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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.
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\textsuperscript{4} and in the Netherlands over 35,000 cases a year\textsuperscript{5}. The skin carcinomas in the general populations are clearly related to solar UV exposure\textsuperscript{6} and so are the skin carcinomas in organ transplantation recipients\textsuperscript{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\textsuperscript{9}. In Europe and the USA 40-61\%\textsuperscript{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\%\textsuperscript{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\textsuperscript{14}. Susceptibility to UV-induced suppression of cellular immunity (contact hypersensitivity) was found to be positively correlated with
skin carcinoma in humans\textsuperscript{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.\textsuperscript{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\textsuperscript{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\textsuperscript{18, 19}. MMF/MPA specifically inhibits inosine monophosphate dehydrogenase, and does not lead to incorporation of pseudo-bases in the DNA\textsuperscript{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)\textsuperscript{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)\textsuperscript{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\textsuperscript{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.

**Immunosuppressant**

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<tr>
<th>Aza</th>
<th>MMF</th>
<th>CsA</th>
<th>CpN</th>
<th>Tac</th>
<th>FKBP</th>
<th>FKBP</th>
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<tr>
<td><strong>Mode of action</strong></td>
<td>Inhibition of purine synthesis</td>
<td>Inhibition of calcineurin and NFAT</td>
<td>Blocking of mTORC1</td>
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<td><strong>Photogenotoxicity</strong></td>
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**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\(^2^4\). CsA was also found to reduce UV-induced apoptosis (sunburn cells) in the skin of BALB/c mice\(^2^5\). Herman et al.\(^2^6\) 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\(^2^7\). 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\(^2^8\)). 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. A similar result of NFAT activity was reported by others for fibroblasts following exposure to low doses of long-wavelength UV-A radiation. 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. 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. In concordance with a blockage of transcription, 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). However, the P53 mutation spectrum in skin carcinomas from an early cohort of renal transplant recipients showed no obvious deviations, 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/PI3-K and leads to activation of mTOR and p70S6 kinase through PI3-K, surprisingly not through Akt. Upon UV exposure mTOR mediates phosphorylation and activation of P53 and BRCA1. And in keratinocytes mTOR relays the UV-induced signaling for TNF-alpha (and IL 10 in murine keratinocytes), involved in UV-induced immunosuppression. 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.

**Angiogenesis:** MMF was developed as an anti-neoplastic agent and was proven to inhibit tumor cell proliferation, to modulate adhesion molecules resulting in impaired invasiveness and to induce terminal differentiation. 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. 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, whereas CsA stimulates VEGF production.

**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. 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, 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 and expression of basal membrane, proliferation and differentiation markers. 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.
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 irradiations49, 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, 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. 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). 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. Also, Rapa could increase apoptotic responses in p53 dysfunctional cells. 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\textsuperscript{16}, whereas other studies showed that CsA decreased UV-induced tumor development\textsuperscript{16} (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.
References


