Cover Page

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Chapter 5

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

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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.

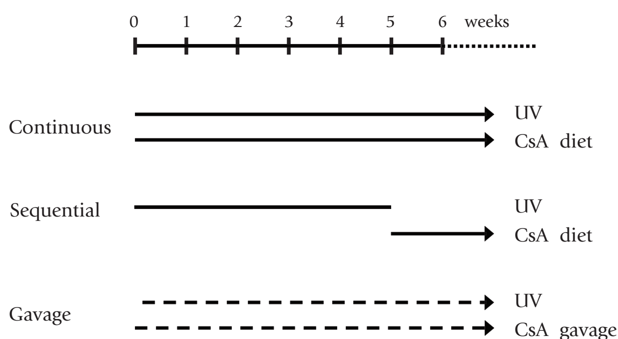

Figure 1: Graphical representation of the different experimental protocols that were used: continuous protocol, sequential protocol and gavage protocol.

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.

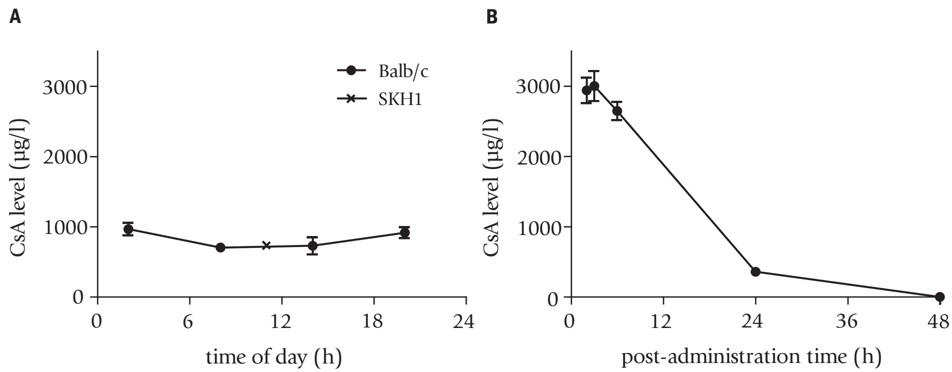

Figure 2: Cyclosporine blood levels over time for different modes of administering the drug. (**A**) cyclosporine blood levels in mice on cyclosporine-containing diet (n=3 per time point); (**B**) cyclosporine blood levels after gavage (n=4-5 per time point). Error bars depict SEM.

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).

