Local effects of immunosuppressants in the skin and impact on UV carcinogenesis

Pieter Voskamp

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Promotor:

Prof.dr. R. Willemze

Co-promotores:

Dr. F.R. de Gruijl

Dr. A. El Ghalbzouri

Dr. C.P. Tensen

Overige leden:

Prof.dr. J. Schalkwijk (UMC St. Radboud, Nijmegen)

Prof.dr. J.W. de Fijter

Prof.dr. L.H.F. Mullenders

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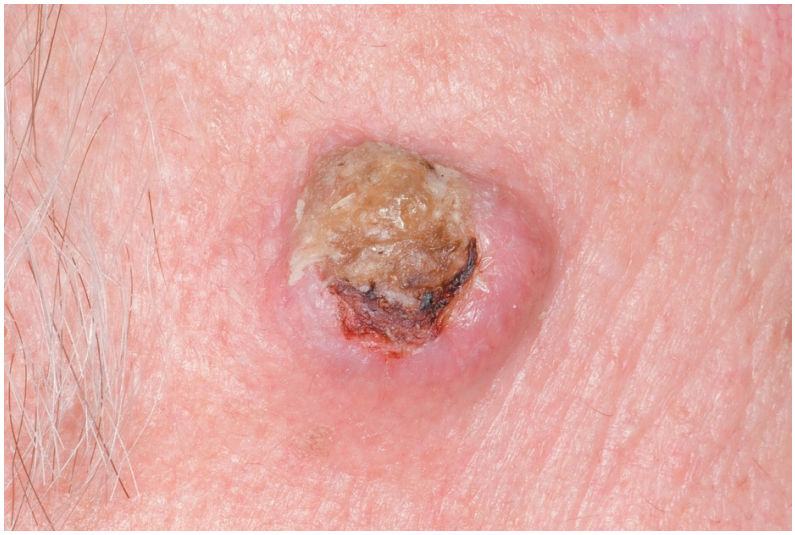
Chapter 1

General introduction

Organ transplants, immune suppression and complications

One of the most remarkable feats of modern Western medicine in the last century has undoubtedly been organ transplantations. The most serious complication which had to be overcome was the rejection of the allograft by the host immune system. As the mortality from immunologic and non-immunologic graft failure diminished and life expectancy of organ transplant recipients increased, other complications such as infections and malignancies rose in frequency. Life-threatening infections were reduced by a more prudent use of immunosuppressive drugs, combined with improved therapies against infections. Inclusion of steroids in the medication of organ transplant recipients have contributed to the occurrence of cardiovascular diseases, but this is ameliorated by aggressive treatment of hypertension and by steroid-free immunosuppressive regimens. A major complication, which worsens with prolonged graft survival, is the increase in malignancies, among which skin carcinomas are most dominant in white-skinned renal transplant recipients[1, 2] (figure 1). The carcinomas cause substantial mortality among organ transplant recipients[3], and the abundance of precursor lesions (actinic keratoses) can have a serious blemishing effect.

A



B

Study	Squamous cell carcinoma SIR (95%CI)
Kidney transplantations	
Hoxtell et al, 1977	36 (10-93)
Kinlen et al, 1979	23 (4-68)
Gupta et al, 1986	18 (17-20)
Hartevelt et al, 1990	253 (172-334)
Gjesvik et al, 2000	49 (33-70)
Moloney et al, 2006	82 (73-91)
Other organs +/- kidney transplantations	
Adami et al, 2003	56 (50-63)
Jensen et al, 1999	65 (53-79)
Lindelof et al, 2000	93 (73-116)
Lindelof et al, 2000	109 (95-123)
Gjersvik et al, 2000	177 (81-336)
Haagsma et al, 2001	70 (28-144)

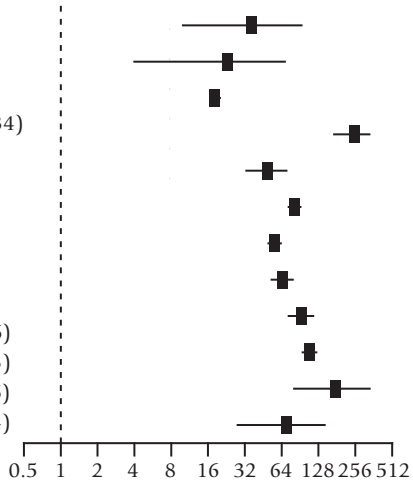


Figure 1: (**A**) Photo of an SCC on the face (**B**) Table showing the increased SCC risk in organ transplant recipients (adapted with permission[57]).

Skin carcinomas and ultraviolet (UV) radiation

Skin carcinomas are the most common form of cancer among Caucasians and are steadily increasing: in the USA over 1.3 million cases per year[4] and in the Netherlands over 35.000 cases a year[5]. The skin carcinomas in the general populations are clearly related to solar UV exposure[6] and so are the skin carcinomas in organ transplantation recipients[2, 7, 8], as reflected by predominant occurrence in sun-exposed skin. A striking observation is that the ratio of squamous cell carcinoma over basal cell carcinoma in the general population is around 1:3, whereas the immunosuppressed population develops these neoplasms in the inverse ratio of 4:1[9]. In Europe and the USA 40-61%[10, 11] of the organ transplant recipients developed skin cancer after 20 years, a figure that is even higher in Australia, where the 20 years prevalence reaches 70-82%[12, 13]. From classic animal experiments it is known that UV-induced skin cancers are antigenic and subject to elimination by the immune system, but a sub-carcinogenic course of UV irradiations can suppress the rejection and even induce specific tolerance toward the tumor[14]. Susceptibility to UV-induced suppression of cellular immunity (contact hypersensitivity) was found to be positively correlated with

skin carcinoma in humans[15]. Hence, the dramatic increase of skin carcinomas in immune suppressed allograft recipients was immediately and automatically attributed to the lack of adequate cellular immunity directed against the skin carcinomas. Experiments by Kelly et al.[16] confirmed that the immunosuppressants azathioprine and cyclosporin A speeded up UV carcinogenesis in a hairless mouse model.

Modes of action of immunosuppressants

Immunosuppressants work through different mechanisms (figure 2).

Blockage of purine synthesis: Azathioprine (Aza) and mycophenolate mofetil (MMF), or rather their respective metabolites 6-mercaptopurine (6MP) and mycophenolic acid (MPA), interfere with the synthesis of purines[17]. The older drug, Aza/6MP, does it rather crudely by competing in the pathway of purine synthesis: as a substrate of thiopurine S-methyltransferase, it leads inhibition of *de novo* purine synthesis or, as a substrate of inosine monophosphate dehydrogenase and guanosine monophosphate synthetase, it competes for incorporation into the DNA and RNA as a 6-thioguanine pseudo-base[18, 19]. MMF/MPA specifically inhibits inosine monophosphate dehydrogenase, and does not lead to incorporation of pseudo-bases in the DNA[20]. T lymphocytes are thought to be particularly vulnerable because they have no salvage pathway for purine synthesis that goes astray. With blocked purine synthesis the T cells are unable to proliferate.

Calcineurin inhibition: Calcineurin is a calcium-inducible phosphatase that serves to expose a part of the nuclear factor of activated T cells (NFAT), thereby causing this transcription factor to translocate to the nucleus; as the name of the factor suggests, this translocation is an essential step in activation of T cells (e.g. in IL2 production after TCR/CD3 activation)[17, 21]. Cyclosporin A (CsA) combines with cyclophilin to inhibit calcineurin, and tacrolimus (Tac, also known as FK506) does the same by combining with FKBP (FK506 binding protein or immunophilin)[22].

Blockage of downstream part of the Akt pathway/mTOR: The mammalian Target of Rapamycin (mTOR, or FKBP 12-rapamycin-associated protein, FRAP) activates p70S6 kinase and indirectly eIF-4E to induce protein synthesis and translation, respectively. The latter is mediated through elimination of p27KIP which also facilitates the G1 to S phase transition. Rapamycin (Rapa, also known as sirolimus) is a structural analogue of Tac and also binds to FKBP[23]. This complex does, however, not target calcineurin, but binds and blocks mTOR specifically in the protein complex mTORC1. Thus, Rapa can obstruct proliferative signaling through the IL2 receptor.

Although these immunosuppressants are used with the aim to inhibit the immune system, it has become clear that they also affect other cell types, among which are skin cells.

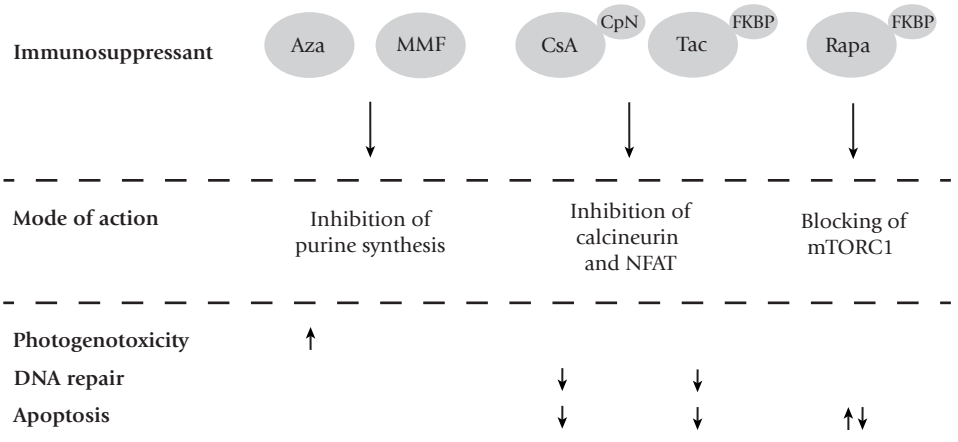


Figure 2: Graphical representation of immunosuppressants, their binding partners, their modes of action, as well as known effects on DNA repair, apoptosis and photogenotoxicity.

Secondary effects of immunosuppressants

DNA repair and apoptosis: In parallel to experiments on the effects of Aza and CsA on UV carcinogenesis in hairless mice, Kelly et al found these immunosuppressants to lower DNA repair as measured by unscheduled DNA synthesis (UDS) in the epidermis of the mice after UV exposure[24]. CsA was also found to reduce UV-induced apoptosis (sunburn cells) in the skin of BALB/c mice[25]. Herman et al.[26] showed that CsA, but not Aza, lowered repair of UV-induced DNA damage in peripheral blood mononuclear cells. More recently, the group of Yarosh showed that the calcineurin inhibitors CsA and ascomycin impaired the repair of UV-induced cyclobutane pyrimidine dimers (CPDs) in the genomic DNA of human keratinocytes, and that the apoptotic reaction of these cells was also lowered[27]. These down-regulated responses can enhance mutagenesis and malignant transformation. The authors emphasize that their data may have serious implications for the topical calcineurin inhibitors on sun-exposed skin (in contrast, the European Dermatology Forum concluded that available data indicated that topical calcineurin inhibitors, e.g. for eczema patients, are safe[28]). Interestingly, Yarosh et al. found that NFAT was translocated to the nuclei of the keratinocytes after short-wavelength UV-B exposure, and that CsA and ascomycin inhibited

this translocation[27]. A similar result of NFAT activity was reported by others for fibroblasts following exposure to low doses of long-wavelength UV-A radiation[29]. Inhibition of this activity by CsA or Tac increased the cytotoxicity of UV-A exposures.

DNA damage: Besides the effect of Aza on DNA repair, Kelly et al already drew attention in the late 80s to the potential photosensitizing effect of Aza on the DNA: the 6-thioguanine pseudo-base absorbs UV-A radiation[30]. More recently, Karran's group found that the UV-A photo-oxidation product, guanine sulfonate, in the DNA is an obstructive lesion that blocks replication and most likely also transcription[31]. In concordance with a blockage of transcription[32], Karran's group found that organ recipients on Aza show an increased erythemal sensitivity to UV-A radiation. The stalling of the DNA replication fork is overcome by switching to error-prone polymerases in a process dubbed 'lesional bypass', which increases the likelihood of mutations. The authors infer that the ensuing photosensitized mutagenesis could (in part) explain the increase in skin carcinomas among organ transplant patients in old cohorts on Aza. If the guanine sulphonate is an important mutagenic lesion, one would expect this to be reflected in mutation spectra such as that of the *P53* tumor suppressor gene in skin carcinomas. In the general population, the *P53* gene is mutated in 50-90% of skin carcinomas. These mutations are typical of UVB radiation (associated with di-pyrimidine sites targets for UV-B induced pyrimidine dimers, and mostly C to T transitions, sometimes even CC to TT tandem mutations)[33, 34]. However, the *P53* mutation spectrum in skin carcinomas from an early cohort of renal transplant recipients showed no obvious deviations[35], and therefore no discernable effect of guanine sulphonate. But this sample with 12 mutations from 23 carcinomas was rather small, and did not include any CC to TT tandem transitions.

Stress response signaling: Besides apoptotic signals, UV irradiation also triggers survival signaling of keratinocytes through EGF-R/PI3-K/AKT/S6K[36] and leads to activation of mTOR and p70S6 kinase through PI3-K, surprisingly not through Akt1[37]. Upon UV exposure mTOR mediates phosphorylation and activation of P53 and BRCA1[38]. And in keratinocytes mTOR relays the UV-induced signaling for TNF-alpha (and IL 10 in murine keratinocytes), involved in UV-induced immunosuppression[39]. Rapa blocks these UV-induced actions of mTOR, and the net outcome is not directly clear. Interestingly, inhibition of survival/anti-apoptotic signaling by Rapa results in apoptosis in p53-null cells, but not in cells with functional p53 which are in G1 phase[40].

Angiogenesis: MMF was developed as an anti-neoplastic agent and was proven to inhibit tumor cell proliferation[41], to modulate adhesion molecules resulting in impaired invasiveness[42] and to induce terminal differentiation[43]. CsA and Rapa were shown to have widely different effects on angiogenesis: while both offered adequate immunosuppression

to maintain a heart transplant in mice, CsA stimulated angiogenesis and outgrowth of inoculated tumor cells, whereas Rapa impaired these processes[44]. A decrease in the release of vascular endothelial growth factor (VEGF) from tumor cells and decreased responsiveness of endothelial cells appears to underlie the anti-angiogenic and anti-tumor growth effects of Rapa[45], whereas CsA stimulates VEGF production[46].

Reduction of skin carcinoma burden in organ transplant patients

As mentioned earlier, the increase in skin carcinomas among organ transplant recipients was considered a logic and inevitable consequence of immunosuppression. But the introduction of novel immunosuppressants with anti-tumor effects altered our thinking. At the outset of the present study in 2006, a clinical trial was already started in which after transplantation of a kidney the recipients were initially kept on a three-months regimen of Rapa-CsA and steroids. After this initial regimen CsA was withdrawn and Rapa increased for half of the group while the other half continued on the original regimen. The former half showed a dramatic 3 fold lower rate of occurrences of skin carcinomas after 5 years[47]. Evidently, there is ample room for improvement in minimizing the skin carcinoma risk, but the possibilities of experimentation with actual organ transplant recipients are, of course, very limited. A systematic approach in appropriate experimental models of UV carcinogenesis is, therefore, called for. To this end, two experimental models are used in this thesis, which are described in the next paragraphs.

The human skin equivalent

Human skin equivalents (HSEs) are three-dimensional tissue cultures containing a dermal matrix seeded with primary fibroblasts and an overlying epidermis consisting of primary keratinocytes. The epidermis is formed spontaneously by keratinocytes seeded on the dermal matrix under specific culturing conditions. Supplementation of culture media with serum or exogenous growth factors is mostly not required when fibroblasts are included into the dermal compartment[48], resulting in a local environment that is similar to that of *in vivo* tissue. HSEs that are formed under these conditions resemble native human skin in structure (figure 3A and B) and expression of basal membrane, proliferation and differentiation markers[48]. Thus, HSEs appear to be suitable models for investigating cellular responses to UV exposure of epidermal keratinocytes in the skin. Due to their limited life-span (maximally around two months), HSEs are not suitable for studying long-term effects of repeated UV exposures.

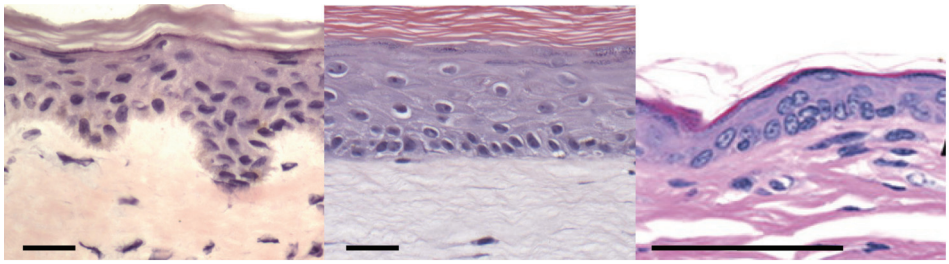


Figure 3: Hematoxylin and eosin stained cross sections of (**A**) native human skin, (**B**) human skin equivalent, (**C**) hairless mouse skin (adapted with permission[58]). Scale bars: 50 µm.

The hairless mouse model

UV radiation is an important factor in the induction of skin cancer in humans. For ethical reasons, UV-carcinogenesis cannot be studied experimentally in humans; therefore epidemiological data are used for risk assessments. These data can be confounded by many factors that cannot be corrected for. For instance, due to continuing development of new immunosuppressive agents patients are differently treated over time, making it impossible to compare older and newer immunosuppressants on skin cancer risk. Experimental studies on UV carcinogenesis are therefore necessary to gain insight in mechanistic effects of immunosuppressants on UV-induced skin cancer development. The immunocompetent hairless mouse strain (SKH1) is one of the mouse models extensively used for studying UV carcinogenesis. Skin of hairless mice morphologically resembles human skin; both consist of a dermis and an epidermis, where the epidermis consists of a basal, a spinous, a granular, and a cornified layer (figure 3). There are however several notable differences between skin of hairless mice and human skin. Human epidermis consists of 5-8 viable cell layers, whereas mouse epidermis comprises only 1-2 cell layers. Unlike human skin, hairless mouse skin contains degenerated hair follicles and no sweat glands or dermal papillae.

When hairless mice are repeatedly UV-irradiated clusters of keratinocytes expressing mutant p53 (mut-p53 cell clusters) develop in the epidermis. In previous studies numbers of mut-p53 cell clusters were correlated with the rate of tumor development after chronic UV irradiations[49, 50]. Mut-p53 cell clusters develop as a clonal expansion of a mutated keratinocyte and occur prior to skin tumors. Skin tumors that are induced in hairless mice are predominantly squamous cell carcinomas (SCCs) and precursor lesions (actinic keratoses). Since most SCCs harbor mutations in the *p53* gene similar to those in mut-p53 cell

clusters[51, 52], *p53* mutations are considered to be early events in SCC formation and mut-p53 cell clusters precursors of SCCs. As stated earlier, SCCs are the type of non-melanoma skin tumor for which the risk is the most increased in transplant patients[10, 53]. In conclusion, the hairless mouse model appears to be the most suitable animal model available to study effects of immunosuppressants on UV-induced skin cancer.

Aim and outline of the thesis

Increased skin cancer risk is a serious problem for organ transplant recipients. Immunosuppressive therapy is considered an important risk factor for skin cancer development. The aim of the present study was to provide experimental data which could contribute to minimize the (skin) carcinogenic risk from immunosuppressive drugs. The main incentive for the study was the recognition that the impact of different immunosuppressants on (UV responses of) skin cells could differ widely, with potentially corresponding differential impacts on UV carcinogenesis. More specifically, it was assessed whether the reported anti- and pro-apoptotic effects of CsA and Rapa, respectively, corresponded directly with possibly different impacts of these drugs on UV-induced mut-p53 cell clusters and skin carcinogenesis. In this respect, it is interesting to note that the mut-p53 cell clusters were proven to be non-immunogenic (in contrast to skin carcinomas)[54]. Their occurrence can therefore be considered to be the result of pure local (UV and drug) effects in the skin, independent of any systemic immunosuppressive effect. Consequently, the experiments presented in this thesis focus on modulating effects of immunosuppressants on the responses of skin cells to UV irradiation, and the UV induction of skin carcinomas and precursor lesions. With reported adverse effects on skin cells and pro-tumor effects of classic immunosuppressants and anti-tumor effects of novel immunosuppressants, Rapa and MMF, the study first focused on the impacts of these novel drugs on the formation of primary skin carcinomas by UV exposures. Subsequently, the study was expanded to include other effects (e.g. on mutation and expression of p53, proliferation and apoptosis) and the main immunosuppressants currently used in organ transplant recipients.

Angiogenesis and outgrowth of tumor implants have previously been shown to be inhibited by the immunosuppressants MMF and Rapa[41, 45, 55]. Also, Rapa could increase apoptotic responses in p53 dysfunctional cells[40]. Hence, these drugs are very interesting as they could lower the skin cancer risk by these local effects in the skin. **Chapter 2** describes the effects of MMF and Rapa on UV carcinogenesis in hairless mice, more specifically on tumor development, p53 mutational spectra and expression of vascular endothelial growth factor (Vegf-a).

Results of chapter 2 showed that skin tumors (>2mm) of Rapa-treated mice harbor an altered *p53* mutational spectrum. In order to assess at which stage in tumor development this altered mutational spectrum develops, mutational spectra of small skin tumors (<2mm) and mut-p53 cell clusters are determined in **chapter 3**. During the analysis, Rapa was found to decrease numbers of mut-p53 cell clusters. Apparently and surprisingly, this had no effect on the onset of tumors (chapter 2). It was therefore investigated whether Rapa inhibits formation of these cell clusters, or whether Rapa induces apoptosis specifically in these cell clusters or decreases p53 protein expression.

Working mechanisms and effects in the skin differ between immunosuppressants. Experiments reported on in **chapter 4** compare the effects of immunosuppressants on the sequence of events leading up to tumor formation, to assess whether early effects on apoptosis and frequency of p53 mutations are predictive of the risk of skin cancer development. The main immunosuppressants (CsA, Tac, Aza, MMF and Rapa) are compared in a human skin model for short term effects of skin cells after UV irradiation and in a hairless mouse model for UV carcinogenesis.

In a pioneering study, the immunosuppressive drug CsA increased UV-carcinogenesis[16], whereas other studies showed that CsA decreased UV-induced tumor development[56] (chapter 4). These conflicting results may be explained by the use of different treatment schemes, i.e. UV exposure and CsA treatment. **Chapter 5** compares the net effect on UV carcinogenesis of three experimental protocols with two ways of administering CsA.

In **chapter 6** all results are summarized and discussed.

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Chapter 2

Early and late effects of the immunosuppressants rapamycin and mycophenolate mofetil on UV carcinogenesis

De Gruijl FR, Koehl GE, Voskamp P, Strik A, Rebel HG, Gaumann A, De Fijter JW, Tensen CP, Bouwes Bavinck JN, Geissler EK

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Abstract

Increased skin cancer risk in organ transplant recipients has been experimentally emulated with enhanced UV carcinogenesis from administering conventional immunosuppressants. However, a newer generation immunosuppressive drugs, rapamycin (Rapa) and mycophenolate mofetil (MMF), have been shown to impair angiogenesis and outgrowth of tumor implants. To ascertain the overall effect on UV carcinogenesis, Rapa and MMF were admixed into the food-pellets of hairless SKH1 mice receiving daily sub-sunburn UV dosages. With immunosuppressive blood levels neither of the drugs affected onset of tumors (<2mm), but in contrast to MMF, Rapa significantly increased latency of large tumors (≥4mm, medians of 190 vs 125 days) and reduced their multiplicity (1.6 vs 4.5 tumors per mouse at 200 days). Interestingly, tumors (>2mm) from the Rapa-fed group showed a reduction in UV-signature *p53* mutations (39% vs 90%) in favor of mutations from putative base oxidation. This shift in mutation spectrum was not essentially linked to the reduction in large tumors as it was absent in large tumors similarly reduced in number when feeding Rapa in combination with MMF, possibly owing to an antioxidant effect of MMF. Significantly fewer tumor cells were Vegf-positive in the Rapa-fed groups, but a correspondingly reduced expression of Hif-1α target genes (*Vegf*, *Ldha*, *Glut1*, *Pdk1*) that would indicate altered glucose metabolism with increased oxidative stress was not found. Remarkably, we observed no effect of the immunosuppressants on UV-induced tumor onset, and with impaired tumor outgrowth Rapa could, therefore, strongly reduce skin carcinoma morbidity and mortality in organ transplant recipients.

Introduction

One of the most remarkable feats of modern Western medicine in the last century has undoubtedly been organ transplantation. However, as the initially leading mortality from immunologic and non-immunologic graft failure diminished and life expectancy of organ transplant recipients increased, other complications have risen. A major complication, which worsens as the graft survival is extended, is posttransplant malignancy, among which skin carcinomas are most prominent[1, 2]. The carcinomas tend to be more aggressive in organ transplant recipients, causing substantial mortality[3], and the abundance of precursor lesions (actinic keratoses) can have serious blemishing effects.

Skin carcinomas among white Caucasians are clearly related to solar UV exposure[4], and so are the skin carcinomas in organ transplant recipients[2, 5], as reflected by predominant occurrence in sun-exposed skin. A striking observation is that the ratio of squamous cell carcinoma (SCC) over basal cell carcinoma (BCC) in the general population ranges around 1:3, whereas the ratio is reversed in organ transplant recipients. In the Netherlands it was estimated that about 40% of the renal transplant recipients will have contracted at least one skin carcinoma by 20 years after transplantation[6]; in Australia this percentage is already exceeded after 9 years[7].

From classic animal experiments it is known that UV-induced skin cancers are antigenic and subject to elimination by the immune system, but a sub-carcinogenic course of UV irradiations can suppress the rejection and even induce specific tolerance toward the tumor[8]. Hence, the dramatic increase of skin carcinomas in immune suppressed allograft recipients was immediately attributed to the lack of adequate cellular immunity directed against the skin carcinomas. Experiments by Australian groups[9, 10] confirmed that the immunosuppressants azathioprine and cyclosporine sped up UV carcinogenesis in a hairless mouse model. However, Kelly et al. also showed that these classical immunosuppressants adversely affected repair of UV-induced DNA damage in skin cells[11]. Moreover, this group recognized that azathioprine led to photosensitization of the DNA to long wavelength UV-A radiation, thus increasing the DNA damage caused by (solar) UV exposure[12]. This photosensitization has been confirmed and studied in greater detail more recently[13]. Besides lowering DNA repair, cyclosporine was found to impair apoptotic responses to UV irradiation in BALB/c mice[14], and these disruptive effects from calcineurin inhibitors, like cyclosporine, were confirmed in human keratinocytes[15]. Aside from immunosuppression *per se*, these drug-specific adverse effects from classical immunosuppressants on skin cells are bound to increase the skin carcinoma risk related to (solar) UV exposure.

