, the number of p53 patches did not appear to be accelerated by immunosuppression of the mice. It was also demonstrated that azathioprine inhibited the DNA repair of UV-induced DNA damage in primary human keratinocytes. Thus, the carcinoma risk in renal-transplant recipients appears to be related -at least in part- to an increase in p53 patches. And the presence of these p53 patches may be attributed to a direct adverse effect of immunosuppressive agents as azathioprine on DNA repair. Additionally, the hypothesis that beta-papillomaviruses decrease apoptosis could not be confirmed in our in-vivo study. We did find a negative correlation between age and UV-induced apoptosis.

In the second part of this thesis it was shown that systemic retinoids are effective in inhibiting skin cancer development in organ-transplant recipients, in contrast to photodynamic therapy, which only had a small (non-significant) effect on the keratotic lesions. Furthermore, it was demonstrated that curettage and electrodessication is an effective treatment with a low recurrence rate for squamous-cell carcinomas in organ-transplant recipients.

Considering the harmful effects of the classic immunosuppressives azathioprine and cyclosporine and the growing evidence of anti-neoplastic properties of the novel immunosuppressive agents mycophenolate mofetil and sirolimus, future studies should be aimed at the effect of these immunosuppressive agents on early UV-induced skin carcinogenesis. Besides p53 patches, these studies should focus on cyclobutane pyrimidine dimers and other photolesions (e.g. caused by phototoxicity) These photolesions are expected to be more persistent in skin of organ-transplant recipients compared to immunocompetent patients.

Future studies should establish the exact role of beta-PV in the early pathogenesis of skin carcinomas in organ-transplant recipients. The impact of beta-PV infection on UV-induced apoptosis in-vivo should be studied specifically in beta-PV carrying cells. Additionally, the role and the possible relation between beta-PV and p53 in tumour development needs to be established.

These studies may finally lead to possible interventions (to prevent skin carcinomas) directed against DNA-damaged cells by administration of repair enzymes. It may also lead to the development of an immunosuppressive regimen with minimum skin cancer risk and improved therapeutic modalities for skin carcinomas in organ-transplant recipients.

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Nederlandse samenvatting

Inleiding

Orgaan transplantatiepatiënten hebben een verhoogd risico op niet-gepigmenteerde huidkankers, in het bijzonder het plaveiselcelcarcinoom, wat gepaard gaat met een aanzienlijke toename van morbiditeit en mortaliteit. De frequentie van huidkanker neemt toe met de tijd na transplantatie. In landen met matige zonexpositie zoals Nederland is het risico op huidkanker 40% 20 jaar na transplantatie. In zonniger oorden, zoals Australië, loopt dit risico op tot 70%. Het plaveiselcelcarcinoom komt ongeveer 65 tot 250 keer zo vaak voor bij transplantatiepatiënten als in de algemene populatie. Bovendien kennen plaveiselcelcarcinomen in deze patiëntenpopulatie een agressiever beloop dan gewoonlijk, vooral als ze in het hoofd-halsgebied zijn gelokaliseerd. Er is een verhoogd risico op lokaal recidief en metastasen. Wratachtige afwijkingen, premaligne keratosen, de ziekte van Bowen en keratoacanthomen gaan gepaard met een verhoogd risico op huidkanker na transplantatie. Andere typen huidkanker komen ook vaker dan normaal voor bij transplantatiepatiënten; het Kaposisarcoom ongeveer 84 tot 113 maal, het basaalcelcarcinoom 10 maal en het maligne melanoom 2 tot 8 maal.

Het eerste deel van dit proefschrift richt zich op vroeg oncogene gebeurtenissen in de huid welke een rol kunnen spelen bij het verhoogde risico op huidkanker bij transplantatiepatiënten. In het bijzonder werd de rol van het p53 tumorsuppressor gen en infectie met beta-papillomavirussen in de vroege carcinogenese van de huid bestudeerd. Het tweede deel van het proefschrift handelt over de preventie van huidkanker bij transplantatiepatiënten door middel van fotodynamische therapie en retinoïden en vervolgens over de behandeling van huidkanker door middel van curettage en coagulatie.