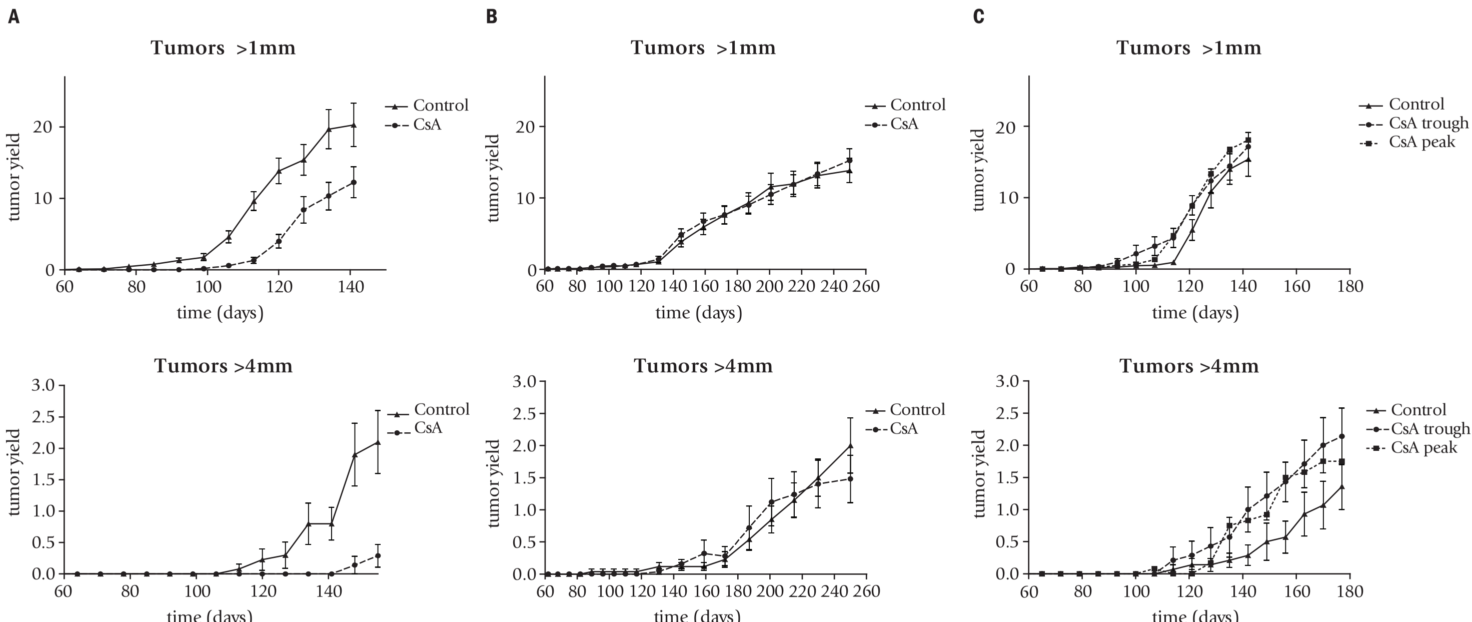

Figure 3: Effect of cyclosporine on yields of tumors >1mm and tumors >4mm in the different experiments on UV carcinogenesis and cyclosporine feeding: In the continuous UV model (**A**) cyclosporine decreased tumor yields, in the sequential model (**B**) there was no effect of cyclosporine on tumor yields, 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).

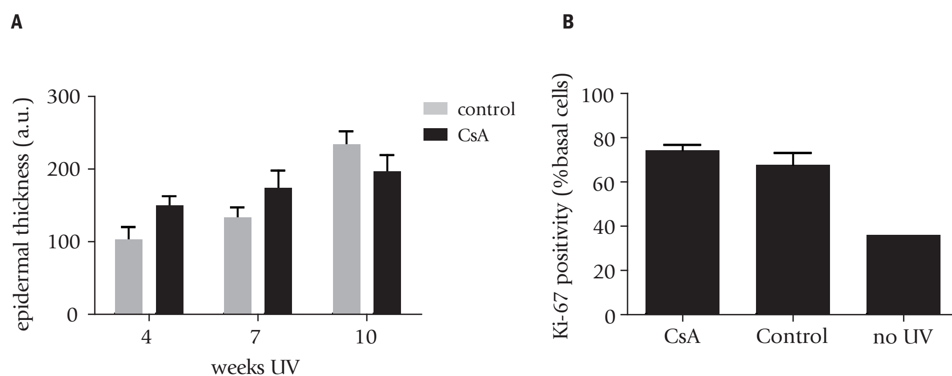

Figure 4: Effects of cyclosporine on epidermal thickness and Ki-67 positive cell fraction. (**A**) Epidermal thickness of mice daily irradiated for 4, 7 and 10 weeks is shown. (**B**) Epidermal proliferation measured by percentage of Ki-67 positive cells after 4 weeks of UV irradiation. N=4 in each measurement,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