A new generation of immunosuppressants may not have these drawbacks. In contrast to the traditional immunosuppressants, mycophenolate mofetil (MMF) and rapamycin (Rapa) impair the outgrowth of tumor inoculations[16-19]. Although MMF (or rather its metabolite mycophenolic acid), like azathioprine, interferes with purine synthesis, MMF does not give rise to incorporation of (6-thio-guanine) pseudo-bases that photosensitize DNA. Furthermore, Rapa operates through an entirely different mechanism, by blocking mTor (mammalian target of rapamycin) up-stream from 4Ebp1 and S6K in the Akt 'survival pathway'[20], thus regulating translation. The anti-angiogenic effect of Rapa is linked to both a reduction in production of Vegf-a in tumor cells and a diminished endothelial response[17]. Transcription of Vegf-a can be driven by Hif-1α which in turn is under translational control of mTor[21-24]. Furthermore, Rapa reportedly causes apoptosis in p53-null cells[25] and impairs tumor outgrowth in p53-null mice[26]. As skin carcinomas raised by chronic UV exposure show an abundance of p53 mutations[27] one might expect a Rapa-driven selective apoptotic response in these p53-mutant cells, which could lower the initiation rate of UV-induced skin carcinomas.

Considering these important differences between classical and novel immunosuppressants, a systematic approach to assess the skin carcinoma risk in appropriate models appears to be urgently needed. Here, we pose the question of how MMF and Rapa would affect the overall process of UV carcinogenesis. Both MMF and Rapa are expected to impair the outgrowth of UV-induced primary skin tumors, but Rapa may also lower the rate of initiation of these tumors. A net beneficial effect would distinguish these novel suppressants from azathioprine and cyclosporine, which have been shown to enhance UV carcinogenesis in experiments with immunocompetent hairless mice[10]. Using the same hairless mouse model, we assessed the effect of Rapa and MMF treatment on UV carcinogenesis.

Materials and Methods

The mice

SKH-1 hairless mice (Charles River, Maastricht, The Netherlands) entered the experiment at 8 weeks of age; both male and female mice were used. The animal room was illuminated with yellow fluorescent tubes (Philips TL40W/16, Eindhoven, The Netherlands) that did not emit any measurable UV radiation. The animals were housed individually in Macrolon type 1 cages (Techniplast, Bugguggiate, Italy) under a 12 h light-12 h dark cycle at 23°C, 60% humidity. Standard chow was supplied in ample amounts (55-60 g/mouse/week), and drinking water was available *ad libitum*. Cage enrichment was absent to prevent shielding

of the animals from UV exposure. All experiments were performed in accordance with legislation and approval of the medical center's ethics committee.

Groups on diets with admixtures of Rapa and MMF

To avoid repeated i.p. injections of the immunosuppressants, exploratory experiments with admixtures of the drugs to standard mouse chow were performed[26]. For the present experiment ssniff *GmbH* (Soest, Germany) supplied the food with Rapa at 20 mg/kg and MMF at 660 mg/kg. Four diet groups were formed: Rapa (n=10), MMF (n=10), Rapa and MMF (n=12) and a control group (n=10) fed the standard chow without admixtures. No apparent differences in food intake were observed between the four groups. With roughly 50 g/wk of food intake by a 30 g mouse, we estimated a weekly intake of Rapa at about 30 mg/kg and MMF at about 1 g/kg. All diets stocks were refrigerated to inhibit degradation.

UV irradiation

The four groups were started on their respective diets 1 week before subjecting them to a regimen of daily UV exposure. TL-12/40W tubes (Philips, Eindhoven, The Netherlands; 54% output in UVB – 280 to 315 nm – and 46% output in UVA – 315 to 400 nm) were used for daily UV exposure. The lamps were mounted over the cages with grid covers to allow undisturbed exposure of the mice. The lamps were automatically switched on daily from 12.30 to 12.50 h. The threshold dose for a sunburn reaction (minimal edemal dose, MED) in the hairless SKH-1 mouse was ~500J/m^2 UV under these lamps. The lamps were dimmed both electronically and by insertion of perforated metal sheets to expose the mice daily to 250 J/m^2 of UV radiation (0.5 MED).

Tumor assessment

The mice were inspected weekly for tumors which were registered for each mouse individually on maps (thus recording location, size, form and coloration/vascularisation). First smallest perceptible (<1mm in diameter) lesion had to be observed at least in two successive checkups to be confirmed and counted. Upon removal of animals from the experiment, tumors were isolated from animals of each group for further analysis as described below.

Histology and Immunohistochemistry

All stainings were performed on formalin-fixed and paraffin-embedded sections (5µm) of skin containing tumors. Hematoxylin and eosin (H&E) staining was performed for tumor staging. The following antibodies were used for immunohistochemistry: rat anti-mouse Vegf-a antibody (RELIA*Tech*, Braunschweig, Germany), rabbit anti-Hif-1α (Novus, Littleton/CO,

USA), rabbit anti-phospho-4Ebp1 (Thr 37/46, 236B4; Cell Signaling, Danvers/MA, USA), rabbit anti-phospho-S6 ribosomal protein (Ser235/236, 91B2; Cell Signaling, Danvers/MA, USA), rat anti-mouse CD34 (clone MEC 14.7; Acris Antibodies, Herford, Germany), using standard protocols and detection by diaminobenzidine. Staging and (immune-)histological evaluation of tumors from each group were performed by a blinded pathologist (Dr. A. Gaumann, University of Regensburg) who is experienced in mouse and human pathology, including diagnosis of actinic keratoses as proper precursors of squamous cell carcinomas[28].

RNA and cDNA preparation

Excised samples of tumors, non-tumor dorsal skin, and ventral skin were treated with RNAlater-ICE (Ambion), snap-frozen in liquid nitrogen and stored in a freezer at -80°C. A biopsy of maximally 8 mm^3 in volume was taken from every tumor by manual excision under a stereomicroscope. The biopsies were homogenized using a rotor stator homogenizer (Ultra-Turrax T8, IKA, Staufen, Germany), followed by RNA extraction with RNeasy mini kit (Qiagen, Valencia, Ca, USA). 0.5µg of total RNA was reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad).

Mutational analysis

Tumor cDNA was used to amplify the entire coding sequences of the p53 transcript by RT-PCR in two overlapping fragments (1st amplicon with primers CCTGGCTAAAGTTCTGTAGC forward and GCCTGTCTTCCAGATACTCG reverse, and 2nd amplicon with CCTGTCATCT-TTTGTCCCTTC forward and GCAGAGACCTGACAACTATC reverse). RT-PCR was performed with iQ SYBR Green SuperMix (Bio-Rad, Hercules, Ca, USA) on an iQ5 thermocycler (Bio-Rad). The reaction mixture was heated to 95°C for 3 min and amplification was carried out for 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. The RT-PCR products were completely sequenced in both directions. Cycle-sequencing was performed using ABI PRISM Big Dye Terminators v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, Ca, USA) and sequencing products were run on an ABI PRISM 3730 Analyzer (Applied Biosystems). Mutation Surveyor (SoftGenetics LLC) software was used for analysis of the sequencing files.

Real-time quantitative RT-PCR analysis

Tumors, as well as dorsal and ventral skin were analyzed for *Ldha*, *Pdk1*, *Glut1*, *Vegf-a* by real-time quantitative RT-PCR[29] (primer pairs: TGTCTCCAGCAAAGACTACTGT forward, GACT-GTACTTGACAATGTTGGGA reverse for *Ldha*; AGGCGGCTTTGTGATTTGTATTATG forward, TGTATTGTCTGTCCTGGTGATTTCG reverse for *Pdk1*; GGGCTGCCAGGTTCTAGTC forward,

CCTCCGAGGTCCTTCTCA reverse for *Glut1*; AAGGAGAGCAGAAGTCCCATGA forward, CACAGGACGGCTTGAAGATGT reverse for *Vegf-a*)[30]. β-actin was used as the cellular housekeeping gene for normalization[31]. PCR reaction settings were 95°C for 3 min, then 40 cycles at 95°C for 15 s and 58°C for 45 s; melting curves were also examined after each run.

Statistical tests

Kaplan Meier plots of tumor-free survival were analysed by χ^2 tests (Graphpad Prism 3.0). Differences in pair-wise comparisons of tumor yields were analysed with Mann Whitney U tests, and Univariate Analysis of Variance was used to test simultaneously for group and gender effects on tumor yields (SPSS 12.0.1). Immunohistological scores and tumor staging was analyzed by pair-wise comparisons using Mann Whitney Rank Sum tests (SigmaPlot9.0/ SigmaStat3.1). Differences in p53 mutations between the diet groups were calculated by χ^2 tests. $p<0.05$ was considered to indicate a significant difference.

Results

Response to diets

Animals in the 4 groups showed no differences in weight, and gained weight from the start of the experiment up to 16 weeks (control males from 30.0 ± 2.4 g [±SD] to 33.8 ± 2.9 g; females from 25.5 ± 1.6 to 27.9 ± 1.8 g). Blood levels of Rapa (33 ± 11 ng/ml, n=6) and MMF (mycophenolic acid at 2.9 ± 0.8 mg/l, n=3) were measured after 1 week on food containing the drugs, and were similar to those previously reported[26].

Tumors

The animals showed no signs of sunburn throughout the experiment. This regimen of daily UV exposure of hairless mice was found to induce endophytic tumors which grow out to carcinomas[28]. Benign papillomas (exophytic – often pedunculated – cauliflower-like tumors) were a small minority (<10%). These earlier findings were confirmed by pathology on tumors from the different groups. In summary, almost all tumors larger than 4mm were (invasive) SCC, whereas a substantial proportion of smaller tumors were SCC precursors, e.g. in situ carcinomas (Figure 1). No basal cell or spindle cell carcinomas were found. Papillomas were not included in the analyses of tumor induction.

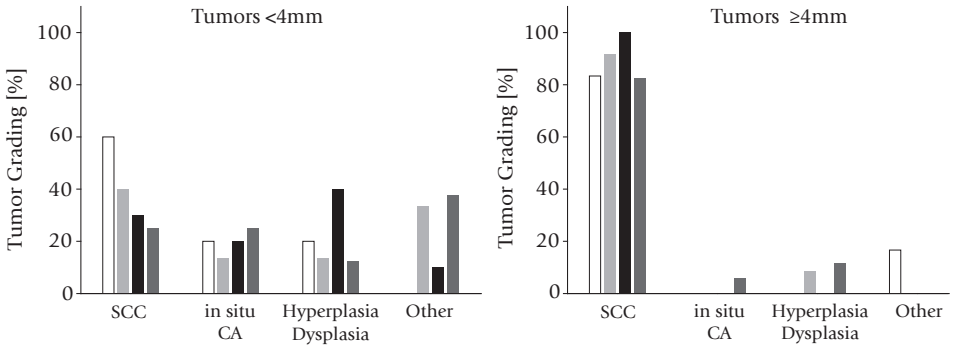

Figure 1: Histopathology of tumors smaller and larger than 4 mm in diameter; quartets of bars representing control (white bar), MMF (light gray), Rapa (black), and Rapa/MMF (dark gray) groups.

Tumor latency times

Figure 2 shows the tumor-free survival in the 4 different diet groups with tumor detection threshold set at 4 different diameters: minimal perceptible at a few tenths of a mm, 1 mm, 2mm and 4mm. No differences were evident between the four groups for tumors with diameters up to 1mm. For tumors ≥2mm, the Rapa group clearly showed an increase in the time of tumor-free survival (p=0.0025 when compared with control). And for tumors ≥4mm the differences between the four groups are very distinct, with the control group developing the tumors most rapidly (with a median latency time, t_m,of around 125 days), followed by the MMF group (t_m=140 d, not significantly different from controls), the MMF/Rapa group (t_m=170 d, p=0.0004 vs controls), and, lastly, the Rapa group (t_m=190 d, p=0.0021 vs controls); i.e., the Rapa group showed a delay of about 50% when compared to the control group. No overall differences in latency times between males and females were detected (p=0.34).

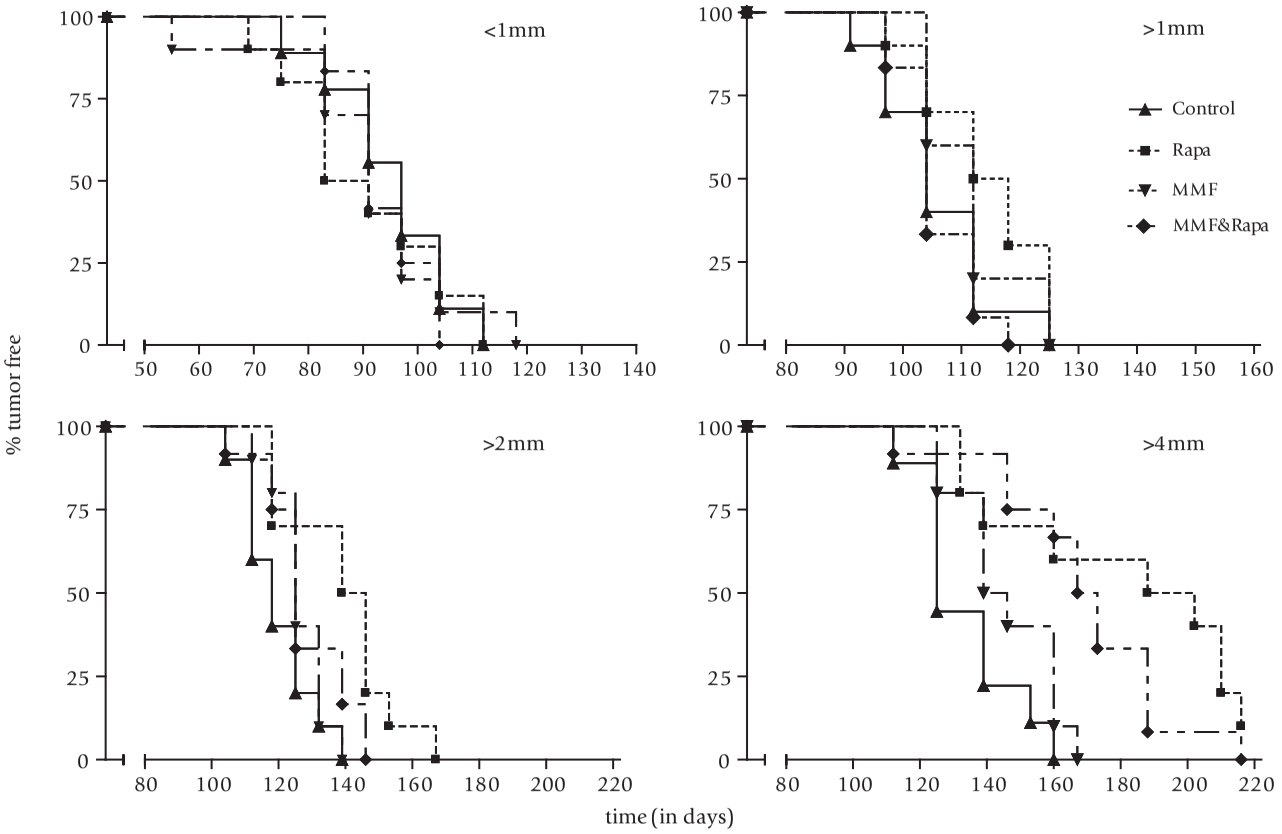

Figure 2: Kaplan Meier plots of tumor free survival in the 4 diet groups with the threshold for tumor detection set at 4 different diameters: upper left panel for minimal perceptible tumors estimated at 0.5 mm in diameter (overall no significant difference between groups), upper right panel for tumors at 1 mm (no significant difference), lower left panel for tumors at 2mm (p=0.0053) and lower right panel for tumors at 4mm in diameter (p=0.0014).

Tumor multiplicity

Aside from how quickly a first tumor appears, the number of tumors that an individual mouse develops is an important measure of severity. And, with small experimental groups like in the present study (n=10-12), counting the number of tumors increases the numerical strength of the analysis. The average number of tumors per mouse is referred to as 'tumor yield'. These yields in the 4 different diet groups are depicted in Figure 3 with the threshold for tumor detection set at 4 different diameters: either minimal perceptible at a few tenths of a mm, 1mm, 2mm or 4mm (i.e. all tumors with diameters at or over the threshold were counted).

With the tumor detection threshold set at minimal perceptible lesions no discernible differences were observed up to 130 days. For tumors ≥1 mm there was a significant difference at 139 days between the Rapa group and the control group (10.4 vs 19.2 tumors/mouse, p=0.003). For tumors ≥2mm there was an even larger relative difference at that time point (1.6 vs 6.9 tumors/mouse, p=0.002) and at subsequent time points. The yields of the largest tumors (≥4mm) showed the clearest differences. At day 202 the Rapa group had developed significantly fewer large tumors than the control group (1.6 vs 4.5 tumors/mouse, p=0.005), as did the Rapa/MMF combination group (1.8 vs 4.5 tumors/mouse, p=0.007). MMF appeared to have little effect as there was only a minor difference between the control group and the MMF group (4.5 and 3.6 tumors/mouse, respectively, n. s.), and virtually no difference between the Rapa and Rapa/MMF groups (1.6 and 1.8 tumors/mouse, n. s.). Again, no overall differences were found between males and females in tumor yields (for diameters ≥1mm p=0.35 at day 139, and ≥4mm p=0.89 at day 202).

Angiogenesis and Vegf-a staining

Although it has been shown that the outgrowth of implanted tumors can be hampered by an anti-angiogenic effect of Rapa or MMF[16, 17, 26], we could not detect any differences in the density of vessels in the tumor samples from the different groups (either by counting the number of vessels or by quantifying vessel endothelial cells stained with CD34; data not shown). Like in the tumor implants, we did, however, observe significantly less expression of Vegf-a in the tumor cells from the Rapa group (Figure 4). The tumor stroma, on the other hand, did not show any difference in Vegf-a staining (data not shown). Interestingly, at this advanced stage of tumor growth (diameters >2-4 mm) we could not detect any clear reduction by Rapa in the levels of activated mTor effector proteins (phospho-S6 ribosomal protein, nuclear 4Ebp1 and Hif-1α; data not shown) which showed substantial variation in immunohistochemical staining between the tumors.

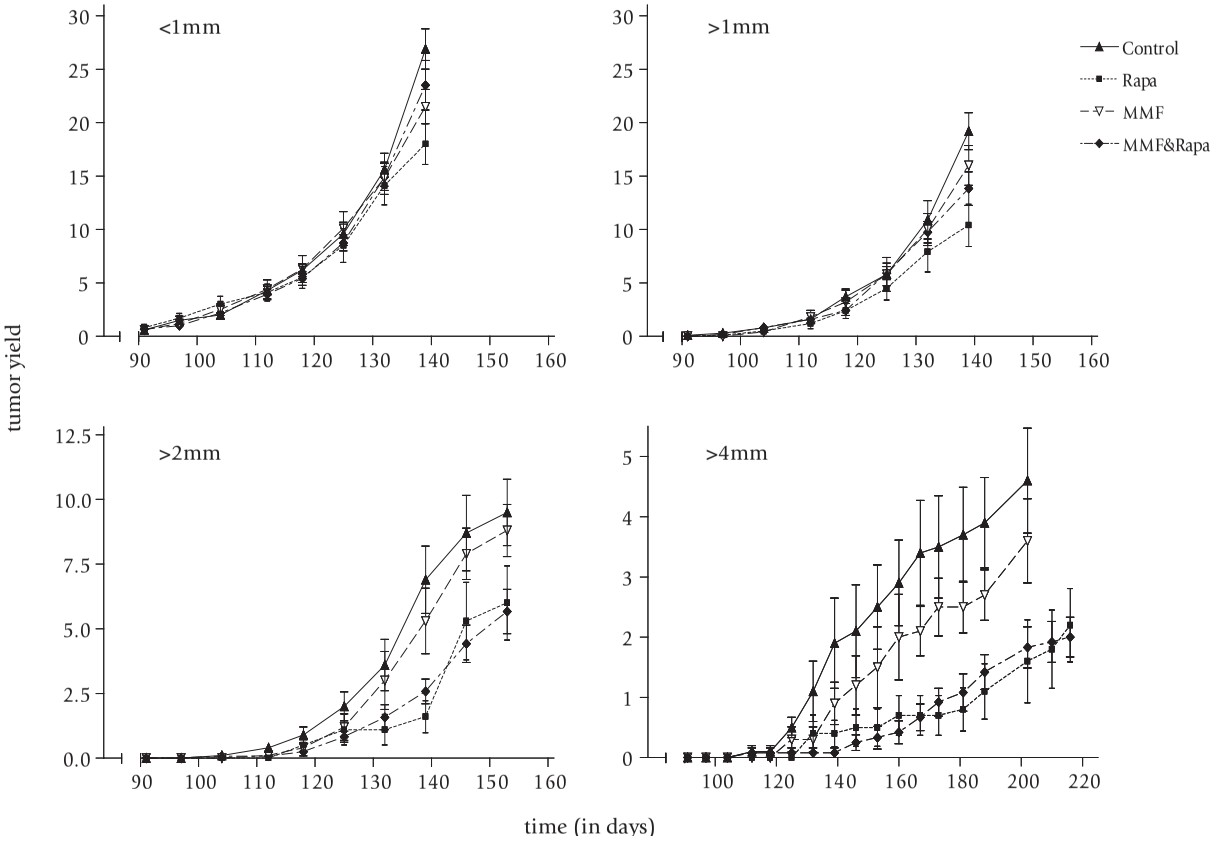

Figure 3: Plots of tumor yields in the 4 diet groups with the threshold for tumor detection set at 4 different diameters: upper left panel for minimal perceptible tumors estimated at 0.5 mm in diameter, upper right panel for tumors at 1mm, lower left panel for tumors at 2mm, and lower right panel for tumors at 4mm in diameter; whiskers depict SEM.

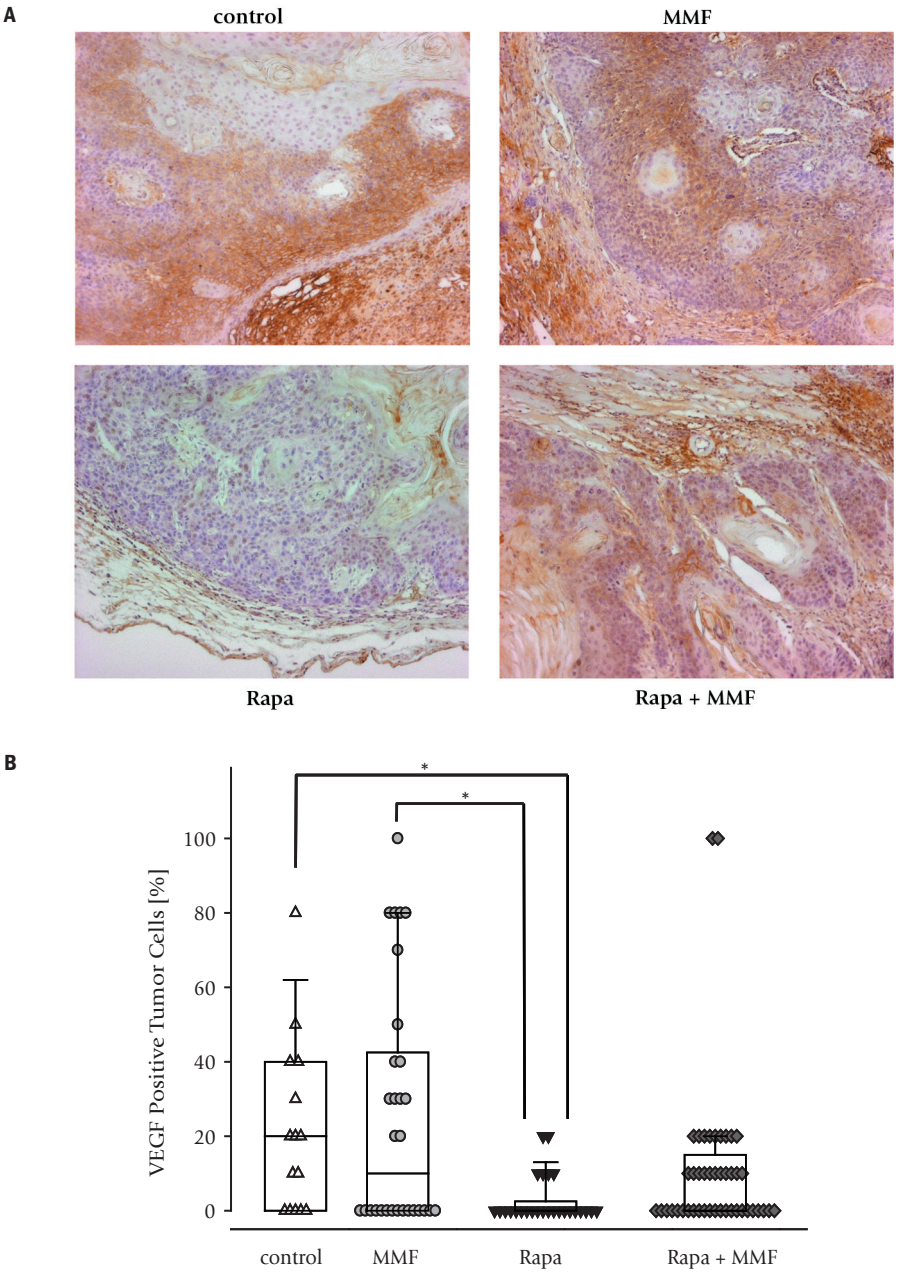

Figure 4: Vegf-a expression in tumors: (A) typical examples of tumor sections histochemically stained for Vegf-a; (B) graphical presentation of Vegf-a expression in tumors from the 4 experimental groups; * p=0.005 vs control.

Mutation analysis of p53 in tumors

Mutations in *p53* occur early in UV carcinogenesis and they show a UV signature, i.e., C to T transitions at dipyrimidine sites[4]. We inspected whether the immunosuppressants could have affected the *p53* mutation spectrum by metabolic effects from Rapa, or by anti-oxidant effects or effects on purine synthesis from MMF. Figure 5 gives a graphical summary of percentages of UV signature mutations in *p53* in tumors (>2mm) from the four experimental groups (table 1 gives a more detailed overview). The control group clearly showed the expected mutation pattern with predominantly UV signature mutations. The mutation spectrum from the MMF group showed no significant change. But the Rapa group showed a clear reduction in percentage of UV signature mutations (39% vs an average of 90% in the other groups, p=0.007 by χ^2 test). The relative increase in G>T and T>G mutations could be caused by oxidative damage[32], and the T>C transitions possibly originate from thymine glycols as oxidation products[33]. Interestingly, combining Rapa with MMF again produced a *p53* mutation spectrum dominated by UV signature mutations, comparable to the control group.

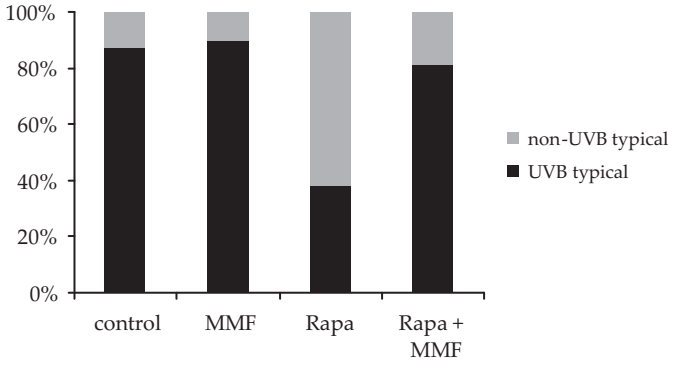

Figure 5: Graph of the percentage of UV signature mutations (C to T transition at dipyrimidine site) among the p53 mutations in tumors from the 4 experimental groups.

