Conversion from Calcineurin Inhibitors to Sirolimus Reduces Vascularization and Thickness of Post-transplant Cutaneous Squamous Cell Carcinomas

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Abstract. *Background: Immunosuppression favors the development of skin cancer. Experimental data suggest that sirolimus (SRL) has antitumoral and antiangiogenic properties. An investigation was undertaken into the effects of SRL on squamous cell carcinoma (SCC) developing in organ transplant recipients (OTR) receiving immunosuppressive treatments, with special emphasis on vascularization. Materials and Methods: SCC that developed in eight OTR before and after conversion from calcineurin inhibitors (CNI) to SRL were compared for thickness, differentiation, ulceration, perineural invasion, density of peritumoral infiltrate, peritumoral vascularization, density of T-regulatory cells and of intratumoral Langerhans cells and growth fraction. Results: SCC developing under SRL showed lower peritumoral vascularization and thickness, and higher growth fraction and density of peritumoral T-regulatory cells. Conclusion: Conversion from CNI to SRL at clinically relevant doses is associated in vivo with a reduced vascularization and thickness of post-transplant human cutaneous SCC. This effect could account for the beneficial effect of SRL on immunosuppression-induced skin carcinogenesis in humans.*

Organ transplant recipients (OTR) are at increased risk for developing cancer because of the chronic immunosuppression necessary to prevent allograft rejection (1). Immunosuppressive drugs indirectly favor the development of tumors through decreased immune surveillance and antitumor defence, and occasionally also *via* direct oncogenic effects. Cutaneous squamous cell carcinoma (SCC) accounts for the majority of *de novo* malignancies developing in OTR. They are usually multiple, may have an aggressive course and cause significant morbidity and mortality. Their management relies on the usual treatment modalities of skin carcinomas and on revision of the immunosuppression (1, 2). In this setting, sirolimus (SRL), a mammalian target of rapamycin (mTOR) inhibitor, is a particularly interesting molecule since it combines immunosuppressive and antitumoral properties. Indeed, recent studies have shown that SRL-based immunosuppressive treatments administered to OTR are associated with a lower risk for developing post-transplant cancer, including cutaneous ones (3). SRL seems to exert antitumoral properties even in combination with cyclosporine A (CSA), although the risk for skin cancer is lower when SRL is given with steroids rather than with CSA (4, 5). Preliminary data also suggest that conversion from calcineurin inhibitors (CNI) to SRL decreases the incidence of skin cancer in renal graft recipients (6). The mTOR signalling pathway, which involves several tumor suppressor genes and proto-oncogenes (including *PTEN, PIK3, Akt* and *elFAE*), is deregulated in several malignancies, and its inhibition by SRL could account for the antitumoral properties of this drug (7-9). At therapeutic doses, SRL inhibits the growth of epidermoid cancer cell lines with an activated Akt signalling pathway *in vitro* and in xenograft models (10). Along with preventing cardiac allograft rejection, SRL also inhibited the growth of CT-26 adenocarcinoma cells and B16 melanoma cells in a mouse model (11). The *in vivo* antitumoral properties of SRL seem to be due to an antiangiogenic effect, mediated *via* vascular endothelial growth factor inhibition (12). On the other hand, SRL has been shown to up-regulate T-regulatory cells *in vitro* and to increase the density of epidermal Langerhans cells in an animal model (3, 14).

In this work we studied several immunopathological features, with particular emphasis on peritumoral vascularization, of SCC developing in OTR prior and after conversion from CNI to SRL.

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Materials and Methods

Patients and tumors. Patients considered for this study included OTR that had developed cutaneous SCC before and after conversion from CNI to SRL (because of multiple skin carcinomas). They belonged to a cohort of over 3,000 OTR followed in our specialized Outpatient Dermatology Clinic, and were examined by the same physician (SE) every three months following the development of a first skin carcinoma. Eight OTR of Caucasian origin were included in the study. Their main demographic data are shown in Table I. SRL introduction was associated with discontinuation of CNI (except for one patient in whom CSA was maintained at low doses of 50 mg/d). In another patient, azathioprine was discontinued.