1. Glover MT, Deeks JJ, Raftery MJ, Cunningham J, Leigh IM. Immunosuppression and risk of non-melanoma skin cancer in renal transplant recipients. Lancet 1997;349:398.
2. Jensen P, Hansen S, Møller B, Leivestad T, Pfeffer P, Geiran O, Fauchald P, Simonsen S. Skin cancer in kidney and heart transplant recipients and different long-term immunosuppressive therapy regimens. Journal of the American Academy of Dermatology 1999;40:177-86.
3. Herman M, Weinstein T, Korzets A, Chagnac A, Ori Y, Zevin D, Malachi T, Gafter U. Effect of cyclosporin A on DNA repair and cancer incidence in kidney transplant recipients. Journal of Laboratory and Clinical Medicine 2001;137:14-20.
4. Hiesse C, Rousseau P, Kriaa F, Larue JR, Charpentier B. Elective vs systematic corticosteroid withdrawal in renal transplant recipients receiving triple drug therapy. Transplant Proc 1995;27:1066-7.
5. Yarosh DB, Pena AV, Nay SL, Canning MT, Brown DA. Calcineurin inhibitors decrease DNA repair and apoptosis in human keratinocytes following ultraviolet B irradiation. The Journal of Investigative Dermatology 2005;125:1020-5.
6. Sugie N, Fujii N, Danno K. Cyclosporin-A suppresses p53-dependent repair DNA synthesis and apoptosis following ultraviolet-B irradiation. Photodermatol Photoimmunol Photomed 2002;18:163-8.
7. Kelly GE, Meikle W, Sheil AG. Effects of immunosuppressive therapy on the induction of skin tumors by ultraviolet irradiation in hairless mice. Transplantation 1987;44:429-34.
8. Canning MT, Nay SL, Peña AV, Yarosh DB. Calcineurin inhibitors reduce nuclear localization of transcription factor NFAT in UV-irradiated keratinocytes and reduce DNA repair. Journal of Molecular Histology 2006;37:285-91.
9. Zamzami N, Larochette N, Kroemer G. Mitochondrial permeability transition in apoptosis and necrosis. Cell Death Differ 2005;12 Suppl 2:1478-80.
10. Norman KG, Canter JA, Shi M, Milne GL, Morrow JD, Sligh JE. Cyclosporine A suppresses keratinocyte cell death through MPTP inhibition in a model for skin cancer in organ transplant recipients. Mitochondrion 2010;10:94-101.
11. Hojo M, Morimoto T, Maluccio M, Asano T, Morimoto K, Lagman M, Shimbo T, Suthanthiran M. Cyclosporine induces cancer progression by a cell-autonomous mechanism. Nature 1999;397:530-4.
12. Wu X, Nguyen B-C, Dziunycz P, Chang S, Brooks Y, Lefort K, Hofbauer GFL, Dotto GP. Opposing roles for calcineurin and ATF3 in squamous skin cancer. Nature 2010;465:368-72.
13. Duncan FJ, Wulff BC, Tober KL, Ferketich AK, Martin J, Thomas-Ahner JM, Allen SD, Kusewitt DF, Oberyszyn TM, VanBuskirk AM. Clinically Relevant Immunosuppressants Influence UVB-Induced Tumor Size Through Effects on Inflammation and Angiogenesis. American Journal of Transplantation 2007;7:2693-703.
14. Wulff BC, Kusewitt DF, VanBuskirk AM, Thomas-Ahner JM, Duncan FJ, Oberyszyn TM. Sirolimus Reduces the Incidence and Progression of UVB-Induced Skin Cancer in SKH Mice even with Co-administration of Cyclosporine A. J Invest Dermatol 2008;128:2467-73.

15. David-Neto E, Araujo LP, Feres Alves C, Sumita N, Romano P, Yagyu EM, Nahas WC, Ianhez LE. A strategy to calculate cyclosporin A area under the time-concentration curve in pediatric renal transplantation. Pediatr Transplant 2002;6:313-8.
16. Niederberger W, Lemaire M, Maurer G, Nassbaumer K, Wagner O. Distribution and binding of cyclosporine in blood and tissues. Transplant Proc 1983;4 (suppl I):2419-37.
17. Takeuchi H, Matsuno N, Senuma K, Hirano T, Yokoyama T, Taira S, Kihara Y, Kuzuoka K, Konno O, Jojima Y, Mejit A, Akashi I, et al. Biological & Pharmaceutical Bulletin 2008;31:90-4.