The Warburg effect

A possible source of reactive oxygen could be the mitochondria by effects of Rapa on the respiration of tumor cells. Tumors have been reported to have shifted their glucose consumption from oxidative phosphorylation to aerobic glycolysis to lactate, which is dubbed the Warburg effect and can be driven by Hif-1α[34]. We hypothesized that Rapa could reduce the Warburg effect by lowering the level of Hif-1α or by interfering with Hif-1α

activation[35], and thus enhance oxidative phorphorylation and the release of reactive oxygen species (ROS) from mitochondria. Therefore, we measured expression of Hif-1α target genes[34]: *Glut1*, *Pdk1*, *Ldha*, and *Vegf-a*, where Glut1 is a glucose transporter, Pdk1 blocks pyruvate's entry into the Krebs cycle, and Ldha catalyses the conversion of pyruvate and NADH to lactate and NAD. We found no differences between mRNA levels from these genes in tumors taken from the Rapa and control groups, as shown in Figure 6. However, we did observe interesting differences between UV-exposed skin, unexposed skin and tumors. In contrast to *Ldha* and *Vefg-a*, both *Glut1* and *Pdk1* were upregulated in UV-exposed dorsal skin when compared to unexposed ventral skin. But in the tumors *Glut1* and *Pdk1* expression was low and *Ldha* and *Vegf-a* expression was high. These data do not reflect any unison effect of Hif-1α on the expression of these four target genes.

Table 1: Mutation analysis of p53 in tumors

Mouse #. Tumor #	Codon	Mutation	Amino acid	Not UV-typical
Control				
1.1	62	ctcCga>ctcTga	R>R/X	
1.2	82	gccCCt>gccTTt	P>P/F	
1.3	124	tCt>tTt	S>S/F	
2.1	142	tTg>tCg	L>S/L	✓
2.1	149	cctCca>cctTca	P> P/S	
3.1	149	cctCca>cctTca	P>P/S	
2.2	210	tttCgc>tttTgc	R>C	
3.2	210	tttCgc>tttTgc	R>R/C	
4.1	210	tttCgc>tttTgc	R>C	
1.2	238	tCc>tTc	S>S/F	
3.2	239	tGc>tTc	C>C/F	✓
5.1	270	gttCgt>gttTgt	R>C	
1.2	270	gttCgt>gttTgt	R>R/C	
1.3	270	gttCgt>gttTgt	R>R/C	
3.1	275	tgcCct>tgcTct	P>P/S	
1.1	373	tCt>tTt	S>S/F	
Rapa				
6.1	110	ggcTtc>ggcCtc	F>L/F	✓
7.1	142	ttGtgg>ttTtgg	L>L/F	✓
8.1	210	tttCgc>tttTgc	R>C	
9.1	239	tGc>tTc	C>C/F	✓

Mouse #. Tumor #	Codon	Mutation	Amino acid	Not UV-typical
9.1	247	cCt>cGt	P>P/R	✓
7.1	261	aacCtt>aacTtt	L>L/F	
10.1	262	cTg>cCg	L>P/L	✓
10.1	267	tTt>tCt	F>F/S	✓
7.1	270	gttCgt>gttTgt	R>R/C	
11.1	272	tgTgcc>tgGgcc	C>W/C	✓
11.1	275	cCt>cTt	P>P/L	
12.1	275	cCt>cTt	P>P/L	
10.2	intron4	AG mutant	heterozyg. del.	✓
MMF				
13.1	149	cctCca>cctTca	P>P/S	
14.1	210	tttCgc>tttTgc	R>C	
13.2	210	tttCgc>tttTgc	R>R/C	
15.1	210	tttCgc>tttTgc	R>R/C	
13.2	270	gttCgt>gttTgt	R>R/C	
13.3	270	gttCgt>gttTgt	R>R/C	
16.1	275	tgcCct>tgcTct	P>S	
16.2	275	tgcCct>tgcTct	P>S	
14.1	294	tgcCct>tgcAct	P>T/P	✓
14.1	307	acCtgc>acTtgc	T>T/T	
MMF + Rapa				
17.1	77	accCct>accTct	P>P/S	
18.1	92	tCt>tTt	S>S/F	
19.1	95	cCt>cTt	P>P/L	
20.1	109-110	ggCTtg>ggTGtg	G>G, F>V/F	✓
18.2	149	cCa>cTa	P>P/L	
21.1	155	gtcCgc>gtcTgc	R>R/C	
19.2	175-176	caCCat>caTAat	H>H, H>N/H	✓
23.1	210	tttCgc>tttTgc	R>C	
21.1	261	aacCtt>aacTtt	L>L/F	
18.1	270	gttCgt>gttTgt	R>R/C	
22.1	270	gttCgt>gttTgt	R>R/C	
21.2	275	tgcCct>tgcTct	P>P/S	
19.1	275	tgcCct>tgcTct	P>P/S	
19.2		heterozyg. splicemutant	exon7-exon12	✓

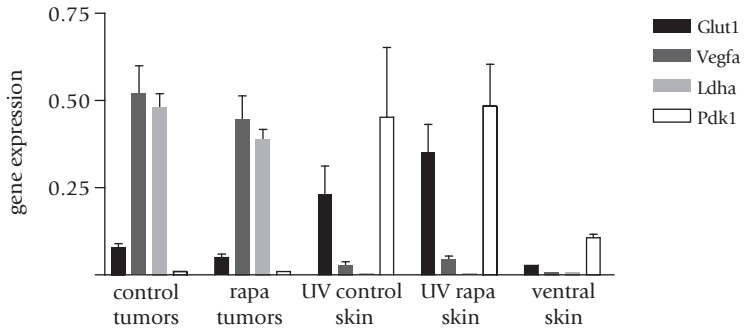

Figure 6: Expression of *Vegf-a*, *Ldha*, *Glut1* and *Pdk1* as target genes of Hif-1α in comparison to β-*actin* in various tissue samples from Rapa-fed and control mice; error bars depict SEM (Rapa and control tumors, each n=9; UV-exposed skin from Rapa n=7 and control group n=4; unexposed control ventral skin n=3).

Discussion

In contrast to earlier findings with azathioprine and cyclosporine[9,10], the newer-age immunosuppressants Rapa and MMF did not enhance UV carcinogenesis. In the case of Rapa, it had the opposite effect of strongly impairing the development of large tumors (≥2mm). This impairment can be attributed to the known anti-tumor, or more specifically, anti-angiogenic effects of Rapa[16,17]. At dosages which maintained allogeneic heart grafts in mice, Rapa thus inhibited outgrowth of tumor implants whereas cyclosporine was found to enhance angiogenesis and strongly stimulate tumor outgrowth[18]. Rapa could even block the pro-angiogenic effect from cyclosporine. In the present study the inhibition of tumor outgrowth by Rapa appeared to be reflected in a significant reduction of Vegf-a positive tumor cells. However, the Vegf-a expression in tumor stroma did not appear to be significantly reduced, which leaves the question whether tumor cells and stroma were equally important in releasing Vegf-a into the interstitial compartment. Interestingly, we did not find any clear effect on the vasculature of tumors that grew in the Rapa-fed groups, but these tumors may be presumed to have been selected for their vascularisation and resistance to Rapa while those that lagged behind were most sensitive to the anti-angiogenic effect of Rapa. MMF was also reported to impair tumor growth and angiogenesis[16], but its effect on tumor implants appeared to be highly variable, possibly owing to differences in bio-availability of its metabolite mycophenolic acid[19]. This may correspond to the slight – but not significant – delay in onset of tumors (≥2mm) we observed in the MMF-fed animals.

Our data differ from those obtained by Duncan et al[36] who found that Rapa (sirolimus) increased the number of tumors by about 50% at the end of the experiment in which Rapa treatment started 10 weeks after weekly UV exposures. They reported some effects of MMF and Rapa on malignancy and average tumor size, but not any dramatic reduction in the number of large tumors, like we report here. In a subsequent study the same group did report a reduction in the number of tumors when Rapa was given after 15 weeks of UV irradiation and irradiation was discontinued[37]; at that point the mice had already developed an average of about 5 tumors per animal. In contrast to our experiments, their experiments were not optimal to detect any effect on tumor onset. Their experimental protocols differed substantially from ours, not only in the timing UV exposures and administration of Rapa, but also in the route of applying Rapa, i.e. by i.p. injection. At effective immunosuppressive dosages (1.5 mg/kg/day), the efficacy of Rapa against tumor growth was found to be much enhanced by regular release (continuous infusion) in comparison to a massive bolus dose delivered by injection (once every 3 days)[38].

The concept that immunosuppressive agents should, by their very nature, enhance UV-induced skin carcinogenesis[8-10] is not supported by the present experiment (nor by the aforementioned experiments in references 36 and 37). Our data show no enhancement at all from immunosuppressive dosages of MMF and Rapa, neither on early stages nor on late stages of tumor development. Early experiments did show some systemic effects on small tumors in hairless mice attributable to altered immunity: prior UV-driven induction of small tumors on a limited area of skin, speeded up later UV tumorigenesis in a distant, formerly shielded, area of skin[39]. It should, however, be noted that the immune effects involved in UV carcinogenesis are complicated: besides a UV-induced (transient) suppression of immunisation, there is also an induction of specific tolerance toward UV-induced tumors[8, 40-43]. UV carcinogenesis was actually delayed in CD80/CD86 double null mice lacking in tolerance induction[42]. Although immune deficient mice did develop skin tumors more rapidly upon UV exposure than their proficient counterparts, these immune deficient mice were found to develop the tumors even faster when they had been thymically-reconstituted[44]. Suppression of T cell-mediated immunity can, therefore, have differential and competing effects on UV carcinogenesis, including both inhibitory and enhancing effects. Whether the lack of effect of Rapa and MMF in the present experiments on UV induction of small tumors is due to a complete lack of immunogenicity of small tumors, or whether it is due to an 'accidental' cancelation of T cell-mediated effects, remains to be determined. Although UV-induced immunosuppression has been reported to be affected by estrogen receptor

signaling[45], we have found no gender effect in our experiments, in agreement with an earlier report[29].

P53 mutations appear to be involved in the earlier stages of tumor development[27]. Our suspicion that Rapa might selectively induce apoptosis in the p53-mutated cells, and thus slow down the rate at which tumors are initiated, is not supported by our data as we found no effect the onset of the smallest perceptible tumors (<1mm). To check further for local effects of the immunosuppressants on the epidermal cells that might have affected UV carcinogenesis (e.g. disturbed metabolism by Rapa) we investigated the *p53* mutation spectrum of the tumors which is normally dominated by the UV signature mutations. We found a dramatic shift in the types of mutations in tumors from the Rapa-fed group. This shift could have been due to an increase in ROS. Rapa did not cause such a shift when combined with MMF, which may be attributable to the known anti-oxidant activity of MMF.[46] We investigated the expression of target genes of Hif-1α to ascertain whether the Warburg effect and its suppression by Rapa could be responsible for changes in oxidative stress in the tumors. Although we did not find any effect of Rapa on the expression of these four marker genes, we did find striking differences in their expression in skin depending on UV exposure and in the tumors. In UV-exposed skin, the *Pdk1* expression was increased, which implies a block of pyruvate entry into the Krebs cycle, shutting down oxidative phosphorylation and associated ROS formation in the mitochondria. Moreover, the increase in *Glut1* may be related to anti-oxidant effects by transport of vitamin C[47]. We found *Ldha* to be highly expressed in the tumors which indicated that the Warburg effect was probably operative as Ldha catalyzes the conversion of pyruvate to lactate. Since *Pdk1* expression was low in the tumors from the control and Rapa groups, oxidative phosphorylation may also have been active in the tumors, which may thus have switched on both glucose-metabolizing pathways.

In conclusion, the present experimental data show that immunosuppressants do not necessarily enhance UV carcinogenesis, and can even lower the tumor burden. It has been already demonstrated that Rapa can impair the outgrowth of tumor implants, but our data provide evidence of the same effect on primary skin carcinomas induced by chronic UV exposure. Clinical data are beginning to emerge that also point to a lowered cancer risk when immunosuppressive medication is switched to Rapa[48-50]. Hence, further experimentation is urgently called for to better understand the pro- and anti-carcinogenic effects of various immunosuppressants, and eventually, to apply adequate immunosuppressive regimens while minimizing the long-term carcinogenic risk in organ transplant recipients.

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Chapter 3

Rapamycin impairs UV induction of mutant-p53 overexpressing cell clusters without affecting tumor onset

Voskamp P, Bodmann CA, Rebel HG, Koehl GE, Tensen CP, Bouwes Bavinck JN, El Ghalbzouri A, Van Kranen HJ, Willemze R, Geissler EK, De Gruijl FR

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Abstract

Because of its anti-tumor effect, the immunosuppressant rapamycin holds great promise for organ transplant recipients in that it may lower their cancer risk. In a mouse model we showed previously that rapamycin inhibits the outgrowth of primary skin carcinomas induced by UV radiation. However, the tumors that did grow out showed an altered *p53* mutation spectrum. Here, we investigated whether this shift in *p53* mutations already occurred in the smallest tumors, which were not affected in onset. We found that rapamycin did not alter the mutational spectrum in small tumors and in preceding microscopic clusters of cells expressing mutant-p53. However, rapamycin did reduce the number of these cell clusters. As this reduction did not affect tumor onset, we subsequently investigated whether rapamycin merely suppressed expression of mutated p53. This was not the case, as we could demonstrate that switching from a diet with rapamycin to one without, or vice versa, did not affect the number of existing mutant-p53 expressing cell clusters. Hence, rapamycin actually reduced the formation of mutant-p53 cell clusters. In wild-type and p53-mutant mice we could not measure a significant enhancement of UV-induced apoptosis, but we did observe clear enhancement in human skin equivalents. This was associated with a clear suppression of HIF1α accumulation. Thus, we conclude that rapamycin reduces the formation of mutant-p53-expressing cell clusters without affecting tumor onset, suggesting that tumors grow out of a minor subset of cell clusters, the formation of which is not affected by rapamycin.

Introduction

Organ transplant recipients have a high incidence of skin cancers. An important risk factor for skin cancer development appears to be immunosuppressive therapy, especially when the conventional immunosuppressants cyclosporine and azathioprine are used[1, 2]. The immunosuppressive drug rapamycin (sirolimus) has now been used for several years in immunosuppressive therapies in organ transplant recipients and appears promising in reducing the post-transplant cancer risk[3].

Rapamycin is an immunosuppressant that exerts its effect through inhibition of the mammalian target of rapamycine (mTOR) pathway, which is a mechanism of action entirely different from that of other immunosuppressants. Rapamycin has been shown to inhibit the mTORC1 complex, preventing p70s6k from becoming activated and thereby preventing S6 phosphorylation and by inhibiting 4EBP1, both processes affecting protein synthesis differently[4]. Furthermore, rapamycin has been shown to inhibit hypoxia-induced HIF1α and VEGF expression[5]. HIF1α accumulates in nuclei of keratinocytes after UV irradiation in a biphasic manner, peaking in protein expression at 10-24 hours after irradiation[6]. Rapamycin increased apoptosis in murine embryo fibroblasts deficient in p53 after serum depletion, while infection with Ad-p53 completely protected against rapamycin-induced apoptosis by inducing G1 cell cycle arrest[7].

Recent studies indicate that rapamycin decreases the rate at which skin malignancies develop. A switch to rapamycin therapy in a small study reduced the development of (pre) malignancies and nonmelanoma skin cancer in renal transplant patients[3]. Studies using hairless mice have also shown inhibiting effects of rapamycin on tumors; it reduced the incidence and progression of UV-induced skin cancer[8]. In a previous study by our group rapamycin did not affect the onset of UV-induced skin tumors <1mm, but showed decreased numbers of tumors >2mm (chapter 2). The latter study also showed that UV-induced tumors >2mm from rapamycin-fed mice harbor a different mutational spectrum of the *p53* gene, with less UV signature mutations (i.e. C to T transitions at dipyrimidine sites), compared with tumors from control-fed mice.

Hence, rapamycin does not affect tumor onset but it decreases development of large tumors, indicating that a specific subset of small tumors is not inhibited in growth by rapamycin. Increased levels of reactive oxygen species have been described in yeast to inhibit binding of rapamycin to its target TORC1, leading to rapamycin insensitivity[10]. The mechanism for rapamycin insensitivity may thus be linked to the altered *p53* mutational spectrum. This

led us to the hypothesis that the altered *p53* mutational spectrum occurs late in tumor development and is not present in the majority of small tumors or precursor lesions. UV-induced skin tumors >2mm typically harbor multiple *p53* mutations (chapter 2). The altered mutational spectrum may therefore appear late in tumor development without affecting tumor onset which is associated with early *p53* mutations, or a minor subset with deviant *p53* mutations may escape inhibition by rapamycin early-on in tumor development. To test this hypothesis on a shift in types of *p53* mutations we have determined *p53* mutations in tumors <2mm and clusters of mutant-p53 overexpressing cells (mut-p53 cell clusters) which arise in the skin after repeated UV exposure and are generally regarded as skin tumor precursors[11-14].

In performing this experiment we found that the number of mut-p53 cell clusters was lower in rapamycin-fed mice than in control mice. The apparent discrepancy between the reduction in mut-p53 cell clusters and unaltered tumor onset led to the hypothesis that rapamycin either decreases expression of the p53 protein or acts in a pro-apoptotic manner on mut-p53 cell clusters. A possible effect on expression of mutant p53 was tested by switching between diets with and without rapamycin, which would also demonstrate whether any disappearance of mut-p53 cell clusters is associated with apoptosis. A possible effect on UV-induced apoptosis was further studied in (conditional) mutant(P275S) p53 knock-in mice and wild-type mice, and more extensively in human skin equivalents. In this investigation we have identified the disparate effects of rapamycin in successive stages of UV carcinogenesis, demonstrating that local effects in the skin culminating in mutant p53-expressing cell clusters need not be predictive of tumor onset.

Materials and methods

SKH1 mouse model

SKH-1 hairless mice (Charles River, Maastricht, The Netherlands) entered the experiment at 8-16 weeks of age; both male and female mice were used. The animal room was illuminated with yellow fluorescent tubes (Philips TL40W/16, Eindhoven, The Netherlands) that did not emit any measurable UV radiation. The animals were housed individually in Macrolon type 1 cages (Techniplast, Bugguggiate, Italy) under a 12 h light-12 h dark cycle at 23°C. Chow was supplied in ample amounts (55-60 g/mouse/week), and drinking water was available *ad libitum*. Cage enrichment was absent to prevent shielding of the animals from UV exposure. All experiments were performed in accordance with legislation and approval of the medical center's ethics committee.

Groups on diets with admixture of rapamycin

To avoid repeated i.p. injections rapamycin was admixed to standard mouse chow resulting in physiologically relevant blood levels as described previously (chapter 2). For the present experiment ssniff *GmbH* (Soest, Germany) supplied the chow with rapamycin at 20 mg/kg, which was stored in a cold room and renewed every month. A study was performed to determine the rapamycin blood levels after starting (t=0, 1, 2 and 3 days after start, n=7-15) and stopping (t=0, 1, 2, 3 and 7 days after stop, n=3-15) the rapamycin diet. Blood of the mice was taken retroorbially between 8 and 9 a.m., and was subsequently analyzed by the Department of Clinical Chemistry (University of Regensburg) by LC-MS/MS for rapamycin concentration. Two diet groups were formed: rapamycin (n=10) and a control group (n=16) fed the standard chow without admixtures. For 10 weeks the mice were kept on their assigned food, then, in some experiments, the groups switched foods, i.e. the rapamycin group was put on control food and vice versa. Before (t=0, n=4), and at different time points after switching food (t=1, 2, 3 and 7 days, n=3), mice were taken out of the experiment (figure 3A).

Mutant $p53^{P275S/+}$ mice

Because both in humans and mice the human codon 278 has been proven a UVB exposure specific mutational hotspot, we have selected the murine equivalent p53P275S mutant for this study. The generation of this conditional point mutant p53 strain of mice was essentially as described for two other p53 point mutant mice with the $p53^{R172H}$ allele and the $p53^{R270H}$ allele[15] and can be briefly summarized as follows. The P275S point mutated allele was constructed by site-directed mutagenesis as described before[16] and has been made conditional by the introduction of a LoxP-flanked transcriptional STOP cassette into intron 1 of the *p53* gene (figure 1). This Lox-STOP-Lox (LSL) cassette was kindly supplied by Dave Tuveson. Detailed characterization of the LSL cassette was provided elsewhere upon the creation of a conditional oncogenic allele of K-ras[17]. This conditional p53P275S allele (18.7 kb) was verified by sequencing the entire construct (data not shown) and targeted into Ola129(E14) embryonic stem cells. Germline transmission was achieved in multiple clones and the presence of the STOP cassette in intron 1 and the presence of the P275S point mutation of these mice was determined by PCR (data not shown). After >5 generations of backcrossing to a C57Bl6 background, $p53^{P275S/+}$ mice were crossbred with EIIa-Cre deleter mice (both strains acquired from the Department of Human Genetics, LUMC, Leiden) yielding offspring with a mutant *p53* allele (C>T mutation changing P to S in codon 275, the UV-mutational hotspot) and wild-type (wt) littermates. As this study is the first to use the mice carrying the $p53^{P275S}$ allele, we checked transcription of mutant

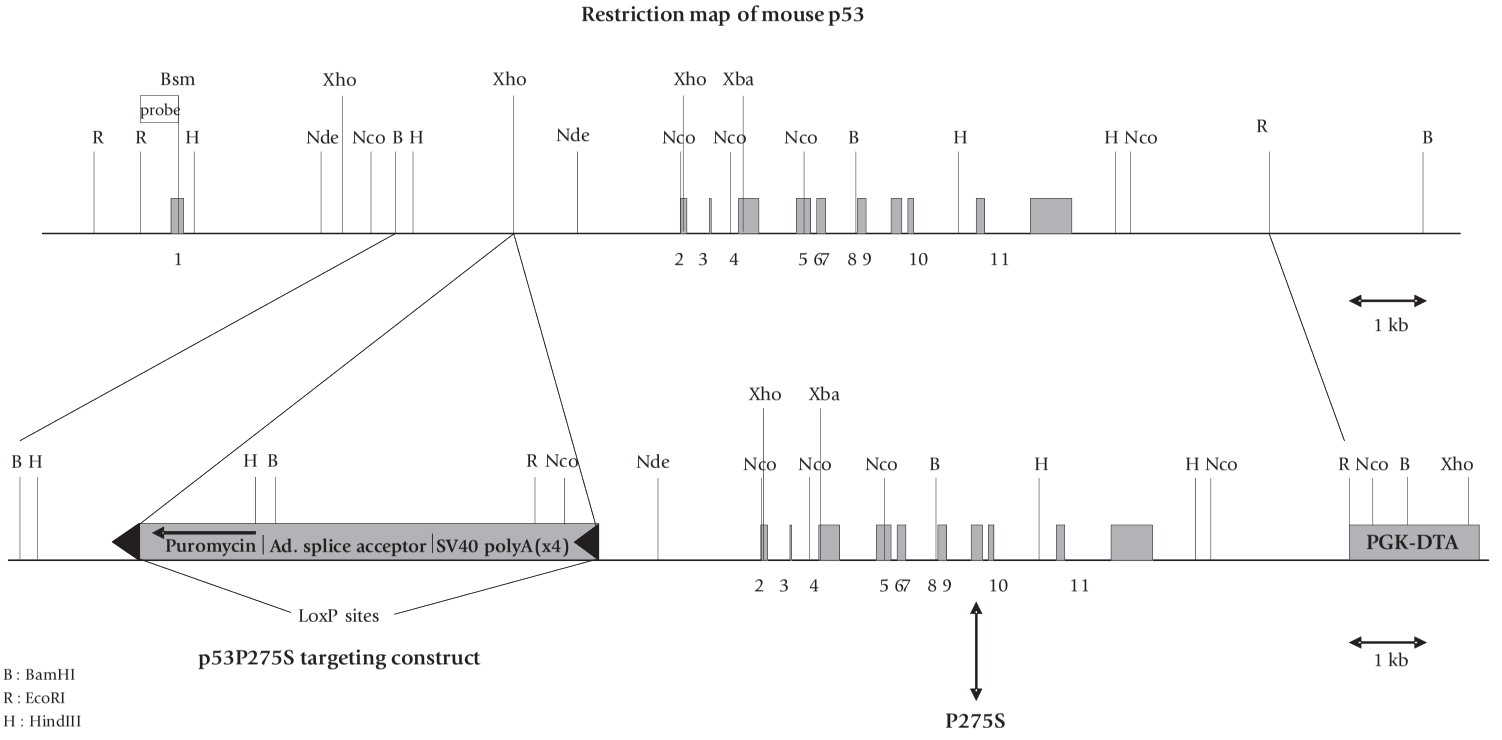


Figure 1: Generation of the p53P275S mutated allele. Insertion of the LoxSTOPLox cassette is shown. The PGK-DTA selection marker cassette was introduced during the construction of the point mutation in codon 275[16].

p53 mRNA in the EIIa-Cre/p53^{P275S} mice. The epidermises of F2 experimental animals were checked on expression of mutant p53 after UV exposure by staining with PAb240, excluding a minority (n=3) of mosaic mice not expressing mutant-p53 in the epidermis. Thus, twelve wt mice and eight mutant-p53 mice contributed to the final data. The mice were started on experimental diets (with or without rapamycin) for two weeks prior to UV irradiation. Mice were shaven one day prior to UV irradiation with 1.5MED (2250J/m^2) or 3MED (4500J/m^2). Forty-eight hours after irradiation mice were sacrificed and biopsies of the skin were taken. Part of each biopsy was snap-frozen in liquid nitrogen, with the other part being fixed in 4% formaldehyde, dehydrated and embedded in paraffin. Sections of the biopsies were stained for active caspase-3, p53 (CM5) and mutant p53 (Pab240).

Human Skin Equivalents (HSE)

Keratinocytes were isolated as described earlier[18]. In brief, surplus skin obtained from cosmetic surgery (obtained in accordance with the Dutch Law on Medical Treatment Agreement) was cut into small fragments and incubated overnight in dispase II (Roche Diagnostics, Almere, The Netherlands). Keratinocytes were isolated from the epidermis through incubation with trypsin at 37°C for 15 minutes. After trypsin inactivation, cells were filtered and cultured in keratinocyte medium at 37°C and 7.3% CO_2 until sub-confluency. Keratinocyte medium consisted of 3 parts Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen, Breda, The Netherlands) and 1 part Ham's F12 medium supplemented with 5% fetal bovine serum (FBS, HyClone/Greiner, Nürtingen, Germany), 0.5 mM hydrocortisone, 1 mM isoproterenol, 0.1 mM insulin (Sigma-Aldrich, Zwijndrecht, The Netherlands), 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Invitrogen, Breda, The Netherlands). For isolation of normal human dermal fibroblasts (NHDFs), human dermis was obtained by overnight incubation of fresh surplus skin from cosmetic surgery with dispase II. Fibroblasts were isolated from the dermis by incubation with a solution consisting of collagenase II (Invitrogen, Breda, The Netherlands) and dispase II (ratio 1:3) at 37°C for 2 hours. The cells were filtered, and cultured in fibroblast medium at 37°C and 5% CO_2 until sub-confluency. Fibroblast medium consisted of DMEM supplemented with 5% FBS, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin. Passages 2-5 were used for the experiments.