Our study was performed on a total of 26 primary SCC excised surgically under local anesthesia from these OTR. Fifteen tumors had developed before (SCC1) and 11 after (SCC2) conversion from CNI (mostly CSA) to SRL. Relevant data on these lesions are shown in Table I. Recurring tumors and SCC that had developed less than 6 months after conversion to SRL were not studied in order to exclude a confounding effect of the previous treatment with CNI.

Pathologic study. The specimens of SCC studied were formalin-fixed and paraffin-embedded. Representative routinely-stained sections of each tumor were re-examined by the same dermatopathologist (JK) blindly as to the group (SCC1 or SCC2), and the following pathological features were assessed: presence of ulceration and perineural invasion, density of the peritumoral cell infiltrate (scored semi-quantitatively as 1: weak, 2: moderate, or 3: dense), micrometric thickness measured (in mm) with an ocular grid, and degree of differentiation (scored as 1: good, 2: moderate, or 3: poor) (Figure 1).

Immunohistochemical study. This was performed on paraffin-embedded tissue sections according to an avidin-biotin amplification immunoperoxidase technique after antigen retrieval. Peritumoral vascularization, T_{reg} cells, Langerhans cells and tumor growth fraction were immunohistochemically assessed with the following monoclonal antibodies respectively: a) clone JC/70A to the endothelial antigen CD31 (Dako, Copenhagen, Denmark); b) clone 236A/E7 to the nuclear transcription factor FoxP3; c) clone 808E10 to CD207/langerin (Dendritics, Dardilly, France); and d) clone MIB-1 to the Ki-67 antigen (Dako) (Figures 2-3).

Evaluation of labeled structures was performed by an automated image analyzer using the Histolab software (Microvision, Haverhill, UK). The areas of the sections to be counted were selected randomly in a blinded fashion as to the group (SCC1 or SCC2) by the same observer (ALRT). Peritumoral vascularization was expressed both as surface of $CD31^+$ endothelial cells and total vascular surface (*i.e.* endothelial cells plus vessel lumen) per surface of dermis, evaluated on 5 fields adjacent to the tumors. Results on $CD207^+$ Langerhans cells and growth fraction ($Ki\text{-}67^+$ tumor cells) were expressed both as number and surface of cells or nuclei, respectively, per tumor surface. The number of infiltrating $FoxP3^+$ T_{reg} cells was counted visually (to exclude occasional cells showing non-specific cytoplasmic staining that could have been counted by the image analyzer) on 10 dermal fields adjacent to the tumor, and the results expressed both per surface of dermis and per total number of infiltrating cells counted by the image analyzer.

Table I. *Demographic characteristics of the patients and tumors studied.*

Sex	6 Men/2 women
Organ grafted	6 Kidney/2 heart
Skin type	II: 1
	III: 7
Sun exposure	Weak: 2
	Moderate: 3
	Heavy: 3
Mean age at first	52.5 years
SCC occurrence (range)	(37-74)
Mean duration of immunosuppression	184 months
at SRL introduction (range)	(53-336)
Treatment before	CNI + steroids: 2
SRL introduction	CNI + steroids + MMF: 4
	Steroids + Aza: 1
	CNI + steroids + Aza: 1
SCC developed before	n: 15 (5 head/neck,
SRL introduction (SCC1)	6 upper limbs, 4 legs)
SCC developed after SRL	n: 11 (3 head/neck,
introduction (SCC2)	4 upper limbs, 4 legs)

CNI, Calcineurin inhibitors (6 cyclosporine A/1 tacrolimus); MMF: mycophenolate mofetil, Aza: Azathioprine.

Statistical analysis. Statistical analysis was performed with SAS® software (version 9.1) (SAS Institute Inc., Cary, NC, USA). Quantitative data were compared with the non-parametric Mann-Whitney test. Qualitative data were compared with Fisher's exact test. A *p*-value of 0.05 or less was considered significant.

