CHAPTER 2

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

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

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Accepted for publication 18 September 2007

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

Please cite this paper as: More epidermal p53 patches adjacent to skin carcinomas in renal transplant recipients than in immunocompetent patients: the role of azathioprine. Experimental Dermatology 2007.

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 specific 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 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).

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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/m² UV) per day, and in the third and fourth week with 1 MED (500 J/m² 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 CO₂ 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 mm²), 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 × 10⁶⁄cm². 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, ³²P 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 harvested.

At the seventh day the PHK cells were rinsed with PBS and irradiated with 300 J/m² from TL-12 lamps. Subsequently the cells were cultured for 6 h with ³H-thymidine, after which the cells were harvested. ³H uptake and ³²P 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 differences 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.
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

<table>
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<th>Patient no.</th>
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<th>Type of skin cancer</th>
<th>Location of skin cancer</th>
<th>Season</th>
<th>P53 patches/cm epidermis</th>
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<tr>
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</tr>
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</table>

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

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Type of skin cancer</th>
<th>Location of skin cancer</th>
<th>Season</th>
<th>P53 patches/cm epidermis</th>
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<td>0.0</td>
</tr>
</tbody>
</table>

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

Acknowledgements

We would like to thank Marjolein Lauwen for assistance with lymphocyte transformation test and Ronald Filon for assistance with the UDS test. We also thank the animal caretakers for assistance and care for the hairless mice.

References