Full-Thickness Models (FTM) using rat-tail collagen were generated as described before[18]. In brief, 80 x 10^3 fibroblasts were seeded into acetic acid extracted rat-tail collagen. The fibroblast-populated matrices were cultured for a week in standard fibroblast medium. FTMs were seeded with 50 x 10^4 normal human epidermal keratinocytes in low passage per model. Cultures were incubated overnight in keratinocyte medium supplemented with

1% FBS, 53µM selenious acid, 10mM L-serine, 10µM L-carnitine, 1mM dL-a-tocopherol-acetate, 100µg ml^{-1} ascorbic acid phosphate, 2.4 x 10^{-5} M bovine serum albumin and a lipid supplement containing 25mM palmitic acid, 15mM linoleic acid and 7mM arachidonic acid (Sigma-Aldrich, Zwijndrecht, The Netherlands). Culture medium was then replaced with supplemented keratinocyte medium as described above, except that serum was omitted and the concentration of linoleic acid was increased to 30 mM. The models were cultured air-exposed from this time onward. Medium was refreshed twice per week. After 2 weeks of air-exposed culture, the HSEs were processed for analysis.

Supplement/Chemicals

Rapamycin (Calbiochem, Canada) dissolved in absolute DMSO was supplemented to HSEs (0.1%) that were cultured for 11 days at the air-liquid interface. Rapamycin concentrations used were 10 or 100nM[19], and were applied two days prior to UV irradiation.

UV-source

Mice: Six groups were started on their respective diets 1 week before subjecting them to a regimen of daily UV exposure. TL-12/40W tubes (Philips, Eindhoven, The Netherlands; 54% output in UVB – 280 to 315 nm – and 46% output in UVA – 315 to 400 nm) were used for daily UV exposure. The lamps were mounted over the cages with grid covers to allow undisturbed exposure of the mice. The lamps were automatically switched on daily from 12.30 to 12.50 h. The threshold dose for a sunburn reaction (minimal edemal dose, MED) in the hairless SKH-1 mouse was ~500J/m^2 UV under these lamps. The lamps were dimmed both electronically and by insertion of perforated metal sheets to expose the mice daily to 250 J/m^2 of UV radiation (0.5 MED).

HSE: Skin models were exposed to UV irradiation from TL-12/20W tubes (Philips, Eindhoven, The Netherlands; 54% output in UVB – 280 to 315 nm – and 46% output in UVA – 315 to 400 nm) at 0.28 mJ/cm^2/sec. UV dosages ranged from 0 to 110 mJ/cm^2 of which the higher dosages yield significant apoptotic response.

Mutant-p53 immunostaining in epidermal sheets

Within 24 hrs after the last irradiation, mice were sacrificed and 11 × 34 mm pieces of dorsal skin were excised and treated with a thermolysin solution, after which the epidermal sheet was separated. A modified procedure of epidermal sheet preparation and subsequent immunostaining and analysis was used[12]. Pab240 antibody (Monosan) was used at a 1:250 dilution, with secondary goat anti-mouse-biotin antibody (Dako) used at a 1:200 dilution.

P53 mutation determination

RNA extracted from tumors <2mm was converted to cDNA and used as a template to amplify and subsequently sequence the entire coding sequences of the *p53* transcript as described previously (chapter 2). Immunostained epidermal sheets were dehydrated and immuno-positive laser dissected mut-p53 cell clusters were lysed by proteinase K. After whole genome amplification (Genomiphi V2, GE Healthcare) of the lysate the DNA was purified by spin columns (DNeasy, QIAGEN, Venlo, The Netherlands) and served as template for PCR amplification of exons 4 – 8 using previously described primers[20]. The PCR products were completely sequenced in both directions.

Morphology and immunohistochemistry

Part of the HSEs were fixed in 4% formaldehyde, dehydrated and embedded in paraffin, whereas the other part was snap-frozen. For morphological analysis, 5µm sections were cut, deparaffinized, rehydrated and stained with haematoxylin and eosin for analysis by light microscopy. For HIF1α staining, sections (5µm) were cut, deparaffinized and rehydrated, and antigen retrieval was performed by boiling the sections in 0.1M citrate buffer (pH 6.9) in an autoclave at 110°C for 10 minutes. Subsequent steps were performed with a CSA amplification kit (Dako, ref 1500). For active-caspase-3 staining of HSE or mouse material, cryosections were cut (5µm), fixed in methanol/acetone (1:1) for 10 minutes and incubated overnight with primary antibody (anti-active-caspase-3, Ab2302, Abcam, Cambridge, UK) at 4°C. Secondary antibody (goat anti-rabbit-biotin, BA1000, Vector, Burlingame, CA) was added for 1 hour at RT and sections were stained with avidin-biotin complex (Vectastain, Vector) incubated with AEC. For *p53* staining of UV-exposed skin in mice, CM5 antibody (Monosan, Uden, The Netherlands) was used as described previously[21]. Deparaffinized sections of skin tumors from a previous study (chapter 2) were stained for mutant-p53 with Pab240 antibody as described previously[21].

Statistics

Differences in *p53* mutational spectra between the diet groups were calculated by $\chi 2$ test. Differences in the number of mut-p53 cell clusters were calculated using ANOVA. The Mann-Whitney test was used to calculate differences between percentages of cells of tumors expressing mutant p53. A P-value of <0.05 was considered as statistically significant difference. All calculations were performed with SPSS 16.0.

Results

Rapamycin alters the mutational spectrum of *p53* late in tumor development

In addition to the earlier measured altered *p53* mutational spectrum in tumors >2mm from rapamycin-fed mice (figure 2) (chapter 2), we investigated the *p53* mutations in tumors <2mm of rapamycin-fed mice from the same experiment. In a new experiment, mut-p53 cell clusters were induced in rapamycin-fed mice under the same conditions as the earlier study, and *p53* mutations in mut-p53 cell clusters were determined. Of the *p53* mutations in tumors <2mm, 80% (20 out of 25) were UV-type (i.e. C to T transition at dipyrimidine sites), and mut-p53 cell clusters contained 82% (9 out of 11) UV-type mutations (figure 2). Mut-p53 cell clusters and tumors have been previously described to harbor 79-89% UV-type mutations[12, 13]. These results show that the altered *p53* mutational spectrum in rapamycin-treated mice is only present in tumors >2mm.

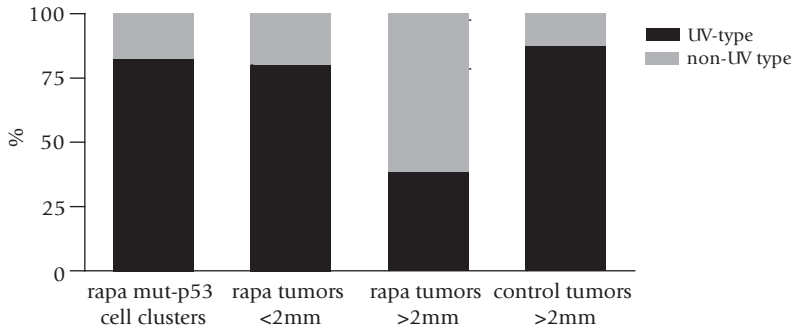


Figure 2: Percentages of UV-typical mutations (C to T transition at a dipyrimidine site) are shown here in mut-p53 cell clusters (n=15), tumors <2mm (n=21) and tumors >2mm (n=7) of rapamycin-fed mice and (n=7) of control mice (two columns for tumors >2mm from chapter 2). The mutational spectrum is only altered in tumors >2mm in rapamycin-fed mice.

Rapamycin impairs induction of mut-p53 cell clusters

In these experiments we observed fewer mut-p53 cell clusters in dorsal skin of rapamycin-treated mice than of control mice (data not shown). This may either be caused by the suppressed expression of (mutant-)p53 protein, enhanced apoptosis, or by decreased formation of mut-p53 cell clusters. To gain more insight into the mechanism, we conducted

an experiment in which mut-p53 cell clusters were induced by daily UV exposure in rapamycin-fed and control mice. Mice were put on experimental diets one week before starting UV exposures. After 10 weeks the diets were switched between the two groups. At different time points after switching food (1 to 7 days) mice were sacrificed and numbers of mut-p53 cell clusters were determined (figure 3A).

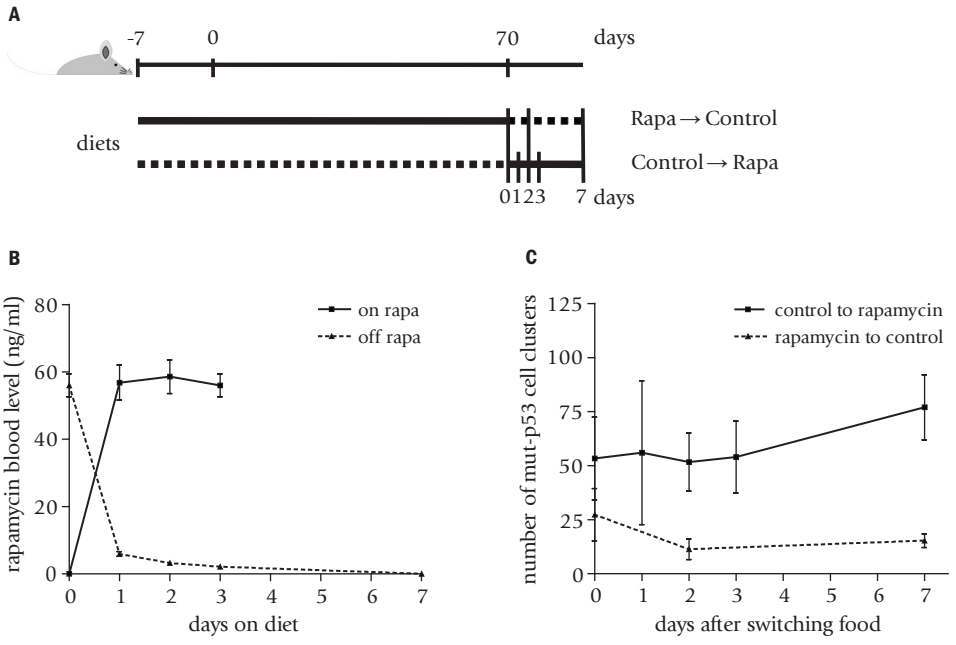


Figure 3: (**A**) Schematic presentation of the time course in the rapamycin diet cross-over experiment; daily UV exposure starts at t=0, dashed line depicts control diet and solid line diet with rapamycin, cross lines depict sampling points of skin to assess frequencies of mut-p53 overexpressing cell clusters. (**B**) rapamycin blood levels in mice started on chow containing rapamycin (solid line), and mice switching from rapamycin-containing chow to control chow (dashed line). (**C**) Numbers of mut-p53 cell clusters in epidermal sheets from mice after switching diet from rapamycin to control (dashed line) or control to rapamycin (solid line). Before switching the diets, mice were daily exposed to UV for 10 weeks. n=3-4 mice per group. Here, time point 0 refers to the time of switching diets. Error bars depict SEM.

We first measured the blood levels of rapamycin after starting or stopping rapamycin diets. Twenty-four hrs after starting the rapamycin diet, the rapamycin concentration in blood had already reached plateau levels (~57 ng/ml). When the rapamycin diet was stopped, drug

blood levels decreased to 10% of plateau after 24 hours, and were below detection limits after seven days (figure 3B).

Next, the numbers of mut-p53 cell clusters were determined in the different groups. Mice that had started on rapamycin diet during 10 weeks of UV-irradiation (n=10) harbored fewer mut-p53 cell clusters than control mice (n=10, p=0.002) Feeding of rapamycin to control mice (carrying pre-induced mut-p53 cell clusters) did not significantly change the numbers of mut-p53 cell clusters at any of the time points tested (figure 3C). Moreover, removal of rapamycin from the diet of mice did not significantly change the number of mut-p53 cell clusters at any of the selected time points (figure 3C).

Rapamycin does not affect the percentage of tumors with *p53* mutations, but does increase the mut-p53 expression in small tumors

Since the development of mut-p53 cell clusters is inhibited by rapamycin without having an effect on onset of tumors (<1mm) (chapter 2), the question arises whether mut-p53 cell clusters are genuine precursors of skin tumors. To gain more insight in the mechanisms involved, we checked whether (a proportion of) skin tumors in rapamycin-treated mice originate from cells without *p53* mutation (*p53* mutation is not obligatory for tumor formation). Therefore, we determined the percentage of tumors <2mm that contained mutations in the *p53* gene across the different groups from a previous study (chapter 2). Seventy-one percent (15 out of 21) of the tumors from mice on control diet, and 73% (11 out of 15) of the tumors from rapamycin-fed mice harbored *p53* mutations. These percentages are in agreement with a previous study[21] and show that the frequency of *p53* mutations is not different in tumors from rapamycin fed mice compared with control mice.

Expression of mutated p53 in tumors <2mm was determined by immunohistochemical staining of tumor sections. Remarkably, a higher fraction of tumor cells expressed mutated p53 in tumors of rapamycin-fed mice (n=16 from 6 mice) than in tumors of control mice (n=10 from 6 mice): 74% vs 34%, respectively, p=0.003 (figure 4). Expression of mut-p53 in tumors >2mm did not differ significantly between rapamycin-fed and control mice (89%, n=6 vs 74%, n=5, respectively).

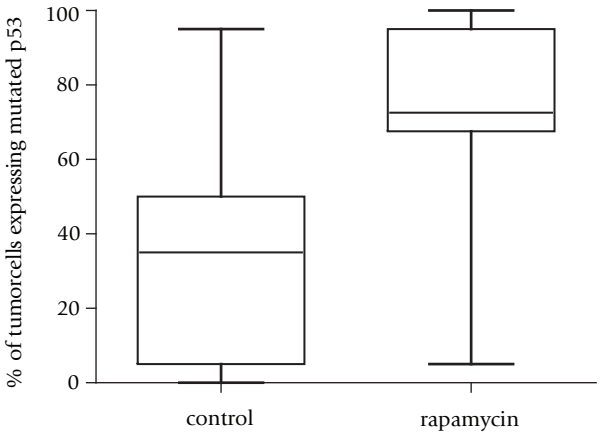


Figure 4: The fraction of mut-p53-expressing cells in tumors <2mm from rapamycin-fed mice is increased compared to the same size tumors from control mice. Staining of mutant p53 was performed on 10 and 16 tumors from six mice per group. Boxes depict upper and lower quartile and whiskers depict the minimum and maximum value.

Effect of rapamycin on active caspase-3 positivity after UV in wild-type and mutant-p53 mice

Using mice that harbor heterozygous mutations in the *p53* gene (275P>S) and littermates that were wild-type for *p53*, we investigated the effect of rapamycin on UV-induced apoptosis in exposed epidermis. Mice were fed a diet with or without admixture of rapamycin for two weeks. Subsequent exposure to 2250 J/m^2 (1.5MED for shaven mice) resulted in induction of the apoptosis marker, active caspase-3. Only mice with positive staining for mutant p53 were included in the analysis to exclude mice without cre-mediated activation of the mutant-p53 construct in the epidermis. The proportion of epidermal cells expressing active caspase-3 tended to be higher in wild-type mice (n=6) compared to mut-p53 mice (n=4) on control diet 48 hours after UV exposure (23% vs 15%, respectively, P>0.05). Rapamycin caused a slight but insignificant increase in apoptosis in wild-type mice (28%, n=6, vs 23%, n=6, respectively), whereas the increase in mutant p53 mice was two-fold, but still not significant (34%, n=4, vs 15%, n=4, respectively). As the variability in staining for apoptosis was substantial in skin samples and between mice (SD between 5 and 20%), a two-fold increase in apoptosis was not significant. Also, expression of p53 (wild-type and mutant) was determined by immunohistochemical analysis and found to be increased after UV irradiation in both mouse strains. There was no clear effect of rapamycin treatment on the fraction of cells overexpressing p53 in the irradiated skin (data not shown). The mutant p53

mice showed no baseline overexpression of mutant p53 in unirradiated (abdominal) skin, i.e. mutation of *p53* did not directly lead to overexpression of the protein.

Rapamycin enhances apoptosis in human skin equivalents (HSEs)

To assess the effect of rapamycin on apoptotic responses in human cells we performed a more elaborate dose-effect experiment using HSEs generated with cells obtained from two different human donors. The HSEs were cultured for 2 days in the presence or absence of 10nM or 100nM rapamycin. Subsequently, HSEs were irradiated with different doses of UVB (0, 30, 60, 85 and 110 mJ/cm^2), and 24 hours later HSEs were processed for assessing active caspase-3 by immunohistochemical analysis. The data shown in figure 5 clearly demonstrates that supplementation of 100nM (91 ng/ml) rapamycin to the cultures consistently results in an increased number of active caspase-3-positive cells in the basal layer of the HSEs at UV doses over 60 mJ/cm^2 (figure 5A). In addition, the number of positive cells for this apoptotic marker differed between HSEs generated with keratinocytes of the two different donors (figure 5B). Similar effects were also observed with 10nM of rapamycin (data not shown).

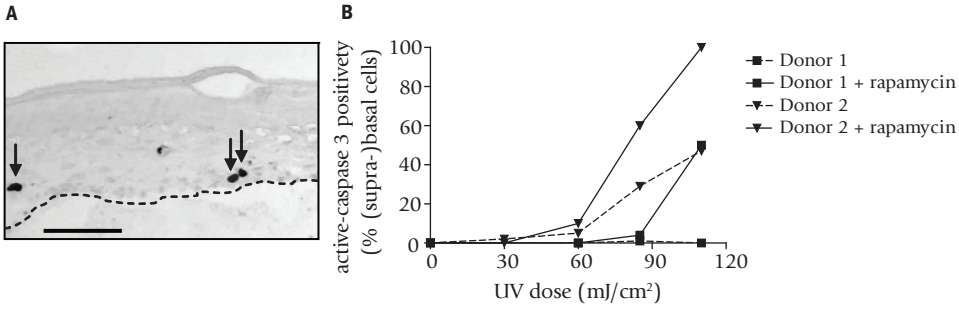
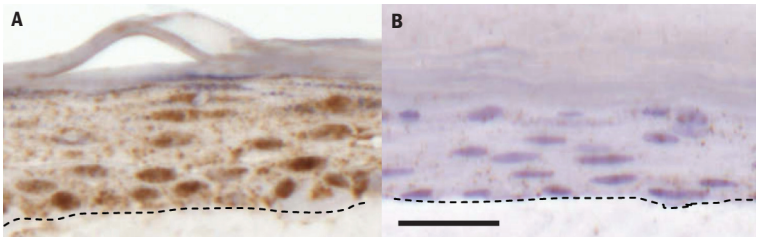


Figure 5: Effect of rapamycin on apoptosis in HSEs. (**A**) cross-section of an HSE treated with 100nM rapamycin and 24 h after irradiation with 90 mJ/cm^2 UV. The stained cells (arrows) are basal cells positive for the pro-apoptotic protein active-caspase-3. The dashed line indicates basement membrane. Scale bar=100 µm. (**B**) The percentage of positive (supra-) basal cells for active caspase-3 after UVB irradiation is increased in HSEs treated with 100nM rapamycin. Results of HSEs from two different donors are depicted. (active caspase-3+ cells in a HSE sample were counted in 2 stretches of 100 basal cells; spread in counts per HSE sample appeared to be within expected 95% confidence interval, based on a simple binomial distribution, i.e. ± 10% around 50% and ± 6% around 10% or 90%).

Rapamycin inhibits HIF1α up-regulation in HSEs after UV irradiation

Rapamycin has been shown to inhibit the accumulation of HIF1α in response to hypoxia[22]. To assess the effect of rapamycin on HIF1α induction after UV irradiation, we determined its expression after UVB irradiation using a dosage of 30 mJ/cm^2 in HSEs supplemented with or without rapamycin. The results obtained demonstrate that HSEs treated with rapamycin do not show any nuclei positive for HIF1α, while the majority of cells in control HSEs showed positive nuclei for this protein at 10 and 24 hrs after UV irradiation (figure 6A-C). The expression of HIF1α was also negative in untreated HSEs 1 hr after UV irradiation, as shown in figure 6C. Having established that HIF1α expression



C

Time after UV-irradiation (hr)	Control	Rapamycin 100nM
1	-	-
10	++	-
24	++	-
No UV	+	-

D

UV dose (mJ/cm^2)	Control	Rapamycin 10nM	Rapamycin 100nM
60	++	-	-
85	++	-	-
110	+	-	-

Figure 6: Effect of rapamycin on HIF1α induction in HSEs after UV exposure (**A**) HIF1α-stained cross section of HSE 10 hours after irradiation with 30 mJ/cm^2 UV in the absence of rapamycin; (**B**) HIF1α-stained cross section of HSE supplemented with 100nM rapamycin 10 hours after irradiation with 30 mJ/cm^2 UV; (**C**) table on HIF1α staining at several time points after UV exposure in rapamycin-treated or mock-treated HSEs. (**D**) table on HIF1α staining at 24 hours after irradiation with different UV dosages in rapamycin-treated or mock-treated HSEs.

Legend: -=<30% positive (supra-)basal nuclei; +=50-80% positive (supra-)basal nuclei; ++=>80% positive (supra-)basal nuclei. The dashed line indicates basement membrane. Scale bar: 50 μm

in HSEs after UV exposure is upregulated after 24 h, we determined the HIF1α expression 24 h after exposing HSEs to higher UV doses (60, 85 and 110 mJ/cm^2). The different UV doses all resulted in upregulated HIF1α expression in the control samples, whereas rapamycin-treated HSEs showed fewer nuclei stained positive for HIF1α (figure 6D). In the rapamycin-treated HSEs irradiated at 85 and 110 mJ/cm^2, the staining was dispersed, possibly due to the high dose of UV irradiation that induced many disintegrated cells positive for active caspase-3.

Discussion

As rapamycin-treated mice developed UV-induced skin tumors with an altered *p53* mutational spectrum, without affecting tumor onset (chapter 2), we speculated that this altered mutational spectrum emerges late in tumor development. Our results show that putative microscopic precursor lesions and early stage tumors indeed harbored a normal UV-related *p53* mutational spectrum, and that the occurrence of an altered mutational spectrum is specific for large tumors. Since we found lower numbers of mut-p53 cell clusters in rapamycin-fed mice, we speculated that rapamycin either decreases expression of the p53 protein or acts in a pro-apoptotic manner on mut-p53 cell clusters. Our results show that rapamycin does not decrease previously induced mut-p53 cell clusters, showing neither an effect on expression of mutant p53, nor any apoptosis in the clusters. From this we conclude that rapamycin truly inhibits the formation of these cell clusters, possibly by enhanced early apoptosis. Such an enhancement of UV-induced apoptosis by rapamycin was not significant in mice in our experimental setup, but could be clearly demonstrated in more elaborate and controlled experiments in HSEs.

The type of *p53* mutations in UV-induced squamous cell carcinomas (SCCs) in mice are normally predominantly of a UV-specific type with a C>T transition on a dipyrimidine site (UV signature mutation). In a previous study (chapter 2) our group has shown that SCCs >2 mm from rapamycin-treated mice displayed an altered mutational spectrum, with a minority of UV-typical mutations. Here we showed that this shift in the type of mutations was not present in mut-p53 cell clusters and skin tumors <2mm from mice treated with rapamycin. It can therefore be concluded that the deviant *p53* mutations were either late events in the development of tumors or only a minor subset of small tumors with an altered mutational spectrum (e.g. tumors with high endogenous oxidative stress) was able to grow out into large tumors, explaining the lower yield of large tumors in rapamycin-fed mice.

Numbers of mut-p53 cell clusters were lower in mice fed rapamycin after 10 weeks of UV irradiation when compared with control mice. The number of mut-p53 cell clusters was not altered after switching the diet without rapamycin to one with rapamycin, or visa versa. Since our study showed that the number of mut-p53 cell clusters was lower when rapamycin was present during UV exposure, and that the number of mut-p53 cell clusters was not decreased after introduction of rapamycin, it can be surmised that rapamycin does not suppress mut-p53 expression or force mut-p53 cell clusters into apoptosis, but genuinely inhibits the formation of mut-p53 cell clusters. This might be attributable to an enhancement of UV-induced apoptosis of DNA damaged cells, thus preventing (*p53*) mutation appearance (see below).

Tumor latency times in UV-irradiated hairless mice were not affected by rapamycin (chapter 2), but rapamycin in the present study impaired formation of mut-p53 cell clusters. This is an interesting finding since previous studies have correlated tumor induction closely with that of mut-p53 cell clusters[12, 23]. The present study shows for the first time that a drug can impair the development of mut-p53 cell clusters without affecting tumor onset. For various reasons mut-p53 cell clusters have previously been considered precursor lesions for skin tumors[12, 21], an assumption that is challenged by this study. It is still likely that most SCCs develop out of mut-p53 cell clusters since mutation of the *p53* gene appears to be a consistent early event in squamous cell carcinoma development, i.e. already present in a majority of precursor lesions and subsequently in a majority of SCCs[11, 21]. But it is now clear that numbers of the p53-mut cell clusters cannot be reliably used as indicator for tumor risk. Since the large majority of mut-p53 cell clusters do not develop into tumors[12], the impaired induction by rapamycin may only affect a majority of mut-p53 cell clusters which are not genuine precursors of tumors (actinic keratoses and SCCs). Hypothetically, a small specific subset of mut-p53 cell clusters might not be inhibited by rapamycin, e.g. due to an additional oncogenic change, and constitute the sole true precursors of the tumors. However, this additional oncogenic change remains to be identified and requires further study.

The percentage of small tumors harboring a mutation in the *p53* gene was not different between the mice on rapamycin and control diet, indicating that mutation of *p53* remained an important early event in the tumors in the rapamycin-treated group. It should, however, be noted that the small tumors were harvested at the end of the experiment and the skin of mice harbored many mut-p53 cell clusters at that time. This would make it more likely for a tumor to start with a mutated *p53* by chance, since many epidermal cells harbor a mutated *p53* in that stage of the experiment. A higher fraction of tumor cells expressed mutated p53 in

tumors <2mm after rapamycin-treatment of mice, compared to the same small-sized tumors of control mice. Rapamycin had, however, no apparent effect on the tumor development at that stage. This difference in expression of mutant p53 between rapamycin-fed mice and controls was not observed in tumors >2mm.