Hoofdstuk 1 geeft een overzicht van het klinische probleem van huidkanker bij orgaan transplantatiepatiënten. De belangrijkste risicofactoren voor huidkanker bij deze patiëntengroep worden besproken; ultraviolet (UV) straling, immuunsuppressieve (afweeronderdrukkende) therapie en infectie met beta-papillomavirussen. Vervolgens wordt ingegaan op de preventieve en therapeutische modaliteiten welke in aanmerking komen bij deze patiëntengroep.

Pathogenese van huidkanker bij transplantatiepatiënten

Een van de belangrijkste risicofactoren voor niet-gepigmenteerde huidkanker bij transplantatiepatiënten wordt gevormd door UV straling. Dit wordt geïllustreerd door het feit dat bij transplantatiepatiënten premaligne afwijkingen, zoals actinische keratosen, en plaveiselcelcarcinomen vooral voorkomen op door de zon beschenen huid.

Mutaties in het p53 tumorsuppressor gen lijken een bijna onvermijdelijke vroege stap te zijn in de ontwikkeling van huidkanker door chronische UV blootstelling. P53 patches (P53 eilandjes), welke gedefinieerd worden als clusters van epidermale cellen die het p53 eiwit tot verhoogde expressie brengen, lijken vroege microscopische voorlopers van actinische keratosen en huidkanker te zijn. **Hoofdstuk 2** beschrijft een studie waarin onderzocht werd of het aantal p53 patches in huid van transplantatiepatiënten verhoogd is in vergelijking met immuuncompetente patiënten (patiënten met een normale afweer). Wij vonden bij onderzoek van de huid rond uitgenomen carcinomen dat p53 patches inderdaad frequenter aanwezig waren in transplantatiepatiënten dan in immuuncompetente patiënten.

Een andere belangrijke factor in de ontwikkeling van huidkanker bij transplantatiepatienten is de immuunsuppressieve therapie. Bij langdurig gebruik ontstaat een situatie waarbij het (vroegtijdig) opruimen van kankercellen door het immuunsysteem is verminderd. Bovendien kunnen geneesmiddelen zoals azathioprine en ciclosporine, onafhankelijk van het immuunsuppressieve effect, een direct kankerverwekkend effect op huidcellen uitoefenen. Aangezien de meerderheid van de transplantatiepatiënten ten tijde van onze studie behandeld werd met het klassieke immuunsuppressieve middel azathioprine,