Rapamycin did not significantly alter the apoptotic response or p53 expression in wild-type mice or mice harboring a germline *p53* mutation. After a single dose of UV the active caspase-3 positive cell fraction in the epidermis of skin cells from mice on rapamycin diet was higher than in mice on control diet, especially in mut-p53 mice, but these rapamycin effects were not significant.. The variability in the staining for active caspase-3 (and p53) appeared to have prevented us from observing significant increases by rapamycin. Nonetheless, in more elaborate and controlled experiments in HSEs we did find a clear increase in UV-induced apoptosis by rapamycin. We found associated HIF1α accumulation in keratinocytes after UV exposure of human skin equivalents to be inhibited by rapamycin. Previously it has been shown that HIF1α is upregulated after hypoxia[24] and UV irradiation[6, 25], and that rapamycin can prevent hypoxia-induced HIF1α accumulation[22]. Here we show a similar effect of rapamycin on UV-induced HIF1α accumulation. HIF1α has been reported to inhibit or induce apoptosis, depending on the cell type[26, 27]. Our data suggest that HIF1α likely moderates UV-induced apoptosis, and that rapamycin neutralizes this HIF1α effect. Early rapamycin-enhanced apoptosis in UV-damaged cells may have reduced the number of p53-mut clusters that develop. Moreover, our previous finding that rapamycin inhibits the development of large tumors (chapter 2) might be attributable to attenuating the HIF1α pathway, thereby impairing the rate at which small tumors grow.

A recent prospective, controlled, clinical trial showed that transplant recipients who were switched to rapamycin treatment had a reduced rate of (pre)malignancies and nonmelanoma skin cancer[3]. In relation to this trial, the present study shows that rapamycin affects specific processes that may play a role in skin tumorigenesis.

To summarize, after UV irradiation apoptosis is increased and HIF1α accumulation is impaired in a human skin model. In our experimental setup we could however not measure such an effect of rapamycin on UV-induced apoptosis in freely moving mice, but rapamycin did reduce mut-p53 cell cluster formation in chronically UV-exposed mice. Although this rapamcyin-related reduction in mut-p53 cell clusters did not result in a reduced onset of tumors, rapamycin treatment did reduce the number of larger tumors. Besides providing insight on the precise impact rapamycin has on UV responses and UV carcinogenesis,

our data unexpectedly demonstrated that fewer mut-p53 cell clusters do not necessarily correspond with a reduced onset of tumors.

Acknowledgements

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Chapter 4

Immunosuppressants do not necessarily enhance UV carcinogenesis in mice and show discordances between mutant-p53 clones and tumor formation

Voskamp P, Bodmann CA, Koehl GE, Rebel HG, Van Olderen MGE, Gaumann A, El Ghalbzouri A, Tensen CP, Bouwes Bavinck JN, Willemze R, Geissler EK, De Gruijl FR

Submitted

Abstract

Immunosuppressive therapy is thought to cause the increased risk of nonmelanoma skin cancer in organ transplant recipients. Considering the different working mechanisms of immunosuppressants, we set out to investigate their effects on the sequence of events leading up to tumor formation, in particular whether early effects on apoptosis and the frequency of *p53* mutations would be predictive of the risk of skin cancer development. To this end, we compared immunosuppressants (azathioprine, cyclosporine, tacrolimus, mycophenolate mofetil and rapamycin) in a human skin model for short term effects and in a hairless mouse model for long term effects on UV carcinogenesis. In human skin models rapamycin was shown to inhibit epidermal regeneration and to increase UV-induced apoptosis, whereas cyclosporine decreased apoptosis. These apoptotic effects appeared to correspond to changes observed in the frequency of clones of cells overexpressing mutant p53 in chronically UV-exposed skin of mice that had immunosuppressants admixed to their diets. Deep sequencing of the mutational hotspots of *p53* (codons 270 and 275) in the UV-irradiated epidermis indicated, however, that none of the immunosuppressants affected the frequency of these hotspot mutations (in about 10% of epidermal cells). Only a minority of p53-mutated cells appeared to overexpress mutated p53. Rapamycin inhibited the outgrowth of tumors, and cyclosporine remarkably delayed the onset of tumors. Thus, we found that apoptosis and the frequency of *p53* mutations were not predictive of the rate at which skin carcinomas occurred. Moreover, none of the dietary fed immunosuppressants enhanced the onset of tumors, suggesting that these immunosuppressive medications per se do not necessarily increase the risk of skin carcinomas.

Introduction

Organ transplant recipients are at an increased risk of developing nonmelanoma skin cancer, especially squamous cell carcinomas (SCC), on sun-exposed body parts. In the Netherlands 40% of renal transplant recipients develop skin cancer within 20 years after transplantation[1]. In Australia where sun exposure is higher, this percentage is substantially higher (70%)[2]. As in the general population, skin carcinomas in organ transplant recipients are clearly related to solar UV exposure[3]. Furthermore, the skin cancers that these patients develop tend to be more aggressive and recur more often than those in immunocompetent patients[4].

From animal experiments it is known that most UV-induced skin carcinomas are antigenic and targeted by the immune system[5]. Therefore, the increased risk of developing skin cancer has long been regarded as an inescapable side effect of immunosuppression, which all transplant recipients are subject to in order to prevent rejection of the transplanted organ. More recently, it has become clear that some of the immunosuppressants commonly used can directly promote cancer growth and/or affect anti-tumorigenic responses such as apoptosis[6-8].

There are several classes of immunosuppressive drugs used in transplantation practice which differ by their mechanism of action. One of the first immunosuppressive drugs that allowed successful organ transplantation in humans is azathioprine (Aza). Its active metabolite 6-mercaptopurine (6MP) inhibits *de novo* purine synthesis and the metabolite 6-thioguanine competes for incorporation into DNA as a pseudo-base, thereby disrupting normal DNA/RNA synthesis[9]. Mycophenolate mofetil (MMF) is a prodrug of mycophenolic acid (MPA) which acts as an immunosuppressant by inhibiting *de novo* purine synthesis, but more specifically than Aza. MPA inhibits the enzyme inosine-5'-monophosphate dehydrogenase (IMPDH) without the incorporation of pseudobases into the DNA[10]. The calcineurin inhibitors cyclosporine (CsA) and tacrolimus (Tac) were thought to act more specifically on immune cells than Aza. CsA functions as an immunosuppressant by inhibiting interleukin-2 (IL-2) gene transcription. It does so by binding to cyclophilin and thus inhibiting the phosphatase calcineurin in activation of the nuclear factor of activated T-cells (NFAT)[11]. Tac also inhibits calcineurin, but via binding to FK-binding protein 12 (FKBP12)[11]. The immunosuppressant rapamycin (Rapa) is a compound that also interacts with FKBP12, but the complex targets and inhibits the mammalian target of rapamycin (mTOR)[12]. mTOR is the central node in signaling pathways that control cell growth, proliferation and metabolism[13]. Since IL-2 signals primarily through this pathway, T cell proliferation is reduced by mTOR inhibitors[14].

Some immunosuppressants have direct adverse effects on skin cells, which can contribute to carcinoma risk. CsA affects DNA repair[6, 7], and Aza enhances DNA damage by photosensitization[8]. Experiments by Australian groups[7, 15] have confirmed that Aza and CsA speed up UV carcinogenesis in hairless mice. CsA enhances angiogenesis and outgrowth of tumor inoculations in a mouse model, whereas Rapa inhibits both processes[16, 17]. Indeed, both Rapa and MMF are interesting with regard to their anti-tumor effects, since they impair growth of tumor inoculations in mice[17, 18] and Rapa inhibits *de novo* tumor development in a p53-null mouse model[19]. Recent research by our group has shown that Rapa increases apoptotic responses after UV irradiation in a human skin model (chapter 3). In a small randomized clinical trial, a switch to Rapa treatment was shown to decrease development of nonmelanoma skin cancers in organ transplant recipients[21]. A previous study by our group has shown that early oncogenic alterations in mutant p53-overexpressing cell clusters (mut-p53 cell clusters) occurred more frequently in the skin of renal transplant recipients using Aza, when compared to immunocompetent patients[22]. As these early oncogenic alterations are not immunogenic[23], it is likely that Aza had direct local effects on skin cells. A recent publication by our group showed that Rapa-fed mice develop fewer mut-p53 cell clusters compared to control mice (chapter 3).

Considering these notable differences between immunosuppressants, we posed the question whether it would be feasible to lower the skin cancer risk in organ transplant recipients by minimizing the non-systemic local impact on the skin. We investigated this by comparing commonly used immunosuppressants in parallel in experimental human skin and mouse models, in contrast to most prior investigations which either only included some immunosuppressants or did not combine early and late effects in UV-induced skin carcinogenesis. First, we systematically compared the impact of different immunosuppressants on epidermis and responses to UV exposure in human skin equivalents. Subsequently, we linked these *in vitro* results to effects of the immunosuppressants on UV carcinogenesis in a hairless mouse model. Thus, we aimed to establish whether local effects of immunosuppressants on specifically UV-induced apoptosis and *p53* mutations are predictive of the risk of skin cancer development.

Materials and Methods

Human Skin Equivalents (HSE)

Generation of human skin equivalents was performed as described earlier[24]. In brief, keratinocytes and fibroblasts were isolated from surplus breast skin obtained from cosmetic surgery of two donors (obtained in accordance with the Dutch Law on Medical Treatment Agreement). 80 x 10^3 fibroblasts were seeded into acetic acid-extracted rat-tail collagen deposited on filter inserts (product no. 3414; Corning, Amsterdam, The Netherlands). The fibroblast-populated matrices were cultured for a week in standard fibroblast medium. These matrices were subsequently seeded with 5 x 10^5 low-passage normal human epidermal keratinocytes and incubated with keratinocyte medium. Medium was refreshed twice a week. After 2 weeks of air-exposed culture, the HSEs were processed for analysis.

Supplements/Chemicals

Immunosuppressants dissolved in absolute DMSO were added to the HSEs at physiologically relevant concentrations[25, 26]: Rapa (Calbiochem, San Diego, SA) at 10nM or 100nM, 6MP (Sigma-Aldrich, St. Louis, MO) at 50nM or 500nM, CsA (Sigma-Aldrich) at 100nM or 1µM, Tac (Calbiochem) at 100nM or 1µM and MPA (Sigma-Aldrich) at 1µM or 10µM, at 0.1%(v/v). 6MP and MPA are used as they are the active metabolites of Aza and MPA, respectively[10]. For effects on morphology, HSEs were supplemented with immunosuppressants or DMSO (as vehicle control) during the air-exposed culture. For effects on the response after UVB irradiation, HSEs were supplemented with immunosuppressants for two days prior to UVB irradiation.

The mice

SKH-1 hairless mice (Charles River, Maastricht, The Netherlands) entered the experiment at 8-16 weeks of age; both male and female mice were used. The animals were housed individually under a 12 h light-12 h dark cycle at 23°C. Experimental chow was supplied in ample amounts (60 g/mouse/week), and drinking water was available *ad libitum*. Cage enrichment was absent to prevent shielding of the animals from UV exposure. All experiments were performed in accordance with legislation and approval of the center's ethics committee for animal experiments.

Groups on diets with admixtures of immunosuppressants

To avoid repeated i.p. injections or oral gavage application of the immunosuppressants, admixtures of the drugs to standard mouse chow were used (Sniff GmbH, Soest, Germany) (chapter 2). For CsA, MMF and Rapa dosages were used that resulted in long-term average blood levels in the mice that were similar to the high end average blood levels in renal transplant patients (CsA at 150 mg/kg: 800 ng/ml, MMF at 660 mg/kg: 2.9 µg/ml MPA, Rapa at 20 mg/kg: 50 ng/ml). Due to toxicity Tac and Aza were admixed at maximal long-term tolerable dosages (Tac 20 mg/kg: 3.2 ng/ml, Aza 30 mg/kg: sub-anaemic white blood cell count). Six diet groups were formed: Rapa (n=26), MMF (n=22), CsA (n=24), Tac (n=24), Aza (n=26) and a control group (n=26) fed the standard chow without admixtures. The tumorigenesis experiment was performed in three parts, 1) Rapa and CsA, 2) Aza and Tac, 3) MMF, each with a control group on chow without admixture. No apparent differences in food intake and body weights were observed between the diet groups.

UVB irradiation

Mice: The six groups were started on their respective diets 1 week before subjecting them to a regimen of daily UV exposure. TL-12/40W tubes (Philips, Eindhoven, The Netherlands; 54% output in UVB – 280 to 315 nm – and 46% output in UVA – 315 to 400 nm) were used for daily UV exposure. The lamps were mounted over the cages with grid covers to allow undisturbed exposure of the mice. The lamps were automatically switched on daily from 12.30 to 12.50 h. The threshold dose for a sunburn reaction (minimal edemal dose, MED) in the hairless SKH-1 mouse was ~500J/m^2 UV under these lamps. The lamps were dimmed both electronically and by insertion of perforated metal sheets to expose the mice daily to 250 J/m^2 of UV radiation (0.5 MED).

HSEs: Skin models were also exposed to UV irradiation from TL-12/20W tubes at 0.28 mJ/cm^2/sec.

UVA irradiation of azathioprine-fed mice

Pigmented C57BL/6 mice on a hairless background (acquired by two rounds of breeding C57BL/6 with SKH1) were put on Aza (n=12) or control diet (n=13) 1 week before subjecting them to a regimen of daily UV exposure. Cleo Professional S R tubes (Philips, Eindhoven, The Netherlands; 1.4% output in UVB – 280 to 315 nm – and 98.6% output in UVA – 315 to 400 nm) were used for irradiation. MED was determined by exposing mice to 7, 14, 21 or 28 kJ/m^2 UV. Mice were daily irradiated at 14 kJ/m^2 (1MED) in the UVA carcinogenesis experiment.

Tumor assessment

The mice were inspected weekly for tumors which were registered for each mouse individually on maps (recording number, location, size and form). For presentation in a single graph of the tumor induction data in Kaplan-Meier plots, the three experiments were adjusted to equal median latency times in the control groups (a time shift of maximally 2 weeks, with an average of 8 days). Upon removal of animals from the experiment, tumors and normal skin were isolated for further analysis as described below.

Epidermal sheet preparation and mutant-p53 immunostaining in epidermal sheets

For quantification of mut-p53 cell clusters, four SKH1 mice were taken from each diet group after 4, 7, or 10 weeks of UV exposures. Within 24 h after the last irradiation, mice were killed and pieces of dorsal skin of 11 × 34 mm were excised and treated with a 100 µg/ml thermolysin solution, after which the epidermal sheet was separated. The procedure of epidermal sheet preparation and subsequent immunostaining and analysis, described elsewhere[28], was followed with two modifications: antigen retrieval in 10mM citrate buffer in an autoclave at 5 min, 110°C and mutant-p53 antibody diluted 1:250 (Pab240, Thermo Fisher Scientific, Fremont, CA). A 1 to 2mm wide mid-dorsal strip of the epidermal sheets was collected per mouse and used for DNA isolation (QIAamp, QIAGEN, Venlo, The Netherlands). Counts of mut-p53 positive cell clusters outside the range of 6-fold over or under the median (in cohorts of 4 mice) were rejected as outliers (with the exception of "0" if the lower limit was <1) to produce figure 2; this led to exclusion of 9 of 84 measurements from this graph. However, none of the measurements were excluded from statistical tests (see below).

P53 mutation hotspot PCR and deep sequencing

DNA from the murine epidermal strips of four mice per group was mixed equimolarly and used in a PCR reaction amplifying the *p53* gene from codon 263 to 276, containing the mutational hotspots at codons 270 and 275. The forward primer contained a sequence-specific part and an adapter sequence at the 5′ end of the primer. The reverse primers were unique for each group containing a sequence-specific part, an index sequence, and an adapter sequence (supplementary table 1). Primers were HPLC-purified (Integrated DNA technologies, Coralville, IA). PCRs were performed using high fidelity Phusion enzyme (Finnzymes, Vantaa, Finland) in a volume of 40 µl with the following program: 98°C for 30 sec, then 28 cycles of 98°C for 10 sec, 64°C for 15 sec, 72°C for 15 sec, closing the program with 72°C for 10 min. PCR products were run on a 1.8% agarose gel, and stained

with Sybr Gold (Invitrogen), after which bands containing the amplicons were excised on a Dark Reader not emitting UV (Clare Chemical Research, Dolores, CO) and purified using QIAquick (QIAGEN) spin columns. Amplicons were analyzed in one lane of an Illumina Sequencer HiSeq2000 (Illumina, San Diego, CA). Reads were filtered for high quality reads containing only bases that were called with a minimal fidelity of 99.9% (phred-score ≥30). Index sequences were used to distinguish the different samples. For the numbering of the bases, the p53 transcript with ID ENSMUST00000108658 from Ensembl was used as reference.

Histology and immunohistochemistry

Biopsies were fixed in 4% formaldehyde, dehydrated and embedded in paraffin or snap-frozen in liquid nitrogen. Stainings for keratin 10 (K10), keratin 16 (K16), keratin 17 (K17) and Ki-67 were performed on formalin-fixed and paraffin-embedded sections (5µm) of skin models, mouse skin or tumors. Immunohistochemical analysis of active caspase-3 was performed using frozen sections (5µm), which after sectioning were fixed in methanol/acetone (1:1) for 10 minutes. Haematoxylin and eosin (H&E) staining was performed on deparaffinized sections for tumor staging. Staging of tumors from each group was performed blinded by a pathologist (Dr. A. Gaumann, University of Regensburg, Germany) who is experienced in mouse and human pathology, including diagnosis of actinic keratoses as proper precursors of squamous cell carcinomas. Antigen retrieval for paraffin-embedded sections was performed by autoclaving the sections in 10mM citrate buffer (pH 6.0) for 10 minutes at 110°C. The primary antibodies used for immunohistochemistry are listed in supplementary table 2. After overnight incubation with the primary antibody at 4°C, sections were stained using standard protocols with AEC.

Estimate of proliferation index

The percentage of Ki-67 positive nuclei in the basal and immediate supra-basal layer was used to determine the proliferation index. A minimum of 100 cells were counted using light-microscopy in three different regions in each section.

Statistical tests

Kaplan Meier plots of tumor-free survival were tested for differences by χ^2 statistics (Graphpad Prism 5.0). Differences in tumor yields were analyzed with randomized block one-way ANOVA, with Bonferroni as post-hoc test (Graphpad Prism 5.0). Differences in number of mut-p53 cell clusters per treatment (without outlier removal) were calculated in a general linear model multivariate analysis with least significant difference as post-hoc test.

Differences in Ki-67 positive cell percentages in HSEs were calculated with one-way ANOVA and Dunett as post-hoc test. Differences in active-caspase 3 positive cell fractions in HSEs were calculated with a one-way ANOVA and Bonferroni as post-hoc test. $P<0.05$ was taken to indicate a significant difference. SPSS 16.0 was used for all statistical analyses, except for the tumor free-survival and yield analyses.

Results

Rapamycin affects epidermal regeneration in HSE

In a human skin equivalent (HSE) the effects of immunosuppressive drugs on epidermal regeneration were studied. HSEs were cultured with immunosuppressants added to the medium for two weeks: Rapa, Tac, MPA, 6MP, CsA or DMSO (vehicle). The skin models cultured in the presence of Rapa (10 and 100nM) showed a much reduced epidermal regeneration; the number of cell layers in control models was 7 to 8, whereas in the Rapa-treated models 3 to 4 layers were formed (figure 1). Rapa treatment resulted in a decreased proliferation index (22% with 100nM Rapa, and 11% with 10nM Rapa versus 43% with DMSO), indicating decreased proliferation ($p<0.05$, supplementary figure 1). Immunohistochemical staining for hyperproliferative markers K16 and K17 showed a decreased expression in the Rapa-treated models. The differentiation marker K10 showed similar expression levels with all treatments, staining all suprabasal cell layers (data not shown). Taken together, these observations show that Rapa decreases the proliferation of keratinocytes in the skin model. All the other treatments with immunosuppressants did not result in significantly altered epidermal regeneration, proliferation or K10, K16 and K17 expression (data not shown), nor were there any other morphological differences.

Cyclosporine decreases and rapamycin increases the active caspase-3 positive cell fraction in HSE after UV irradiation

To examine the effects of immunosuppressants on a possible apoptotic response, HSEs from two donors were irradiated with UV (140mJ/cm^2) two days after supplementation of immunosuppressants to the media. The percentage of active caspase-3 positive cells 24 hours after irradiation was determined by immunohistochemistry. Of the five immunosuppressants, only Rapa at 10nM and 100nM increased the percentage of active caspase-3 positive (supra-) basal cells (average 20%, SEM = 4%, vs 7%, SEM = 6%, in controls) in skin models of one donor with a low apoptotic response, whereas only CsA at 1µM was found to decrease the

apoptotic percentage (35%, SEM = 10%, vs 100%, SEM = 0%, in controls) in skin models of the donor with a high apoptotic response.

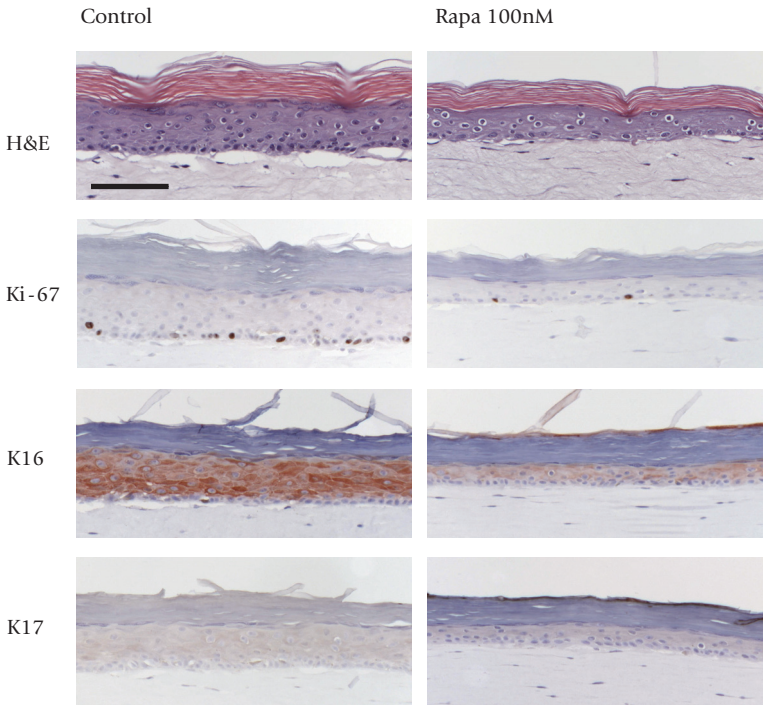


Figure 1: Rapa alters epidermal regeneration, proliferation, K16 and K17 expression in HSEs. Representative pictures are shown. Scale bar: 100µm.

Cyclosporine and azathioprine increase numbers of mut-p53 cell clusters

Mut-p53 cell clusters develop in chronically UV-irradiated skin, which are generally thought to be precursor lesions of squamous cell carcinomas that arise after continued UV exposure. Since HSEs are not suitable for generating mut-p53 cell clusters due to their limited lifespan, we examined the effects of immunosuppressants on generation of mut-p53 cell clusters in mice. Hairless mice were fed with immunosuppressants admixed to the chow at dosages resulting in blood levels that are at the high end of the range of typical blood levels in renal transplant recipients. Due to toxicity of Tac and Aza, dosages of these drugs were limited to maximal long-term tolerable levels. The mice were daily irradiated with 0.5MED UVB for 4, 7, or 10 weeks, at which time numbers of mut-p53 positive cell clusters were determined. CsA and Aza treatment increased the number of mut-p53 cell clusters ($p<0.001$ and $p<0.05$,

respectively) (figure 2). Rapa tended to inhibit their formation at all time points (p=0.07). The proliferation index of dorsal skin from 4 week-irradiated mice was not affected by any of the immunosuppressants (data not shown). For comparison with *p53* mutational frequencies (described below) we determined in 10 week-irradiated control mice the percentage of surface covered by the mut-p53 cell clusters in the epidermal sheet bordering the epidermal strip that was removed for mutational analysis; mut-p53 cell clusters covered about 6% of the surface area.

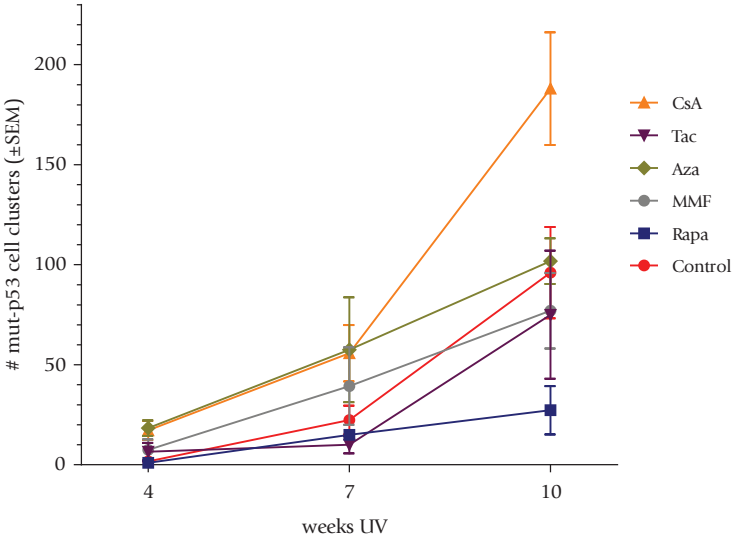
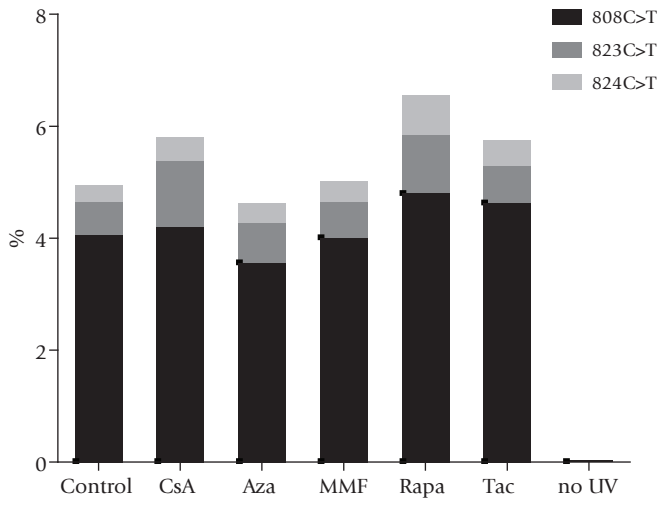


Figure 2: Effects of immunosuppressive drugs on the induction of epidermal mut-p53 cell clusters after 4, 7 and 10 weeks of chronic UV treatment. CsA and Aza-treated mice developed increased numbers of mut-p53 cell clusters; Rapa-treated mice developed a lower number of mut-p53 cell clusters compared to control mice.

Frequency of p53 mutations in UV-exposed skin

The question arose whether the number of mut-p53 cell clusters corresponded with the frequency of mutated alleles in the *p53* gene in the epidermis. To answer this question, DNA from epidermal strips of mice UV-irradiated for 10 weeks and unirradiated control mice was used as template to amplify part of the *p53* gene containing two mutational hotspots (codons 270 and 275). PCR products (pooled for four mice per group) were analyzed by deep-sequencing, with a sequencing depth between 2 million and 8 million for the different samples. Three mutations were common in samples of the UV-irradiated mice: 808C>T = aa270 R>C, 823C>T = aa275 P>S, 824C>T = aa275 P>L, with numbers cumulating to a total

between 4.6% and 6.6% of the PCR products; samples from un-irradiated mice contained these mutations far less frequently at 0.03% (figure 3). There were no clear differences in mutational frequencies between the samples from different immunosuppressive treatments and controls.



Base number	mutation	Amino acid change
808	C>T	270 R>C
823	C>T	275 P>S
824	C>T	275 P>L

Figure 3: Frequency of hotspot mutations in the p53 gene. DNA isolated from epidermal strips of mice daily UVB-irradiated for 10 weeks was analyzed by deep-sequencing the fragment-spanning codons 263 to 276 of the p53 gene. The three most commonly occurring mutations are presented in the table and in the graph as percentages of all analyzed PCR products.

Cyclosporine delays tumor induction and rapamycin inhibits tumor growth

To examine the effects of immunosuppressants on UVB-induced tumor formation, mice were fed immunosuppressants in the food and daily exposed to 0.5MED of UV. Tumor development was assessed weekly. CsA feeding resulted in increased tumor latency times for tumors >1mm (figure 4A). Confirming earlier results (chapter 2), Rapa-fed mice showed no change in onset of small tumors (diameters around 1mm) but did show a lowered rate of formation of larger tumors, i.e. an inhibition of tumor outgrowth. Rapa and CsA groups showed significantly lower yields of tumors >4mm compared with controls ($p<0.01$ and

p<0.001, respectively) (figure 4B). Near the end of the experiment, several mice had to be taken out of the different groups because they extensively scratched their tumors. After 155 days, 7 of the 12 mice of the CsA group were still in the experiment compared with 9 out of 13 mice in the control group. None of the other immunosuppressants in the food of the mice appeared to affect the onset or outgrowth of the UV-induced tumors. Histopathology showed all tumors >4mm to be SCCs. No effect of immunosuppressive drugs on tumor type or grading was found.

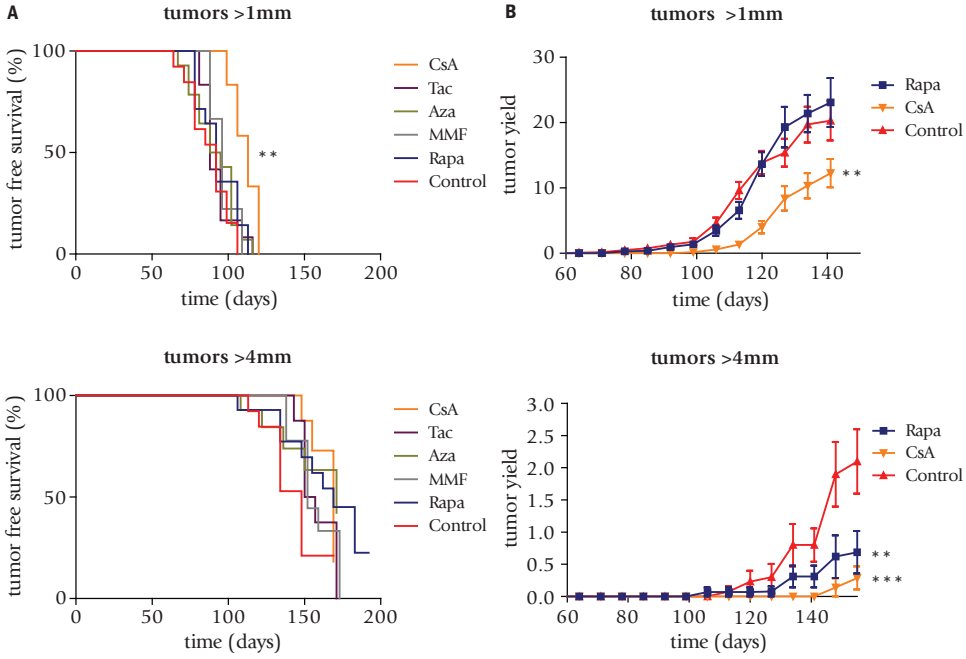


Figure 4: (**A**) Tumor-free survival (Kaplan-Meier) plots of the different treatment groups, with different thresholds for tumor detection: diameters >1mm or >4mm. (**B**) Tumor yields (average number of tumors per mouse) are shown for control, Rapa and CsA treatment. Error bars depict SEM. **p<0.01 ***p<0.001

Azathioprine did not affect UVA-induced tumor induction

Aza has been previously reported to increase the sensitivity of the skin to UVA light[9]. To assess whether Aza sensitizes mice for UVA carcinogenesis, mice were put on an Aza-containing diet or control diet for two weeks. No difference in minimal edemal dose was observed

between the two groups 24 hours after irradiation, both for albino hairless SKH1 mice, and for pigmented SKH1/BL6 mice. In the pigmented mice, 72 hours after irradiation, the Aza-fed mice showed more extensive skin burning compared with control mice (supplementary figure 2). UVA carcinogenesis was therefore studied in pigmented mice. In these mice Aza showed no significant effect on tumor yields for tumors >1mm or >2mm (supplementary figure 3). Since some mice started scratching their tumors after 19 weeks in the experiment they had to be removed from the experiment; as a result, tumor yield data for tumors >4mm could not be measured reliably.

Discussion

In this study we aimed to establish whether short-term effects of immunosuppressants on UV-induced apoptosis and medium term effects on *p53* mutations are predictive of the risk of skin cancer development. Of the immunosuppressants tested in this study, Rapa showed an inhibitory effect on epidermal regeneration and increased UV-induced apoptosis in human skin models, whereas CsA decreased apoptosis. Despite corresponding effects on the formation of clusters of cells overexpressing mutant-p53 in mice – i.e. a decrease by Rapa, an increase by CsA – the effects on tumor onset were discordant. CsA was unexpectedly shown to delay tumor onset and Rapa had no effect on tumor onset, but inhibited tumor growth. Remarkably, none of the dietary fed immunosuppressants increased UV-induced skin carcinogenesis in the mouse model employed in this study; an overview of the results is presented in table 1. In the following paragraphs we discuss the effects of immunosuppressants on UV-responses and the different stages in tumor development, *viz.* cellular responses in human skin models, mut-p53 cell clusters, p53 mutational frequencies and tumor development in mice.

Table 1: Summary of the results obtained for the different immunosuppressants.

		CsA	Tac	Aza	MMF	Rapa
Human skin model	Epidermal regeneration	–	–	–	–	↓
	Proliferation	–	–	–	–	↓
	Apoptosis after UV	↓	–	–	–	↑
Mouse model	Proliferation	–	–	–	–	–
	Number of mut-p53 cell clusters	↑	–	↑	–	↓
	P53 mutational frequency in non-tumor epidermis	–	–	–	–	–
	Yield of small tumors (>1mm)	↓	–	–	–	–
	Yield of large tumors (>4mm)	↓	–	–	–	↓

Legend: – = no effect, ↓ = inhibitory effect, ↑ = positive effect

Rapa increased the apoptotic response of human skin models to UV exposure, whereas CsA inhibited this apoptotic response, which is in agreement with earlier studies in cell cultures and mice[6, 29]. Epidermal regeneration in reconstructed human skin was impaired by Rapa. HSEs treated with Rapa showed a thin epidermis with only a few cell layers, as well as a decreased proliferation index and reduced expression of the hyperproliferation markers K16 and K17. This decreased proliferation in HSEs may be related to wound-healing problems associated with Rapa treatment in transplantation patients[30]. In HSEs, CsA pre-treatment caused a decrease in the apoptotic response after UV irradiation. This decrease in apoptotic response was only evident in HSEs with a high percentage of apoptotic cells, leaving the possibility that only at high UV doses CsA decreases apoptotic responses, as suggested by Flockhart et al.[31].

CsA and Aza increased the formation of mut-p53 cell clusters in mice, whereas Rapa tended to inhibit mut-p53 cell cluster formation. The lower number of UV-induced mut-p53 cell clusters formed in the skin of Rapa-treated mice is apparently not due to decreased proliferation, since the fraction of Ki-67 positive cells was not decreased in Rapa-treated mice. A previous study from our group[22] showed no effect of Aza on mut-p53 cell cluster formation in mice, but it should be noted that Aza in these experiments was administered by i.p. injections, and variations in counts of these clusters were very large.

Immunosuppressants did not affect the mutational frequency of *p53* in chronically UV-irradiated skin in mice. Deep sequencing analysis of the mutational hotspots in the *p53* gene showed that many cells harbor a mutated allele of *p53*. There was no clear effect of the immunosuppressive treatments on the frequencies of mutated *p53* alleles, contrasting with effects on mut-p53 cell cluster formation. On average, 5% of the sequenced alleles were mutated, corresponding to 10% of the cells assuming heterozygous mutations. As the hotspot mutations at codons 270 or 275 comprise about half of the mutations in the p53 gene[28, 32], the mutational frequency suggests that approximately 20% of the cells in the skin would harbor mutated *p53*. These results are in agreement with a recent deep sequencing study of human skin from middle aged individuals revealing that persistent *p53* mutations had accumulated in 14% of the epidermal cells[33]. The percentage of skin area containing mut-p53 cell clusters was much lower, approximately 6%. It can therefore be concluded that a minority of the cells harboring a mutated *p53* show overexpression of mut-p53 in cell clusters. This puts the effects of Rapa, CsA and Aza in a different perspective, indicating that a mutation in *p53* is apparently not sufficient to cause overexpression of the p53 protein in mut-p53 cell clusters[34] (chapter 3), and that tumors may arise from a larger

pool of p53-mutated cells that do not overexpress the protein. Evidently, the local effects of immunosuppressants in the skin affecting the mut-p53 cell clusters are not paralleled by corresponding effects on p53 mutations and tumor onset.

None of the immunosuppressants increased UV-induced tumor development, and CsA even delayed tumor onset. Rapa had no effect on the development of small tumors, but decreased the outgrowth of tumors. Growth inhibition was reported earlier by our group (chapter 2), but the growth inhibition by Rapa was more prominent in that experiment. Other UV carcinogenesis studies with different treatment schemes have shown either lower tumor yields[35] or higher tumor yields[36] in Rapa-treated mice, indicating that treatment schemes may have important effects on the outcomes of the tumorigenesis experiments. Proliferation of epidermal keratinocytes was not affected by CsA, making it unlikely that reduced keratinocyte proliferation was causing the tumor delay. Toxicity of CsA on tumors is not very likely either, as a three-fold lower concentration of CsA in the diet still exerted the same effect (See supplementary figure 4). In contrast to CsA, Tac did not have any measured effects in this study, possibly due to the lower dose of Tac that was used to avoid toxicity problems. Blood levels of Tac were approximately four times lower than trough Tac blood levels in organ transplant recipients[37]. Alternatively, the effects of CsA on tumor development are caused by a an effect not exerted by Tac[38]. A pioneering study of the effect of CsA on UV-carcinogenesis[7] showed a shorter tumor latency time compared to controls. In that experiment mice were administered CsA in oil by gavage three times a week, after which they were exposed to UV at 2 to 4 hours (corresponding to the expected peak drug levels). The mode of administrating CsA in our experiment results in relatively stable CsA blood levels over time; while using gavage results in a peak of CsA blood levels in the first hours and decrease thereafter. With a different experimental setup, Wulff et al also showed that CsA treatment by repeated i.p. injections reduced the rate of development of new tumors in mice[35]. The different ways of administrating CsA result in pharmacologically different profiles; stable exposure to CsA when administered in the food may have different effects when compared to bolus injection. Further experimentation on the effect of CsA treatment schemes on tumor formation is called for.

No correlation was found in this study between development of mut-p53 cell clusters and SCCs. Numbers of mut-p53 cell clusters had always correlated with the rate of tumor development in previous studies [5, 28, 39]. Mut-p53 cell clusters develop as a clonal expansion of a mutated keratinocyte. Since most SCCs and actinic keratoses harbor mutations in the *p53* gene, *p53* mutations are considered as early events in SCC formation and therefore

mut-p53 cell clusters are thought to be precursors of SCCs[40, 41] (figure 5A). However, an earlier retrospective study did not find any differences in numbers of mut-p53 cell clusters between skin from patients with solitary versus multiple skin carcinomas[42]. As we found that compounds (Rapa and CsA) can have opposing effects on mut-p53 cell clusters and SCC formation, it follows that mut-p53 cell cluster formation cannot be used as a simple indicator of tumor risk. These results open up an alternative perspective on the developmental stages of skin carcinomas, one in which SCC development is independent of mut-p53 cell cluster formation (figure 5B).

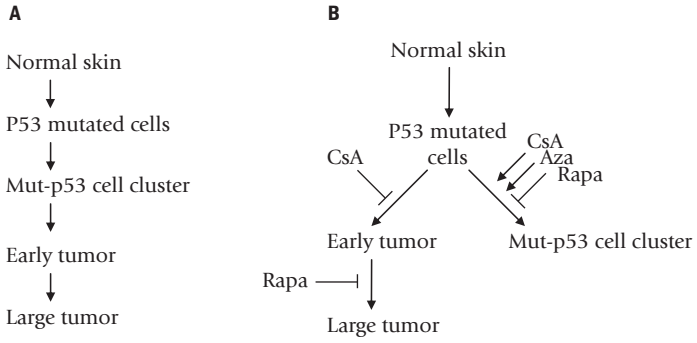


Figure 5: Schematic overview of potential developmental stages in UV-carcinogenesis. (**A**) Previous model adapted from Boukamp[43]. (**B**) Model based on present results. Effects of immunosuppressants on different stages as described in the article are depicted.

Using the present mouse model and experimental setup, the effect of the immunosuppressive drugs on tumor risk appears not to be in agreement with the increased skin cancer risk in transplant recipients. Moreover, we did not find consistent links between effects on UV-induced apoptosis, frequency of *p53* mutations and the ultimate formation of skin tumors. These discrepancies between the effects on mut-p53 cell clusters and tumors, and the striking overall lack of enhanced UV carcinogenesis in the present mouse experiments imply that the mouse model does not properly emulate skin cancer development in organ transplant recipients. Elucidation of these discrepancies, whether they are caused by the experimental setup or physiological differences between mice and humans, should contribute to a better understanding of the risk of skin carcinogenesis in organ transplant recipients.

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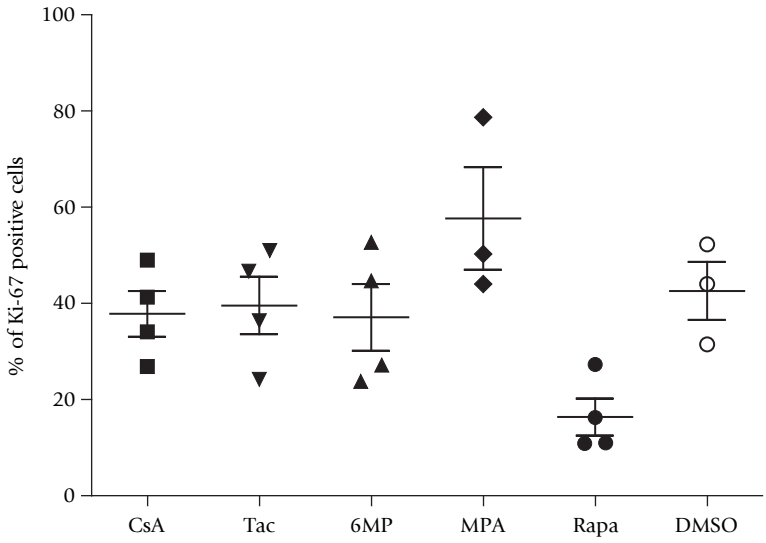
Supplementary material

Supplementary table 1: Primer sequences; * indicates phosphorothioate bond.

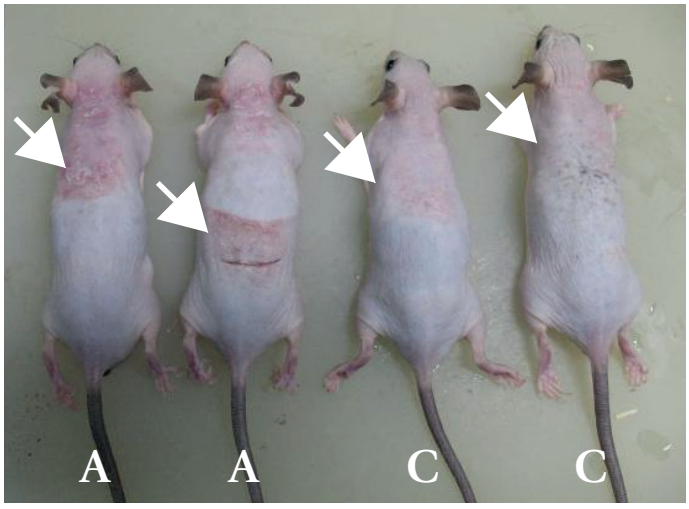
Primer	Sequence
Forward primer	AATGATACGGCGACCACCGACCGGATAGTGGGAACCTTC*T
Reverse primer1	CAAGCAGAAGACGGCATACGAAAGCCCCTTCTTCTGTACGGCGGTC*T
Reverse primer2	CAAGCAGAAGACGGCATACGACACACCCTTCTTCTGTACGGCGGTC*T
Reverse primer3	CAAGCAGAAGACGGCATACGACCCCTACTTCTTCTGTACGGCGGTC*T
Reverse primer4	CAAGCAGAAGACGGCATACGACATCGGCTTCTTCTGTACGGCGGTC*T
Reverse primer5	CAAGCAGAAGACGGCATACGATCGTTGCTTCTTCTGTACGGCGGTC*T
Reverse primer6	CAAGCAGAAGACGGCATACGAGGGCACCTTCTTCTGTACGGCGGTC*T
Reverse primer7	CAAGCAGAAGACGGCATACGACCAGACCTTCTTCTGTACGGCGGTC*T

Supplementary table 2: Primary antibodies used for immunohistochemical staining of tissue sections.

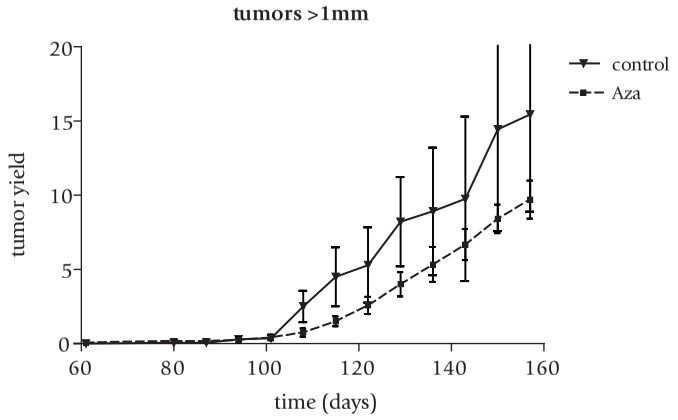
Antibody	Dilution
Ki-67 (MIB1; Dako)	1:100
K10 (DE-K10; Neomarkers)	1:100
K16 (LL025; Serotec)	1:50
K17 (CK-E3; Novus Biologicals)	1:25
active caspase-3 (Ab2302; Abcam)	1:300
mutant-P53 (Pab240; Monosan)	1:250

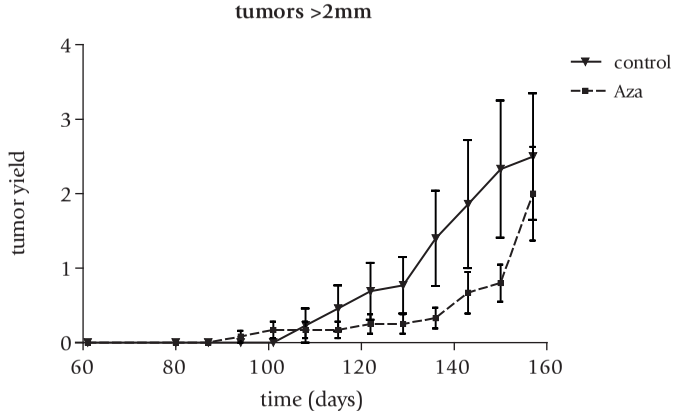


Supplementary figure 1: Effects of immunosuppressive drugs on epidermal proliferation in HSEs (n=3-4) as % of Ki-67 positive (supra-)basal cells. ANOVA shows only Rapa to differ from control (DMSO) ($p<0.05$). Results for two concentrations of immunosuppressants are combined. Error bars depict SEM.

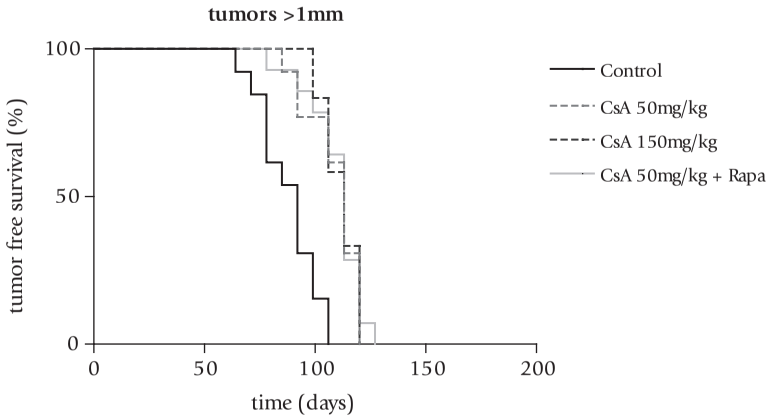


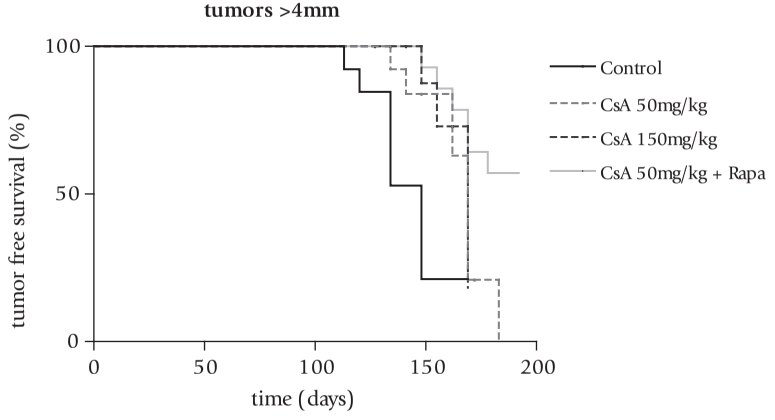
Supplementary figure 2: Mice on Aza diet (**A**) show more extensive burning 72 hours after UV irradiation compared with mice on control diet (**C**). Arrows indicate area on the mice that was irradiated with 28 J/m^2 UV.





Supplementary figure 3: No effect of Aza on tumor yields induced by daily UVA irradiation. Average tumor yields per mouse for tumors >1mm and >2mm are shown.





Supplementary figure 4: Tumor-free survival (Kaplan-Meier) plots of the different treatment groups (n=12-13), with different thresholds for tumor detection: diameters >1mm or >4mm. Three groups of mice receiving CsA in the diet (at 50 and 150mg/kg) show significantly delayed tumor induction.

Chapter 5

Dose scheduling of cyclosporine determines the impact on UV-induced tumor development in mice

Voskamp P, Bodmann CA, Koehl GE, Tensen CP, Bouwes Bavinck JN,
Willemze R, Geissler EK, De Gruijl FR

Abstract

Organ transplant recipients using the immunosuppressant cyclosporine have an increased risk for developing nonmelanoma skin cancer. Disparate effects of cyclosporine have, however, been reported on UV-induced skin carcinogenesis in mouse experiments. Therefore, we set out to compare three experimental protocols using hairless mice with the aim to emulate most closely the increased skin cancer risk in organ transplant recipients. Using an experimental protocol with chronic UV exposure and continuous dietary administration of cyclosporine, it was shown to inhibit tumor formation. Using an experimental protocol in which mice were treated with dietary cyclosporine after a period of UV exposures, cyclosporine did not affect ensuing UV carcinogenesis. Thus, the treatment schemes where mice were fed cyclosporine via their chow, resulting in stable cyclosporine blood levels, did not show increased skin cancer development. An increase in tumor development was found in UV-exposed mice that were force-fed cyclosporine, resulting in strongly varying blood levels of cyclosporine. There was no difference in tumor development between mice UV irradiated during cyclosporine peak or trough blood levels. Time-averaged cyclosporine blood levels in these mice were similar to those with cyclosporine in the diet. The difference in tumor development in mice with these experimental treatments shows that cyclosporine in bolus doses increases skin cancer risk in contrast to even cyclosporine administration. Extrapolation to transplant patients suggests that the mode of administrating cyclosporine may be crucial for the increased skin cancer risk and that this risk might be lowered with a more steady release of cyclosporine in the body.

Introduction

Organ transplant recipients (OTRs) taking immunosuppressive drugs (including cyclosporine) to prevent rejection of the transplant have an increased risk for developing non-melanoma skin cancer[1-4].

The immunosuppressant cyclosporine has direct adverse effects on skin cells, which may contribute to carcinoma risk. It inhibits repair of UV-induced DNA damage and apoptosis of overly damaged cells in the skin[3, 5-8]. The latter is possibly due to inhibition of mitochondrial permeability and pore opening[5, 9, 10]. In immune compromised mice, cyclosporine induced phenotypic changes, including invasiveness of non-transformed cells, which was TGF-β dependent[11]. Squamous cell carcinomas from OTRs on cyclosporine harbor less senescent cells than those from OTRs on other immunosuppressants[12]. These findings indicate that – aside from systemic immunosuppression – local effects of cyclosporine in the skin may contribute to the increased skin cancer risk in transplantation patients.

In order to systematically compare different immunosuppressive drugs on their skin cancer risk, several mouse studies have been performed. Mouse experiments aimed at mimicking the skin cancer promoting effect of cyclosporine on transplantation patients have yielded different outcomes. A pioneering study showed enhanced UV-induced skin carcinogenesis when cyclosporine was administered by gavage and UV exposure was performed twice weekly[7]. A more recent study with mice UV exposed thrice weekly showed that larger tumors developed with daily i.p. injections of cyclosporine[13]. Another study from this group showed, however, that UV-induced tumor development was impaired when cyclosporine treatment was started and UV exposure discontinued when the first tumors had occurred[14]. A recent study from our group surprisingly showed that tumor formation by daily UV exposures was inhibited when cyclosporine was administered in the food (chapter 4). In all these studies the hairless SKH1 mice were used. The main differences between these studies were the experimental procedures regarding UV irradiation and cyclosporine administration, which suggests that the exact experimental protocol determines the net effect of cyclosporine on UV-induced skin cancer development. These different experimental outcomes led us to the hypothesis that UV carcinogenesis is enhanced when cyclosporine is force fed in bolus dosages and UV irradiation occurs during peak levels of cyclosporine in the blood.

In this study we therefore set out to compare three experimental protocols with the aim to emulate most closely the increased skin cancer risk in organ transplant recipients. The protocols were:

a) cyclosporine is administered in the food of mice that are daily UV irradiated (part of our earlier study mentioned above, chapter 4),
b) mice are daily UV irradiated for only five weeks, and subsequently had cyclosporine administered in the food, and
c) cyclosporine is administered through gavage thrice weekly each time followed by UV irradiation during peak or trough levels in the blood.