werden vervolgens in **Hoofdstuk 2** twee mogelijke mechanismen bestudeerd waarmee azathioprine het aantal p53 patches zou kunnen verhogen: immuunsuppressie en een vermindering van DNA herstel.
Wij bestudeerden allereerst het effect van azathioprine op de experimentele inductie van p53 patches in de haarloze muis. Eerdere experimenten hebben laten zien dat azathioprine de huidkankervorming door UV-straling in de haarloze muis kan versnellen. In onze studie werd echter geen verhoogd aantal p53 patches gevonden in de dagelijks met UV bestraalde haarloze muis, waarbij de afweer onderdrukt werd met azathioprine, in vergelijking met controle muizen zonder azathioprine. Dit veronderstelt dat, in tegenstelling tot huidkanker, de vorming van p53 patches niet lijkt te worden versneld door immuunsuppressie in de haarloze muis. Dit is in overeenstemming met resultaten van een eerdere studie waarbij geen verschil werd gevonden in de snelheid van ontwikkeling van p53 patches tussen immuungecompromiteerde RAG-1 muizen en wild-type controle muizen. Een mogelijke verklaring voor deze bevinding zou kunnen zijn dat p53 patches niet onderhevig zijn aan herkenning of eliminatie door het immuunsysteem.
We toonden vervolgens aan dat azathioprine een remmende werking heeft op het herstel van schade geïnduceerd door UV straling in primaire humane keratinocyten, welke werd gemeten door middel van DNA herstel synthese (UDS) **(Hoofdstuk 2)**. In de mens wordt UDS gedomineerd door herstel van cyclobutaan pyrimidine dimeren (CPD), in tegenstelling tot de muis, in welke dieren deze carcinogene fotoproducten veel slechter worden gerepareerd en voornamelijk de minder aanwezige DNA laesies, 6-4 fotoproducten, worden gerepareerd. In de mens zou een verlaagd herstel van CPD, gerelateerd aan azathioprine, het verhoogde aantal p53 patches bij transplantatiepatiënten kunnen verklaren. In de muis echter, is het herstel van de CPD al minimaal en heeft azathioprine waarschijnlijk nauwelijks effect.
Onze bevindingen doen veronderstellen dat het verhoogde huidkankerrisico bij transplantatiepatiënten tenminste ten dele toe te schrijven valt aan het verhoogde aantal p53 patches. Deze patches kunnen op hun beurt weer deels worden toegeschreven aan een direct nadelig effect op het DNA herstel door middelen als azathioprine. In tegenstelling tot de klassieke immuunsuppressieve middelen azathioprine en ciclosporine, hebben de nieuwere immuunsuppressiva mogelijk een remmende werking op tumorgroei. Het effect op vroeg oncogene veranderingen als p53 patches is echter nog niet onderzocht.

Overexpressie van mutant p53 kan een immuunreactie tegen p53 tot gevolg hebben, zoals bekend is bij patiënten met darmkanker. De aanwezigheid van antistoffen tegen p53 voorspelde een slechte prognose bij deze patiënten. Wij onderzochten of een verhoogde p53 expressie in huidlaesies ook zou kunnen resulteren in de aanwezigheid van deze antilichamen. **Hoofdstuk 3** beschrijft de prevalentie van p53-specifieke antilichamen bij zowel niertransplantatiepatiënten als immuuncompetente personen met en zonder een plaveiselcelcarcinoom in de voorgeschiedenis. We vonden echter geen associatie tussen p53-specifieke antilichamen en plaveiselcelcarcinomen in niertransplantatiepatiënten en ook niet in immuuncompetente personen. Evenmin werd er een verschil gevonden in de hoeveelheid p53-specifieke antilichamen tussen de transplantatiepatiënten en immuuncompetente patiënten.

Een derde factor die mogelijk een rol speelt in de ontwikkeling van huidkanker bij transplantatiepatiënten is een infectie met betapapillomavirussen (beta-PV). Naast epidemiologische gegevens die een samenhang tonen tussen beta-PV infecties en plaveiselcelcarcinomen zijn er recentelijk ook dierexperimentele aanwijzingen voor een verband. Ook is onlangs in vitro aangetoond dat E6 eiwitten van enkele beta-PV typen het eiwit Bak kunnen remmen. Dit eiwit stimuleert apoptose, geprogrammeerde celdood. De gedachte is dat infectie met beta-PV de UV-geïnduceerde apoptose remt, waardoor de betreffende cel niet wordt opgeruimd en