Materials and methods

The mice

SKH-1 hairless mice (Charles River, Maastricht, The Netherlands) entered the experiment at 8-16 weeks of age; both male and female mice were used. The animals were housed individually under a 12 h light-12 h dark cycle at 23 °C. Standard chow was supplied in ample amounts (60 g/mouse/week), and drinking water was available *ad libitum*. Cage enrichment was absent to prevent shielding of the animals from UV exposure. All experiments were performed in accordance with legislation and approval of the center's ethics committee for animal experiments.

UV irradiation

Mice that received cyclosporine in the diet during the entire experiment were started on their diet 1 week before subjecting them to a regimen of daily UV exposure. TL-12/40W tubes (Philips, Eindhoven, The Netherlands; 54% output in UVB – 280 to 315nm – and 46% output in UVA – 315 to 400nm) were used for daily UV exposure. The lamps were mounted over the cages with grid covers to allow undisturbed exposure of the mice. The lamps were automatically switched on daily from 12.30 to 12.50 h. The threshold dose for a sunburn reaction (minimal edemal dose, MED) in the hairless SKH-1 mouse was ~500 J/m^2 UV under these lamps. The lamps were dimmed both electronically and by insertion of perforated metal sheets to expose the mice daily to 250 J/m^2 of UV radiation (0.5 MED).

Experimental protocols

A previous study by our group (chapter 4) has shown that admixing cyclosporine 150mg/kg to the standard mouse chow resulted in immunosuppressive average cyclosporine blood levels of 0.8 mg/L. No apparent differences in food intake and body weights were observed between the diet groups. In two of the experimental protocols cyclosporine was administered in the diet and in one protocol cyclosporine was administered by gavage (figure 1):

Continuous protocol: Two diet groups were formed: one with cyclosporine admixed to the food (n=12) and a control group (n=14) without any admixture. These mice were started on their diets one week prior to starting daily UV exposures (0.5MED/d). Until the end of the experiment, the mice were daily UV-exposed and kept on their diets. This experiment was part of an earlier study of ours. (chapter 4).

Sequential protocol: Mice were first daily exposed to UV irradiation (0.5MED/d) for a period of 5 weeks, and subsequently left unexposed and divided into two diet groups: one with cyclosporine (n=25) and a control (n=26) group without cyclosporine.

Gavage protocol: Three groups of mice were formed. Two groups received cyclosporine dissolved in peanut oil by gavage three times a week. One group (n=12) was UV-exposed 3 hours after gavage and another group (n=14) 24 hours after gavage. A control group of mice received peanut oil administered by gavage three times a week and was UV-exposed 3 hours after gavage (n=14). Cyclosporine (LC labs, Woburn, MA) was dissolved in peanut oil (Sigma-Aldrich, St.Louis, MO) at a concentration of (1.5% w/v) and administered in 200µl volumes.

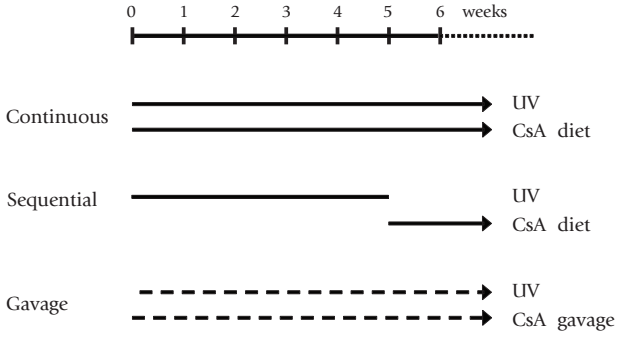


Figure 1: Graphical representation of the different experimental protocols that were used: continuous protocol, sequential protocol and gavage protocol.

Tumor assessment

The mice were inspected weekly for tumors which were registered for each mouse individually on maps (thus recording location, size and form). Upon removal of animals from the experiment, tumors and normal skin were isolated for further analysis as described below.

Histology and immunohistochemistry

Biopsies were fixed in 4% formaldehyde, dehydrated and embedded in paraffin or snap-frozen in liquid nitrogen. Staining for Ki-67 was performed on deparaffinized sections (5µm) of mouse skin. Haematoxylin and eosin (H&E) staining was performed on deparaffinized sections for tumor staging. Staging of tumors from each group was performed blinded by a pathologist (Dr. A. Gaumann, University of Regensburg, Germany) who is experienced in mouse and human pathology, including diagnosis of actinic keratoses as proper precursors of squamous cell carcinomas. Antigen retrieval was performed by autoclaving the sections in 10mM citrate buffer (pH 6.0) for 10 minutes at 110°C. Primary antibody against Ki-67 (MIB1, Dako) was used at 1:100 dilution and goat-anti-mouse secondary antibody (E0433, Dako) was used at 1:200 dilution. After overnight incubation with the primary antibody at 4°C sections were processed according to standard protocols and stained by AEC.

Epidermal thickness

Epidermal thickness was determined in H&E stained sections of dorsal skin acquired from mice on cyclosporine diet and daily UV exposed for 4, 7 and 10 weeks. These samples originated from a previous study where mice were similarly treated as in the continuous model in the present study regarding cyclosporine diet and UV exposures (chapter 4). Three photos were taken of each section with standard 40 x magnification and these were subsequently analyzed by ImageJ software. Epidermal thickness was determined as the average of the number of pixels of epidermal thickness perpendicular to the surface.

Cyclosporine bloodlevel determination

Cyclosporine blood levels and other responses to the cyclosporine diet were first assessed in Balb/c mice. Blood samples were taken retroorbially at indicated time points during the day, and were subsequently analyzed by the Department of Clinical Chemistry (University of Regensburg) by LC-MS/MS alongside patient samples. Blood levels of hairless mice on cyclosporine diet were subsequently checked and found to be similar. Blood was taken around 11 a.m. Blood levels were also determined in hairless mice that were fed cyclosporine by gavage. Blood was collected by tail bleeding at different time points after gavage. Concentration of cyclosporine in these samples was determined by Fluorescence Polarisation Immuno Assay (AxSYM CYCL, Abott) by the Central Laboratory for Clinical Chemistry of the Leiden University Medical Center.

Results

Similar time-averaged blood levels of cyclosporine after gavage or feeding in diet

Feeding cyclosporine in the diet resulted in relatively stable cyclosporine blood levels during the day (0.7-1.0mg/L) (figure 2A). Administering cyclosporine by gavage resulted in a peak level after three hours (3.0mg/L), after which the level decreased and became undetectable (<25µg/L) after 48 hours (figure 2B). Averaging over time yielded mean levels of 0.8mg/L cyclosporine both for mice on a diet containing cyclosporine and mice fed cyclosporine by gavage.

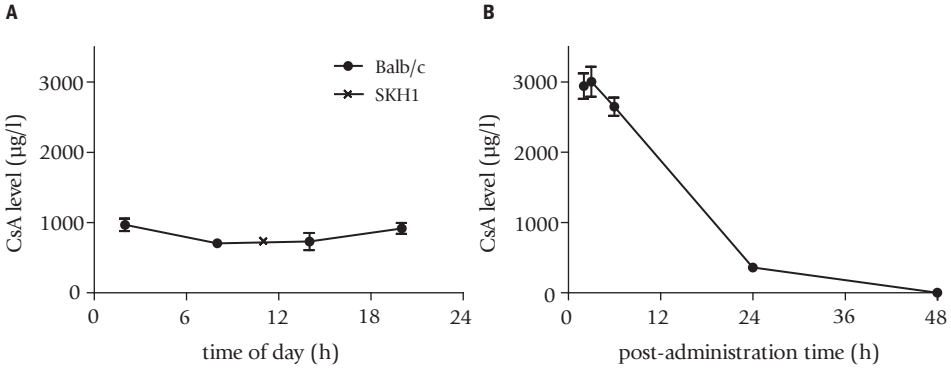


Figure 2: Cyclosporine blood levels over time for different modes of administering the drug. (**A**) cyclosporine blood levels in mice on cyclosporine-containing diet (n=3 per time point); (**B**) cyclosporine blood levels after gavage (n=4-5 per time point). Error bars depict SEM.

Decreased tumor development with cyclosporine in the diet during chronic UV exposure

In the first experiment (part of an earlier study, chapter 4) mice were fed cyclosporine in the food and daily exposed to mild UV irradiation at half of the threshold dose for a sunburn (0.5MED). Treatment with cyclosporine resulted in delayed tumor onset and lower yields of tumors >1mm and >4mm compared with controls ($p<0.001$) (figure 3A).

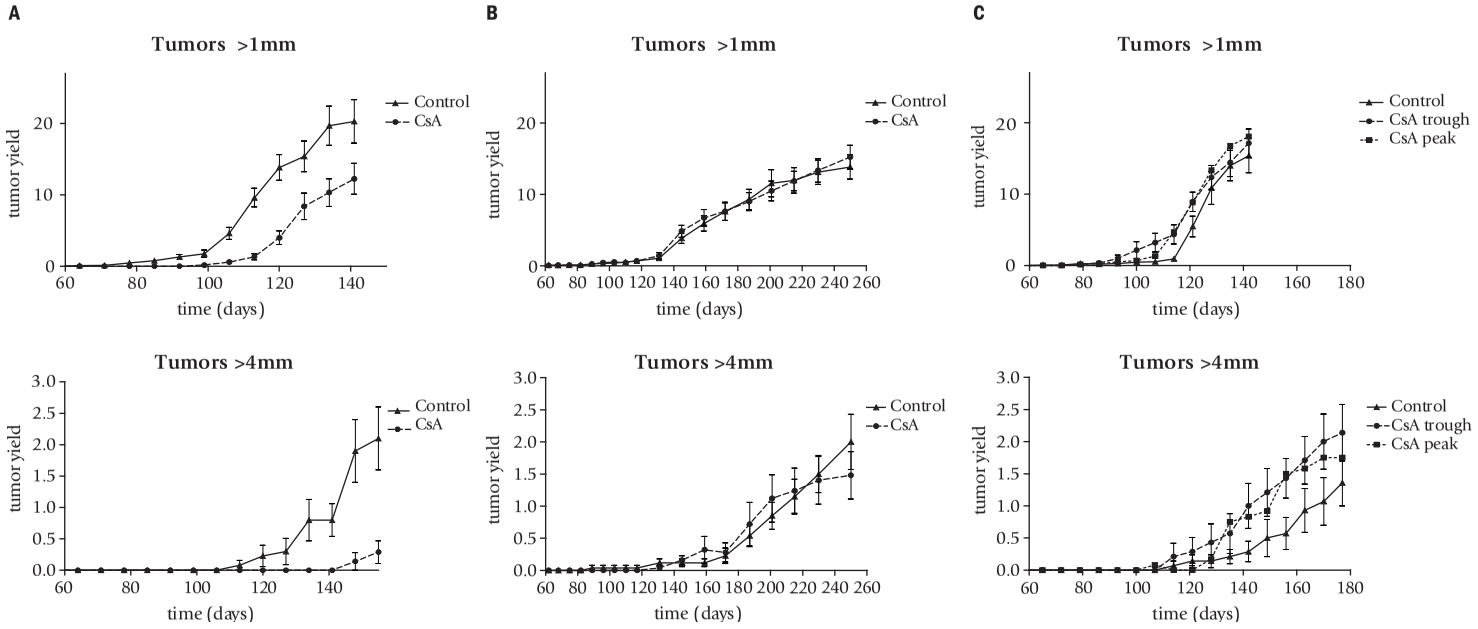


Figure 3: Effect of cyclosporine on yields of tumors >1mm and tumors >4mm in the different experiments on UV carcinogenesis and cyclosporine feeding: In the continuous UV model (**A**) cyclosporine decreased tumor yields, in the sequential model (**B**) there was no effect of cyclosporine on tumor yields, and in the gavage model (**C**) tumor yields were increased by cyclosporine, both with UV exposure during peak and trough levels in the blood. Error bars depict SEM.

No effect on tumor development with cyclosporine diet after discontinuation of UV exposure

In the second experiment the cyclosporine-containing diet was started after a period of 5 weeks of daily UV exposure (0.5MED). No skin tumors were present when the UV exposures were discontinued. Subsequent tumor development was assessed biweekly. Treatment with cyclosporine did not affect onset and yields of tumors >1mm and >4mm when compared with controls (figure 3B).

Increased tumor formation with cyclosporine administrated by gavage

In the third experiment cyclosporine was administered by gavage three times a week. One group of mice receiving cyclosporine was UV exposed (1MED) three hours after gavage with cyclosporine blood levels at 3.0mg/L, the other group of mice receiving cyclosporine was UV exposed (1MED) 24 hours after gavage, with cyclosporine blood levels at 0.4mg/L (figure 2B). Treatment with cyclosporine resulted in higher yields of tumors >4mm (and tended to be higher for tumors >1mm) compared with controls (figure 3C). Tumor development in mice irradiated 3 hours after gavage did not differ from that in mice irradiated 24 hours after gavage. But both of these groups differed significantly from controls in yields of tumors >4mm ($p<0.05$ and $p<0.001$ respectively).

Tumor pathology

Tumors >4mm from the different treatment groups were examined. All tumors were classified as invasive squamous cell carcinomas, without any discernable differences between the groups in tumor type and grading.

Epidermal thickness or proliferation index was not affected by cyclosporine

We showed that cyclosporine protects against UV-induced skin carcinogenesis when mice were fed cyclosporine during UV irradiations. To determine whether cyclosporine decreases epidermal proliferation, the epidermal thickness and Ki-67-positive cell fractions of mice UV irradiated for 4, 7 and 10 weeks from this earlier experiment (chapter 4) were determined. No effect of cyclosporine on epidermal thickness or Ki-67-positive cell fraction was found (figure 4).

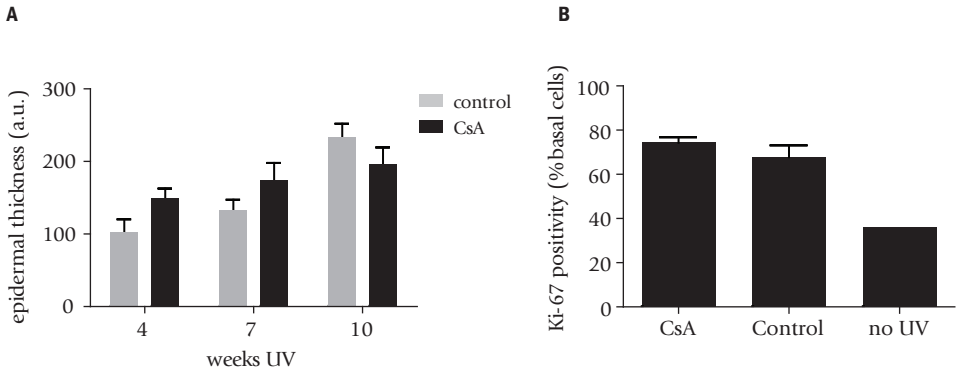


Figure 4: Effects of cyclosporine on epidermal thickness and Ki-67 positive cell fraction. (**A**) Epidermal thickness of mice daily irradiated for 4, 7 and 10 weeks is shown. (**B**) Epidermal proliferation measured by percentage of Ki-67 positive cells after 4 weeks of UV irradiation. N=4 in each measurement, Error bars depict SEM.

Discussion

In this study we set out to assess the effects of cyclosporine on UV carcinogenesis in mice using different experimental procedures. In the first experiment with chronic UV-exposure and continuous dietary immunosuppressive treatment we surprisingly found cyclosporine to inhibit tumor formation. In a second experiment we simulated treatment with the immunosuppressant after a period of early life UV exposure and further avoidance of UV exposure. No effect of cyclosporine on UV-carcinogenesis was apparent in this experiment with mice on dietary cyclosporine after a period of 5 weeks of daily UV exposure. In the third experiment we assessed the effect of administering bolus dosages cyclosporine leading to highly variable blood levels of cyclosporine. An increase in tumor development occurred in UV-exposed mice that received cyclosporine by gavage. However, our hypothesis that UV irradiation with high cyclosporine blood levels would most strongly increase UV carcinogenesis was disproved. There was no difference in tumor development in mice UV-irradiated during either peak or trough levels of cyclosporine in blood, indicating that cyclosporine blood level during UV exposures bore no relevance to UV carcinogenesis.

Dietary cyclosporine treatment inhibited tumor development only when it was given simultaneously with UV irradiation. Cyclosporine in the diet after a period of damage induction by UV did not affect tumor formation. Hence, the tumor inhibiting effect of cyclosporine in the diet was only present in the continuous model and not in the sequential

model. The tumor inhibiting effect of cyclosporine (possibly related to TGF-β[14]) could not be explained by decreased epidermal proliferation as measured by Ki-67 positivity and epidermal hyperplasia.

Cyclosporine administration by gavage resulted in increased tumor formation, confirming the early experiments by Kelly et al.[7]. Total cyclosporine exposure in blood was similar in mice on a cyclosporine-containing diet and mice fed cyclosporine by gavage, as determined by time-averaged cyclosporine blood levels (also used as measure of cyclosporine exposure in cyclosporine-treated patients[15]). Average cyclosporine blood levels in mice were 0.8mg/L with the two methods of administration. In kidney transplant recipients, average cyclosporine blood levels of 0.28-0.45mg/L are aimed for (Therapeutic Drug Monitoring – Dutch Society of Hospital Pharmacists; http://www.2nvza.nl/layout/raadplegen.asp?atoom=5665). However, the pharmacological dynamics with the two methods of administering cyclosporine differed widely. Cyclosporine in the diet resulted in stable cyclosporine blood levels (0.7-1.0mg/L), whereas feeding cyclosporine by gavage resulted in widely varying cyclosporine blood levels with a peak after three hours (3.0mg/L) and undetectable levels after 48 hours. In the skin though, cyclosporine levels may have fluctuated much less, as half-lives of cyclosporine in organs have been reported to vary between 60 and 120 hours[16]. Thus, the skin may become loaded with cyclosporine depending on its exposure to the drug. Despite similar average blood levels, the uptake and levels of cyclosporine in the skin may have differed importantly between the two methods of administering cyclosporine.

The blood level of cyclosporine at the time of UV exposure is not of importance for tumor formation. UV irradiation of mice 24 hours after feeding cyclosporine by gavage (trough blood level) or three hours after gavage (peak blood level) did not result in differences in tumor formation. Immunosuppression in these two groups of mice may be assumed to be the same and to contribute equally to the increased tumor development when compared to controls. However, the difference in tumor development between mice with cyclosporine by diet or gavage did show that evenly administered cyclosporine differed in effect from cyclosporine in bolus doses. An explanation for this differential effect may lie in a difference in immunosuppressive effect of the two methods of administering cyclosporine, or may be due to different cyclosporine skin levels due to the different pharmacokinetic profiles. Elucidation of the mechanism responsible for the differences in impact oft the modes of administering cyclosporine on UV carcinogenesis evidently needs further study.

Organ transplant recipients that use cyclosporine as immunosuppressant mostly take the drug twice daily. This results in a pharmacokinetic profile of cyclosporine blood levels peaking 2-3 hours after cyclosporine intake and decreasing afterwards[17]. This pharmacokinetic profile is similar in the mice in this study that were administrated cyclosporine per gavage. We have shown that cyclosporine increases skin cancer induction by UV radiation in these mice. Mice receiving cyclosporine in their chow, resulting in stable cyclosporine blood levels, showed less skin cancer induction by UV radiation. Extrapolation to transplant recipients, in which skin cancer risk is greatly increased, suggests that the mode of administrating cyclosporine might play an important role in the increased skin cancer risk. Methods resulting in more gradual and constant release of cyclosporine in the body may result in lower skin cancer risk.

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Chapter 6

Summary and general discussion

The overall aim of the study was to provide experimental data which could contribute toward minimizing the (skin) carcinogenic risk from immunosuppressive drugs. The immediate objective of the experiments described in this thesis was to determine modulating effects of immunosuppressants on the responses of skin cells to UV irradiation, and UV induction of skin carcinomas and precursor lesions. Hence, we have determined the effects of the immunosuppressants at several stages of tumor development. Comparison of the different immunosuppressants on their effects on tumor development should contribute to lowering skin cancer risk from immunosuppressive regimens. Unexpectedly, none of the immunosuppressants fed in long-term tolerable dosages (assessed by blood cell counts and/or condition of the animals) enhanced UV carcinogenesis (chapters 2 and 4). CsA even delayed UV carcinogenesis (chapter 4). Evidently, these mouse experiments did not simulate the enhanced risk of skin carcinogenesis in human organ transplant recipients. Our experiments suggest that immunosuppressants do not necessarily accelerate UV carcinogenesis. Feeding CsA in bolus dosages (by gavage) instead of evenly in the diet reproduced the speeding up of UV carcinogenesis as found by Kelly et al.[1] (chapter 5). Thus, our experiments demonstrated the potential importance of the dose schedule and ensuing profiles in blood levels of the immunosuppressive drug. However, even in bolus dosages of CsA the impact on experimental UV carcinogenesis did not appear to match the dramatic increase in risk of skin carcinoma in human organ transplant recipients (60- to 100-fold increase[2]). This suggests that the mouse model we used was not quite adequate, either because mice react differently (e.g. lower rate of DNA repair) or the model is incomplete, e.g. most prominently by the absence of an actual graft (absence of continuous antigenic stimuli and possible tolerance).

Interestingly, the close correspondence between the induction of mut-p53 cell clusters and skin carcinomas reported earlier by our group[3, 4] was not observed when feeding immunosuppressants like CsA and Rapa (chapters 3 and 4). These results indicate that the large majority of mut-p53 cell clusters are probably not potential precursors of skin carcinomas, in contrast with earlier conclusions[3, 5]. Consequently, the frequency of mut-p53 cell clusters in the skin is not a reliable predictor of skin carcinoma risk, again invalidating earlier inferences[3, 4].

Thus, our study has yielded four unanticipated major findings:

a) Immunosuppressants *per se* do not necessarily enhance UV carcinogenesis and in this respect dose scheduling can be of major importance.

b) The mouse model with administration of immunosuppressants appears inadequate in simulating the increase in risk of squamous cell carcinomas in human organ transplant recipients.

c) Mut-p53 cell clusters in majority are not potential precursors of squamous cell carcinomas.

d) Early local carcinogenic effects from various immunosuppressants as expressed by *p53* mutation frequencies in the UV-exposed mouse skin (or rather the lack of such effects) are not predictive of the overall effects on UV carcinogenesis. This latter finding indicates that systemic and not local effects are of major importance for the effects of immunosuppressants on UV carcinogenesis.

In the following paragraphs the effects of immunosuppressive drugs on UV-response in normal skin, *p53* mutations, mutant-p53 overexpressing cells and skin tumors are discussed in more detail. Consequences of these effects for the model of UV-induced tumor development are discussed, followed by concluding remarks and future directions.

Effects of immunosuppressants on epidermal regeneration and responses to UV in human skin equivalents

Some immunosuppressants have direct adverse effects on skin cells, which could contribute to carcinoma risk. CsA affects DNA repair[6, 7] and has been shown to impair apoptotic responses induced by UV in normal human keratinocytes[6] and mice[8]. Aza enhances DNA damage by photosensitization[9]. In chapter 3 and 4 we systematically compared the impacts of the immunosuppressants on epidermis and responses to UV exposure in human skin equivalents (HSEs). Epidermal regeneration (formation of an epidermis by seeded keratinocytes) in HSEs was impaired by Rapa. HSEs treated with Rapa show a thin epidermis with only a few cell layers, as well as a decreased proliferation index and reduced expression of the hyperproliferation associated markers K16 and K17. Decreased proliferation may be relevant to wound-healing problems associated with Rapa treatment in transplantation patients[10]. None of the other immunosuppressants showed any effect on epidermal regeneration.

The apoptotic response after UV exposure in HSEs was increased after Rapa treatment. In our mouse experiments (chapter 3) we did, however, not see any significant increases in apoptosis in Rapa-treated mice which could be attributable to a wide variation in responses. In HSEs CsA pre-treatment caused a decrease in the apoptotic response after UV irradiation, confirming earlier results in cell cultures[6].

We found HIF1α accumulation to be inhibited by Rapa in keratinocytes after UV exposure of HSEs, while the apoptotic response was increased by Rapa. Previously it has been shown that HIF1α is upregulated after hypoxia[11] and UV irradiation[12, 13] and that Rapa can prevent hypoxia-induced HIF1α accumulation[14]. Here we have shown that Rapa is also able to inhibit UV-induced HIF1α accumulation (chapter 3). HIF1α is known to inhibit or induce apoptosis depending on the cell type, and inhibition of HIF1α accumulation may therefore be involved in the pro-apoptotic effect of Rapa in HSEs[15, 16].

Immunosuppressants and UV-induced p53 mutations

In chronically UV-irradiated skin, clusters of cells expressing mutated p53 (mut-p53 cell clusters) develop. These cell clusters harbor similar mutations in the *p53* gene as the skin tumors that arise later[17]. Also the development of these cell clusters correlated with skin tumor development in several studies[3, 4], therefore these cell clusters are generally regarded as precursor lesions for squamous cell carcinomas that arise after continued UV exposure[3, 17-19]. Immunosuppressants do not affect the mutational frequency of *p53* in chronically UV irradiated non-tumor skin in mice (chapter 4). Deep sequencing of two mutational hotspots in the *p53* gene (codons 270 and 275) showed that many cells harbor a mutated allele of *p53*. There was no clear effect of the immunosuppressive treatments on the frequencies of mutated *p53* alleles. On average, 5% of the alleles sequenced were mutated in a stretch of 40 bp, corresponding to 10% of the cells when assuming heterozygously mutated cells (total mutations in *p53* were therefore estimated to amount 20%). These results are in agreement with a recent deep sequencing study of human skin from mid-life individuals that revealed that persistent *p53* mutations had accumulated in 14% of the epidermal cells[20]. In contrast to the mutational frequencies, immunosuppressants did affect formation of mut-p53 cell clusters. CsA and Aza increased the formation of mut-p53 cell clusters in mice, whereas Rapa inhibited mut-p53 cell cluster formation (chapters 3 and 4). The lower number of UV-induced mut-p53 cell clusters formed in the skin of Rapa-treated mice might be caused by early enhanced apoptosis or decreased proliferation, but Ki-67-positive cells were not decreased in Rapa-treated mice. A previous study by our group showed no effect of Aza on mut-p53 cell cluster formation in mice[21], but Aza was administered by i.p. injections in those experiments, and the measurement was limited to one time point with relatively large error margins in the counts. The discrepancy between *p53* mutational frequencies and mut-p53 cell cluster formation is discussed below.