waardoor genetische instabiliteit kan accumuleren in de celkernen. **Hoofdstuk 4** presenteert onderzoek naar beta-PV in voorheen niet door de zon beschenen huid bij zowel transplantatiepatiënten als gezonde vrijwilligers. In het bijzonder werd het effect van beta-PV op UV-geïnduceerde apoptose onderzocht. Daarnaast werd het effect van UVB blootstelling op de aanwezigheid van beta-PV geëvalueerd. In onze studie werd echter geen effect gevonden van beta-PV op de hoeveelheid cellen die in apoptose gaan. Een waarschijnlijke verklaring hiervoor is dat de hoeveelheid virus, welke werd gevonden in onze samples, zeer laag was, meestal beneden de detectie limiet van de kwantitatieve PCR. Bovendien is het niet noodzakelijk dat elk beta-PV type een hoog-risico type is en een vergelijkbaar effect op UV-geïnduceerde apoptose uitoefent. De spaarzame hoeveelheid cellen geïnfecteerd met beta-PV werd hoogstwaarschijnlijk overschaduwd door de aanwezigheid van vele niet-geïnfecteerde cellen. Het is dan ook aan te bevelen het effect van beta-PV op apoptose op cellulair niveau te onderzoeken. In onze studie vonden we evenmin een effect van een eenmalige UV blootstelling op de aanwezigheid van beta-PV. Dit kan verklaard worden door het feit dat één UV blootstelling niet afdoende is om een eventueel effect op beta-PV replicatie te bewerkstelligen. We vonden namelijk een tendens tot een verhoogde prevalentie van beta-PV infectie in de langdurig aan zon blootgestelde huid van de onderarmen met wratachtige huidafwijkingen en actinische keratosen.
Een opmerkelijke bevinding van onze studie in zowel transplantatiepatiënten als gezonde vrijwilligers was een verlaging van UV-geïnduceerde apoptose met toenemende leeftijd.

Preventie en therapie van huidkanker bij transplantatiepatiënten

Het belangrijkste onderdeel van de preventie van huidkanker bij transplantatiepatiënten is patiënteneducatie betreffende de risico's van blootstelling aan UV-straling en zonbeschermingsadviezen. Patiënten dienen voorafgaand aan de transplantatie al geïnformeerd te worden over het verhoogde risico op huidkanker. Educatie met betrekking tot zelfonderzoek van de huid is belangrijk, opdat (pre)maligne afwijkingen vroeg worden herkend en de patiënt tijdig de arts bezoekt.
Controle en behandeling van premaligne en maligne huidafwijkingen in een vroeg stadium zijn van groot belang. Transplantatiepatiënten met premaligne huidafwijkingen, zoals actinische keratosen, dienen dan ook naar een dermatoloog verwezen te worden. Voor chemopreventie van huidmaligniteiten zijn systemische retinoïden een optie. In **Hoofdstuk 5** wordt een overzicht gegeven van de werkzaamheid van topicale en systemische retinoïden in de preventie van huidkanker bij transplantatiepatiënten. Hieruit kan geconcludeerd worden dat retinoïden in lage doseringen, vooral acitretine, redelijk effectief zijn in het remmen van de ontwikkeling van huidkanker. Hierbij dient opgemerkt te worden dat langdurig gebruik van systemische retinoïden nodig lijkt. Dit wordt echter vaak bemoeilijkt door de bijwerkingen. Wij zijn van mening dat systemische retinoïden dan ook voorbehouden moeten zijn aan patiënten met meerdere hyperkeratotische laesies en minimaal één plaveiselcelcarcinoom in de voorgeschiedenis.
Hoofdstuk 6 beschrijft een gerandomiseerde gecontroleerde studie met gepaarde waarnemingen bij 40 transplantatiepatiënten, bij wie het effect van fotodynamische therapie op het ontstaan van nieuwe plaveiselcelcarcinomen op de zonblootgestelde huid werd onderzocht. In deze studie werd echter geen preventief effect gevonden van fotodynamische therapie met violet licht. Er werd een klein (niet-significant) effect gevonden op het aantal hyperkeratotische laesies. Een mogelijke verklaring hiervoor is de locatie van behandeling; het is bekend dat de dikkere laesies op de armen minder goed reageren op fotodynamische therapie vergeleken met de doorgaans vlakkere laesies op de schedel. Ons behandelprotocol is mogelijk niet optimaal geweest, een tussenpoos van 6 maanden is mogelijk te lang. Bovendien zou

voorbehandeling van de hyperkeratotische laesies met curettage, het gebruik van methylaminolevulinezuur en rood licht kunnen resulteren in een hogere penetratie en dientengevolge een beter effect. Meer onderzoek naar fotodynamische therapie bij immuungecompromiteerde patiënten lijkt dan ook nuttig.

Chirurgische excisie met postoperatieve snijrandbeoordeling is de eerste keus behandeling van huidkanker. Handrugtransplantaties, een procedure waarbij de gehele, actinisch zwaar beschadigde huid van de handrug wordt vervangen door weinig of niet beschadigde huid van bijvoorbeeld het bovenbeen of de nates, kunnen nuttig zijn bij patiënten bij wie veel plaveiselcelcarcinomen ontstaan in dit gebied. Deze behandeling heeft mede een preventief effect. Curettage en coagulatie vormt een behandelingsoptie bij geselecteerde tumoren op romp of extremiteiten. Echter over de werkzaamheid van deze behandeling bij transplantatiepatiënten was weinig bekend. In **Hoofdstuk 7** wordt een serie plaveiselcelcarcinomen van transplantatiepatienten beschreven die behandeld werden door middel van curettage en coagulatie, met het doel om het risico op een recidief of hernieuwde uitgroei na niet volledige verwijdering na deze behandeling te bepalen en deze voor verschillende locaties te vergelijken. Uit deze studie blijkt dat curettage en coagulatie een effectieve behandeling is voor plaveiselcelcarcinomen bij transplantatiepatiënten waarbij 94% in een periode van 50 maanden was genezen. Het cosmetisch resultaat was goed en een substantiële follow-up periode werd bereikt.

Hoofdstuk 8 geeft een samenvatting van de resultaten beschreven in de voorgaande hoofdstukken en bediscussieert de bevindingen. Mogelijkheden voor toekomstig onderzoek worden ten slotte besproken, waarvan de belangrijkste is om te onderzoeken wat het effect is van de nieuwere immuunsuppressiva mycofenolaat mofetil en sirolimus op de vroege huidkanker ontwikkeling. Bovendien dienen de rol van beta-PV in de vroege pathogenese van huidkanker bij transplantatiepatiënten, en een eventuele relatie met p53, nader geëvalueerd te worden.

Curriculum vitae

Leontien de Graaf werd op 16 juni 1976 te ’s Gravenhage geboren. In 1994 behaalde zij het VWO diploma aan het Alfrink College te Zoetermeer. In hetzelfde jaar begon zij met de studie Biomedische Gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen. Aan dezelfde universiteit startte zij in 1996 met de studie Geneeskunde. In deze periode verrichtte zij onderzoek naar roken en interacties met geneesmiddelen (Prof. dr. P. Smits, Afdeling Farmacologie & Toxicologie, Katholieke Universiteit Nijmegen). Voor de studie Biomedische Gezondheidswetenschappen deed zij haar afstudeeronderzoek naar het gebruik van teerpreparaten en het risico op huidkanker (dr. P. Pasker-de Jong en dr. P.G.M. van der Valk, Afdeling Klinische Epidemiologie en Biostatistiek en Afdeling Dermatologie, Academisch Ziekenhuis Nijmegen, St. Radboud). Het doctoraalexamen Biomedische Gezondheidswetenschappen, afstudeerrichting Epidemiologie, werd afgelegd op 26 april 2001. Na een keuze co-assistentschap in El Salvador behaalde zij het artsexamen op 5 oktober 2001.
In december 2001 werd zij aangesteld als AGIKO (assistent-geneeskundige in opleiding tot klinisch onderzoeker) op de Afdeling Dermatologie van het Leids Universitair Medisch Centrum en begon zij met promotieonderzoek onder begeleiding van dr. J.N. Bouwes Bavinck en dr. F.R. de Gruijl en werd de basis gelegd voor dit proefschrift. In januari 2005 startte zij in Leiden met de opleiding tot dermatoloog (opleider Prof. dr. R. Willemze).

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Submitted.

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