Immunosuppressants and UV-induced tumor development in mice

Effects of immunosuppressants on UV-induced skin cancer were determined in daily UV-irradiated mice fed with drug-containing chow (chapters 2 and 4). Surprisingly, none of the immunosuppressants increased UV-induced tumor development. Remarkably, tumor induction was delayed by CsA treatment. Proliferation of epidermal keratinocytes (e.g. through TGF-β) was not affected by CsA, making it unlikely that reduced keratinocyte proliferation caused the tumor delay. A pioneering study on the effect of CsA on UV carcinogenesis showed a shorter tumor latency time compared to controls[1]. With a different experimental setup, Wulff et al. showed that CsA treatment (by repeated i.p. injections) reduced the rate of new tumor development in mice[22], in agreement with our results. We found that Rapa had no effect on the development of small tumors, but impaired the development of large tumors (chapter 2). This impairment can be attributed to the known anti-tumor, or more specifically, antiangiogenic effects of Rapa[23, 24]. At dosages that maintained allogeneic heart grafts in mice, Rapa was described to inhibit outgrowth of tumor implants whereas CsA enhanced angiogenesis and strongly stimulated tumor outgrowth[25]. Rapa could even block the proangiogenic effect from CsA. In chapter 2 the inhibition of tumor outgrowth by Rapa appeared to be reflected by a significant reduction of Vegf-a positive tumor cells. Interestingly, we did not find any clear effect on the vasculature of tumors that grew in the Rapa-fed groups, but these tumors may be presumed to have been selected for their vascularization and resistance to Rapa whereas those that lagged behind were most sensitive to the antiangiogenic effect of Rapa. Other UV carcinogenesis studies with different treatment schemes have shown either lower tumor yields[22] or higher tumor yields[26] in Rapa-treated mice, indicating that treatment schemes can have important effects on the outcomes of the tumorigenesis experiments.

P53 mutational spectra in SCCs

P53 mutations appear to be involved in the early stages of tumor development[3]. Our hypothesis that Rapa might selectively induce apoptosis in the *p53*-mutated cells, and thus slow down the rate at which tumors are initiated, was not supported by our data because we found no effect on the onset of the smallest perceptible tumors (<1mm) (chapter 2). To check further for local effects of the immunosuppressants on the epidermal cells that might have affected UV carcinogenesis (e.g., disturbed metabolism by Rapa), we investigated the *p53* mutation spectrum of the tumors, which is normally dominated by the UV signature mutations (i.e. C>T transition on a dipyrimidine site)[27]. We found a dramatic shift in the types of mutations in tumors from the Rapa-fed group. Since Rapa-insensitivity can be acquired via increased cellular oxidative stress[28] it is possible that only tumors with high

levels of endogenous oxidative stress could grow into large tumors, hence causing the altered mutational spectrum. The antioxidant effect of MMF[29] may have prevented formation of non-UV-type mutations in tumors of mice on a combination diet with Rapa and MMF, although tumor development was not different in mice on combined MMF and Rapa diet than on Rapa diet. We further determined in chapter 3 that this shift in the type of mutations occurs only in tumors >2mm and not in mut-p53 cell clusters or skin tumors <2mm in mice treated with Rapa. It can therefore be concluded that the altered mutational spectrum is a late event in tumor development. Either it occurred after the initial development of small tumors, or a specific selection of small tumors with altered mutational spectra grew into large tumors, corresponding to the lower yield of large tumors in Rapa-fed mice.

Discrepancies between effects of immunosuppressants at different stages of tumor development in mice

There was no clear effect of the immunosuppressive treatments on the frequencies of mutated *p53* alleles, in contrast with effects on mut-p53 cell cluster formation. Deep sequencing of the mutational hotspot in the *p53* gene (codons 270 and 275) showed that many cells (~20%) harbor a mutated allele of *p53* before the occurrence of tumors. The percentage of skin area containing mut-p53 cell clusters was much lower, approximately 6% in control mice. It can therefore be concluded that a minority of the cells harboring a mutated *p53* show overexpression of mut-p53 in cell clusters. This puts the effects of Rapa, CsA and Aza in a different perspective. A mutation in *p53* is apparently not sufficient to cause overexpression of the p53 protein in mut-p53 cell clusters[30], in agreement with what we found in mut-p53 mice (chapter 3). Tumors may arise from a larger pool of *p53*-mutated cells that do not overexpress the protein. Development of mut-p53 cell clusters and SCCs was not strictly correlated in our experiments. When mice were treated with CsA, numbers of mut-p53 cell clusters were clearly increased compared to controls, but CsA delayed tumor onset. Rapa on the other hand reduced mut-p53 cell clusters without any effect on the onset of small tumors. Numbers of mut-p53 cell clusters had in previous studies always shown a good correlation with the rate of tumor development with chronic UV irradiation[3, 4, 31]. Since most SCCs harbor mutations in the *p53* gene, *p53* mutations were considered early events in SCC formation and therefore mut-p53 cell clusters were thought to be precursors of SCCs (Chapter 4 figure 5A)[18, 32]. However, a retrospective study did not establish differences in numbers of mut-p53 cell clusters between skin from patients with solitary versus multiple skin carcinomas[33]. As we found that immunosuppressants can have opposing effects on mut-p53 cell clusters and SCC formation, this suggests that mut-p53 cell cluster formation cannot be used as a simple indicator of tumor risk. As most mut-p53 cell clusters are

not genuine precursors of SCC, another oncogenic early event is likely to drive early UV carcinogenesis. The necessity of another oncogenic event might also explain the absence of an increased SCC risk among Li-Fraumeni patients, i.e. people heterozygous in germline mutations in *p53*. These results open up an alternative perspective on the developmental stages of skin carcinomas, in which mut-p53 cell clusters do not contribute to skin carcinoma risk (Chapter 4 figure 5B).

Dose schedule of CsA determines effect on experimental UV carcinogenesis

In order to systematically compare different immunosuppressive drugs on their skin cancer risk, several mouse studies have previously been performed. Mouse experiments aimed at mimicking the skin cancer promoting effect of CsA on transplantation patients have resulted in different outcomes. A pioneering study showed enhanced UV-induced skin carcinogenesis with CsA when the drug was administered through force feeding and UV exposure was performed twice weekly[1]. A more recent study showed that CsA administration resulted in larger UV-induced skin tumors compared to control when CsA was administered via i.p. injections daily and UV exposures thrice weekly[26]. Another study from this group showed that CsA decreased UV-induced tumor development when CsA treatment is started and UV treatment was discontinued when the first tumors had occurred[22]. In chapter 4 we showed that CsA inhibited UV-induced tumor formation when CsA was administered in the food and with daily UV exposures. In all these studies the hairless SKH1 mice were used. The main differences between these studies were the experimental procedures regarding UV irradiation and CsA administration, which suggests that the exact experimental protocol determines the effect of CsA on UV-induced skin cancer development. These differential outcomes led us to the hypothesis that when mice are force-fed CsA and repeatedly UV irradiated at times when they have high CsA blood levels that this would increase UV carcinogenesis. In chapter 5 we set out to assess the effects of CsA on UV carcinogenesis in mice using different experimental procedures. No effect of CsA on UV carcinogenesis was apparent in mice treated with dietary CsA after a period of UV exposures. An increase in tumor development occurred in UV-exposed mice that were CsA treated by gavage. This confirmed the results of the earlier experiments by Kelly et al.[1]. However, there was no difference in tumor development in mice UV irradiated during CsA peak blood levels or trough blood levels, indicating that CsA blood level during UV exposures bore no relevance to the increased UV carcinogenesis. Evidently, a more evenly administered CsA lowers (the risk of) UV carcinogenesis.

Concluding remarks and future perspectives

The studies described in this thesis confirm the potential that Rapa, or Rapa analogs like everolimus, may have for decreasing skin cancer in organ transplant recipients. Inhibition of tumor growth by Rapa should be further exploited in immunosuppressive treatment of organ transplant recipients, and by generating proper analogs this beneficial effect might be retained while eliminating adverse side effects.

We have shown that most mut-p53 cell clusters are not genuine precursors of SCCs. It remains likely that SCCs develop from mut-p53 cell clusters, since mutation of the *p53* gene is an early event in tumor development[34]. The effects of immunosuppressants on the development of mut-p53 cell clusters without correspondingly altering tumor onset make it possible to use these drugs in identifying a novel oncogenic event that drives early UV carcinogenesis.

The studies described here have shown that the mouse models of UV carcinogenesis with immunosuppressants may be basically unsuitable to emulate the increased risk of cutaneous SCC in organ transplant recipients. But it is also possible that the models lack an essential component like the graft. Considering the strong immunogenic and tolerogenic effects from a graft and likely interference thereof with skin tumor immunity, it would appear probable that inclusion of a graft in the model will have a great impact.

Table 1: Effects of immunosuppressants on cellular processes in non-immunological cells, based on majority view of literature.

	CsA	Tac	Aza	MMF	Rapa
Proliferation	↓ 35-37	↓ 37, 38		↓ 39-42	↓ 43, 44
Photogenotoxicity			↑ 9		
DNA repair	↓ 6, 7, 45-47	↓ 6, 47			
Apoptosis	↓ 6, 8	↓ 6			↑/↓ 48-52
Angiogenesis	↑ 24, 25	↓ 53, 54		↓ 39, 55, 56	↓ 24, 25, 53

Legend: ↓ = inhibitory effect, ↑ = positive effect

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Chapter 7

Nederlandse samenvatting

Orgaantransplantatie en huidkanker

Sinds de jaren zestig zijn orgaantransplantaties tussen mensen steeds vaker succesvol. Een belangrijke rol daarin hebben medicijnen die het afweersysteem onderdrukken (immuunsuppressiva) en daarmee afstoting van het getransplanteerde orgaan voorkomen. Naarmate de levensduur van patiënten met een donororgaan toenam bleek ook het risico op kanker bij deze patiënten dramatisch toe te nemen. Vooral het risico op het plaveiselcelcarcinoom van de huid is sterk verhoogd in deze patiëntengroep vergeleken met de algemene populatie. Risico op huidkanker is duidelijk gerelateerd aan ultraviolet (UV) licht blootstelling. Dat geldt ook voor transplantatiepatiënten, waarbij de huidkankers met name op zonbeschenen lichaamsdelen ontstaan. In Europa en de VS hebben 40-61% van de orgaantransplantatiepatiënten huidkanker ontwikkeld binnen 20 jaar, in het zonnige Australië ligt dat percentage zelfs op 70-82%.

Immuunsuppressiva

De immuunsuppressiva waarmee transplantatiepatiënten behandeld worden, kunnen ingedeeld worden in drie categorieën: remmers van calcineurine (cyclosporine en tacrolimus), purine synthese (azathioprine en mycofenolaat mofetil (MMF)) of mTOR (rapamycine). Deze immuunsuppressiva remmen de activatie en deling van T-cellen. De immuunsuppressieve therapie voor orgaantransplantatiepatiënten was aanvankelijk gericht op een optimale onderdrukking van immuunreacties tegen het donororgaan, waarbij het toegenomen risico op kanker als min of meer onvermijdelijk werd geaccepteerd. Door onderzoek is echter ook bekend geworden dat een aantal van deze medicijnen niet uitsluitend op cellen van het immuunsysteem effect heeft. Cyclosporine remt herstel van beschadigd DNA en kan evenals azathioprine geprogrammeerde celdood verhinderen (belangrijke processen om het lichaam te beschermen tegen gemuteerde cellen). In tegenstelling hierop hebben de nieuwere immuunsuppressiva MMF en rapamycine juist een remmende werking op tumorontwikkeling. Dit geeft aan dat het risico op huidkanker bij personen met orgaantransplantaties mogelijk sterk verminderd kan worden door aanpassing van de immuunsuppressieve behandeling. Om ethische en praktische redenen kunnen de effecten van verschillende immuunsuppressiva op processen betrokken bij kankerontwikkeling niet experimenteel onderzocht worden bij patiënten. Bij de experimenten beschreven in dit proefschrift is daarom voornamelijk gebruik gemaakt van twee modellen: een huidmodel en een muismodel.

Doel van de studies

Het centrale doel van de studies beschreven in dit proefschrift was om experimentele gegevens te verkrijgen die bij kunnen dragen aan het verminderen van het huidkankerrisico dat wordt veroorzaakt door immuunsuppressieve therapie. Dit is verder toegespitst op het bepalen van effecten van immuunsuppressiva op de reactie van huidcellen op UV blootstelling en effecten op UV-geïnduceerde huidkankerontwikkeling. We hebben hiervoor effecten van immuunsuppressiva op verschillende stadia van huidkankerontwikkeling in kaart gebracht. Het op deze wijze vergelijken van effecten van immuunsuppressiva kan bijdragen aan de vermindering van het risico op huidkanker.

Humaan huidmodel

Het humane huidmodel dat is gebruikt bestaat uit een dermale collageenmatrix waarin fibroblasten zijn gezaaid met daarboven een epidermis bestaande uit keratinocyten. Dit huidmodel bestaat uit primaire humane cellen en is goed vergelijkbaar met de humane huid wat betreft morfologie en expressie van diverse karakteristieke eiwitten. Dit model is daarom geschikt om de reactie van keratinocyten op UV blootstelling te bepalen. De meeste huidmodellen kunnen slechts een beperkte tijd in kweek worden gehouden en zijn daarom niet geschikt voor het bepalen van lange termijn effecten van herhaalde UV blootstelling.

Haarloze muis

De haarloze muis (SKH1) is een veelgebruikt model bij studies naar huidkankerontwikkeling door UV. Deze muizen hebben een functionerend immuunsysteem en de huid lijkt in veel opzichten op die van de mens. Wanneer haarloze muizen herhaaldelijk blootgesteld worden aan UV ontwikkelen zich in de epidermis clusters van cellen die gemuteerd p53 tot expressie brengen (mutant-p53 celclusters). Deze mutant-p53 celclusters ontstaan als een clonale expansie van een gemuteerde keratinocyt en zijn al aanwezig voordat huidkankers zich ontwikkelen. De huidtumoren die ontstaan zijn voornamelijk plaveiselcelcarcinomen en goedaardige voorlopers daarvan (actinische keratosen). Aangezien de meeste plaveiselcelcarcinomen een gemuteerd p53 bevatten met vergelijkbare mutaties als in mutant-p53 celclusters, wordt het muteren van p53 als een vroege gebeurtenis beschouwd in tumorvorming en worden mutant-p53 celclusters als voorlopers van plaveiselcelcarcinomen gezien. Zoals eerder genoemd zijn plaveiselcelcarcinomen het type kanker waarvan de incidentie het meest verhoogd is in orgaantransplantatiepatiënten. Haarloze muizen lijken daarom geschikt om effecten van immuunsuppressiva op UV-geïnduceerde huidkanker te onderzoeken.

Effecten van immuunsuppressiva op huidmodellen

In **hoofdstukken 3 en 4** zijn de effecten van immuunsuppressiva op de epidermis en reactie van de huid op UV-blootstelling onderzocht in humane huidmodellen. De opbouw van de epidermis werd geremd door rapamycine, zichtbaar door onder andere een verminderd aantal levende cellagen en een verminderd percentage delende cellen. Het aantal cellen dat apoptotisch werd (geprogrammeerde celdood ondergaand) na UV blootstelling was verhoogd in huidmodellen met rapamycine. De apoptotische reactie na UV blootstelling werd juist geremd door cyclosporine, in overeenstemming met eerdere bevindingen. Het eiwit HIF1α, dat een belangrijke rol speelt in het reguleren van metabole processen in een cel, accumuleerde in keratinocyten in UV bestraalde huidmodellen. Rapamycine remde deze accumulatie van HIF1α (hoofdstuk 3). HIF1α kan apoptotische reacties van cellen beïnvloeden en kan daarom betrokken zijn bij het pro-apoptotische effect van rapamycine in huidmodellen. In tegenstelling tot cyclosporine kan rapamycine dus de vorming van kwaadaardige cellen ondervloed van UV tegengaan door de groei te remmen en door de sterfte onder beschadigde cellen op te voeren.

Immuunsuppressiva en UV-geïnduceerde p53 mutaties en mutant-p53 celclusters

P53 mutaties zijn vroege gebeurtenissen in het ontstaan van huidkanker. Om effecten van immuunsuppressiva op het ontstaan van deze mutaties te onderzoeken hebben we mutatiefrequenties in p53 bepaald in muizenhuid die gedurende 10 weken was blootgesteld aan UV, nog voordat tumoren ontstonden (**hoofdstuk 4**). Door middel van deep-sequencing (hiermee wordt de sequentie van miljoenen DNA moleculen tegelijk bepaald) is bepaald dat veel cellen een gemuteerd allel van p53 bevatten (naar schatting 20%). Er was geen effect van immuunsuppressiva op de mutatiefrequenties.

In tegenstelling tot deze mutatiefrequenties hadden immuunsuppressiva wel effect op de ontwikkeling van mutant-p53 celclusters. Cyclosporine en azathioprine versnelden de ontwikkeling hiervan in muizen, terwijl rapamycine deze juist vertraagde (hoofdstukken 3 en 4). De discrepantie tussen de effecten van immuunsuppressiva op p53 mutatie-frequenties in de huid en de ontwikkeling van mutant-p53 celclusters wordt verderop in dit hoofdstuk behandeld.

Immuunsuppressiva versnelden huidkankerontwikkeling niet

Effecten van immuunsuppressiva op UV-geïnduceerde huidkanker zijn bepaald in muizen die dagelijks zijn blootgesteld aan UV en die immuunsuppressiva via het voer kregen

toegediend (**hoofdstukken 2 en 4**). Onverwacht bleek dat geen van de immuunsuppressiva de huidkankerontwikkeling versnelde. Muizen op cyclosporinedieet vertoonden zelfs een vertraagde huidkankerontwikkeling. Epidermale proliferatie was niet verminderd door cyclosporine, waardoor het onwaarschijnlijk is dat dit de oorzaak was van de vertraagde tumorontwikkeling. Eerdere studies hebben verschillende effecten van cyclosporine op huidkanker laten zien, zowel vertragend als versnellend. Rapamycine had geen effect op het ontstaan van de eerste kleine tumoren maar remde de ontwikkeling van grote tumoren (hoofdstuk 2). Dit kan veroorzaakt zijn door de bekende tumorgroei remmende eigenschappen (remming van bloedvatvorming) van rapamycine.

Rapamycine verandert het p53 mutatiespectrum in huidkankers

Het vermoeden dat rapamycine selectief apoptose induceert in p53-gemuteerde cellen en daarom tumorontwikkeling remt lijkt onwaarschijnlijk, omdat rapamycine het ontstaan van kleine tumoren (<1mm) niet remt (**hoofdstuk 2**). Om andere lokale effecten van immuunsuppressiva te onderzoeken is het spectrum van mutaties in het p53 gen bepaald van tumoren. Normaliter is de meerderheid van deze mutaties van het UV-type (C>T transitie op een dipyrimidineplaats), maar in de tumoren van muizen op rapamycinedieet was dit sterk veranderd. Een grote verschuiving in het type mutaties was opgetreden waardoor de meerderheid van de mutaties niet van het UV-type waren. Deze verandering in mutaties kan het gevolg zijn van een verhoogd niveau reactief zuurstof. In tumoren van muizen op een combinatiedieet van rapamycine en MMF was het mutatiespectrum echter onveranderd ten opzichte van controles, wat veroorzaakt kan zijn door het antioxidante effect van MMF. In **hoofdstuk 3** hebben we laten zien dat dit veranderde mutatiespectrum niet optrad in kleine tumoren en mutant-p53 celclusters van muizen op rapamycinedieet. Het veranderde mutatiespectrum is daarom een late gebeurtenis in tumorontwikkeling die pas optreedt na de ontwikkeling van kleine tumoren, of veroorzaakt wordt door een selectiemechanisme waardoor alleen een specifieke selectie van kleine tumoren kon uitgroeien. Aangezien ongevoeligheid voor rapamycine kan worden verkregen door verhoogde oxidatieve stress is het denkbaar dat alleen tumoren met verhoogde oxidatieve stress snel door kunnen groeien, waarbij ook het veranderde mutatiespectrum in grote tumoren is ontstaan.

De meeste mutant-p53 celclusters hebben niet de potentie om zich tot huidkanker te ontwikkelen

In **hoofdstukken 2, 3 en 4** worden de effecten van immuunsuppressiva op verschillende stadia in huidkankerontwikkeling beschreven. Er zijn discrepanties tussen effecten van immuunsuppressiva in de verschillende stadia:

- Geen van de immuunsuppressiva had effect op het percentage cellen met gemuteerd p53 in dagelijks UV-bestraalde huid (tumorvrije huid).
- De ontwikkeling van mutant-p53 celclusters werd verlaagd door rapamycine en verhoogd door cyclosporine.
- Tumorontwikkeling werd door cyclosporine vertraagd.
- Tumorgroei werd door rapamycine vertraagd.

Met name de effecten op ontwikkeling van mutant-p53 celclusters zijn anders dan verwacht kan worden op basis van het bestaande algemene model voor huidkankerontwikkeling door UV. Daarbij worden mutant-p53 celclusters als directe voorlopers van huidtumoren beschouwd met gelijke ontwikkelingsdynamiek. Dit is vooral duidelijk bij cyclosporine, waarbij geen effect op het percentage p53 gemuteerde cellen optreedt, de ontwikkeling van mut-53 celclusters wel verhoogd is, maar tumorontwikkeling vertraagd is. Ook de resultaten voor rapamycine laten zo'n discrepantie zien voor mutant-p53 celclusters. Aan de hand van deze resultaten concluderen we dat ontwikkeling van mutant-p53 celclusters niet direct samenhangt met huidkankerontwikkeling, maar dat deze celclusters naast en onafhankelijk van huidtumoren ontstaan uit een grote poule van mutant-p53 cellen.

Experimenteel protocol is bepalend voor het effect van cyclosporine op huidkankerontwikkeling door UV

In het verleden zijn verschillende muizenstudies gedaan om de effecten van immuunsuppressiva op huidkankerontwikkeling te onderzoeken. Studies die onderzoek naar het effect van cyclosporine op huidkanker beschrijven hebben verschillende uitkomsten. Eén van de eerste studies laat zien dat huidkankerontwikkeling verhoogd wordt door cyclosporine en een recente studie beschrijft dat cyclosporine ook de groei van tumoren verhoogt. Een andere studie laat daarentegen zien dat cyclosporine tumorontwikkeling remt, in overeenstemming met hoofdstuk 4 van dit proefschrift. Al deze studies maken gebruik van dezelfde stam haarloze muizen en huidkankerinductie door UV, ze verschillen echter in het protocol waarmee de cyclosporine wordt toegediend en in welke periode de muizen worden blootgesteld aan UV. **Hoofdstuk 5** beschrijft de verschillen tussen drie verschillende protocollen in de effecten van cyclosporine op huidkankerontwikkeling door UV. Cyclosporine had geen

effect op huidkankerontwikkeling in muizen die op cyclosporinedieet werden gezet na een periode van UV blootstelling. Daarentegen vond verhoogde tumorontwikkeling plaats in muizen die cyclosporine toegediend kregen door middel van gavage (sondevoeding). Dit bevestigde dat cyclosporine de huidkankerontwikkeling door UV kan bevorderen. Er was geen verschil in huidkankerontwikkeling tussen muizen die UV-bestraald werden na gavage op het moment dat cyclosporine bloedniveaus piekten of op het moment dat ze laag waren. Deze experimenten geven aan dat bloedniveaus van cyclosporine op het moment van UV blootstelling niet van belang zijn op het huidkankerbevorderende effect. Het toedienen van cyclosporine in bolus doseringen (via gavage) in plaats van een gelijkmatige toediening via het voer resulteerde in een versnelde huidkankerontwikkeling. Hieruit blijkt dat de manier van toediening van immuunsuppressiva belangrijk kan zijn voor effecten op de ontwikkeling van huidkanker. Maar zelfs de toediening van cyclosporine in bolus doseringen resulteerde niet in een verhoogde huidkankerontwikkeling in de mate die bij transplantatiepatiënten wordt gezien (60- tot 100-voudige toename). Dit suggereert dat het muismodel dat gebruikt is niet optimaal was, omdat muizen anders reageren dan mensen (bijvoorbeeld langzamer herstel van beschadigd DNA) of omdat het model niet compleet is door bijvoorbeeld het ontbreken van een orgaantransplantaat.

Ontwikkelingen

De studies in dit proefschrift bevestigen het potentieel dat rapamycine (of analogen zoals everolimus) heeft om het verhoogde huidkankerrisico bij transplantatiepatiënten te verminderen. Remming van tumorgroei door rapamycine-analogen met minder bijwerkingen dient verder onderzocht en ontwikkeld te worden. Dit zou voor de patiënt een verbetering op kunnen leveren.

De meerderheid van de mutant-p53 celclusters zijn geen voorlopers van plaveiselcelcarcinomen. Toch is het waarschijnlijk dat de meeste plaveiselcelcarcinomen ontstaan uit cellen met gemuteerd p53 aangezien mutatie van p53 een vroege gebeurtenis is in de ontwikkeling van deze tumoren. Een nog onbekende oncogene gebeurtenis zou echter vooraf kunnen gaan aan de p53 mutatie.

De hier beschreven studies laten zien dat de gebruikte muismodellen voor huidkankerontwikkeling door UV niet het door immuunsuppressiva verhoogde huidkankerrisico in transplantatiepatiënten nabootsen. Het is mogelijk dat het muismodel een belangrijk element mist, namelijk het transplantaat, en daarmee ook de mogelijke tolerantie hiervoor. Als belangrijk en logisch vervolg op het hier beschreven onderzoek zou dus een transplantaat

en de daarbij behorende tolerantie in het UV carcinogenese model met de haarloze muis moeten worden opgenomen.

List of publications

Voskamp P, Bodmann CA, Rebel HG, Koehl GE, Tensen CP, Bouwes Bavinck JN, El Ghalbzouri A, Van Kranen HJ, Willemze R, Geissler EK, De Gruijl FR. Rapamycin impairs UV induction of mutant-p53 overexpressing cell clusters without affecting tumor onset. Int J Cancer. 2011 Dec 9. [Epub ahead of print]

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List of abbreviations

6MP	6-mercaptopurine
Aza	Azathioprine
BCC	Basal-cell carcinoma
CsA	Cyclosporine A
CPD	Cyclobutane pyrimidine dimer
HIF1α	Hypoxia-inducible factor 1, alpha subunit
HSE	Human skin equivalent
MED	Minimal erythemal/edemal dose
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T-cells
NMSC	Non-melanoma skin cancer
OTR	Organ transplant recipient
PCR	Polymerase chain reaction
Rapa	Rapamycin (Sirolimus)
SCC	Squamous-cell carcinoma
Tac	Tacrolimus
UV	Ultraviolet light
VEGF	Vascular endothelial growth factor

Curriculum Vitae

Pieter Voskamp was born on 18 May 1981 in Woerden, The Netherlands. After graduating from "Driestar College" (VWO) in 1999 he started with the study Life Science and Technology at the Leiden University and Delft University of Technology. As part of his study he performed research internships at the Department of Human and Clinical Genetics of the Leiden University Medical Center (atherosclerosis) and the Department of Biological Chemistry of the Leiden University (cancer biology). He received his Master's degree cum laude in 2006.

In 2006 Pieter started as a PhD-student at the Department of Dermatology of the Leiden University Medical Center under supervision of Dr. F.R. de Gruijl and Prof.dr. R. Willemze. During this project he collaborated with Dr. G.E. Koehl and Prof.dr. E.K. Geissler from the University of Regensburg (department of Surgery) in Germany.

As of November 2011, Pieter is working as a consultant at the consultancy firm Stratelligence in Leiden, The Netherlands.

Nawoord

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