Results

The immunopathological features of the two SCC groups studied are shown in Table II. Compared with SCC1, SCC2 showed a statistically significantly lower thickness (1.02 *vs*. 2.3 mm, $p=0.003$) and peritumoral vascularization, expressed both as endothelial (5.35×10^{-2} *vs*. 8.2×10^{-2}, $p=0.02$) and total vascular surface (6.30×10^{-2} *vs*. 9.72×10^{-2}, $p=0.03$) per dermal surface. SCC2 contained significantly higher numbers of peritumoral T_{reg} cells, expressed both per total infiltrating cells (1.85×10^{-2} *vs*. 1.24×10^{-2}, $p=0.02$) and per dermal surface (1.41×10^{-4} *vs*. $9.2\times10^{-5}/m^2$ $p=0.02$). The growth fraction (expressed both as number and surface of $Ki\text{-}67^+$ nuclei per tumor surface) was higher in SCC2 *vs*. SCC1 (respectively 13.81×10^{-2} *vs*. 4.75×10^{-2}, $p=0.01$, and 18.35×10^{-4} *vs*. 8.02×10^{-4}, $p=0.004$). The remaining features studied (density of intratumor Langerhans cells, ulceration, degree of differentiation, perineural invasion, density of the peritumoral infiltrate scored semi-quantitatively) did not show statistically significant differences between SCC1 and SCC2.

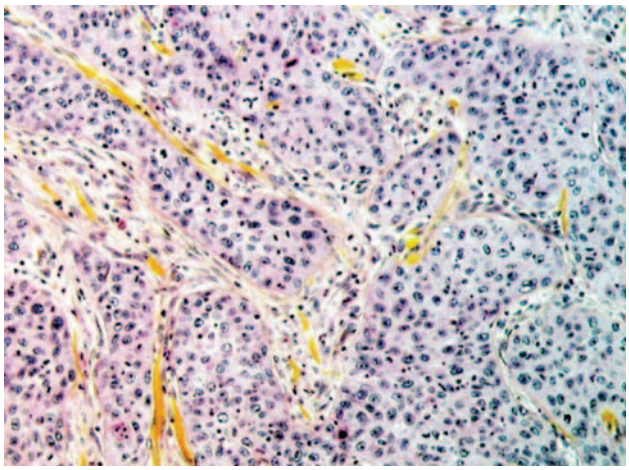

Figure 1. *Squamous cell carcinoma of moderate differentiation (grade 2) surrounded by a weak (grade 1) peritumoral infiltrate (hematoxylin-eosin stain, original magnification ×100).*

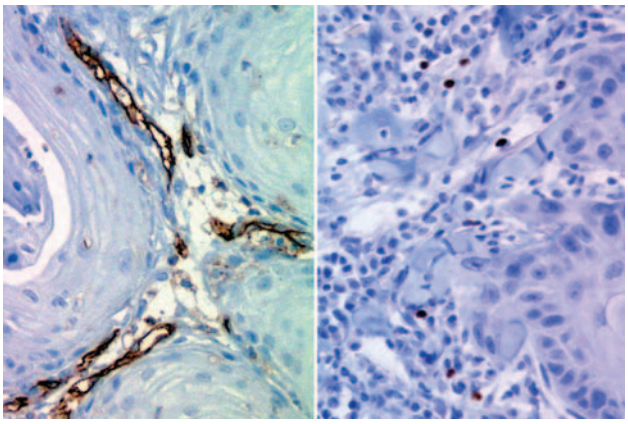

Figure 2. *Squamous cell carcinoma. Left panel: peritumoral vessels revealed with immunolabeling for the CD31 endothelial antigen. Right panel: tumor infiltrating FoxP3+ T regulatory cells (immunoperoxidase, original magnification ×200).*

Table II. *Immunopathological features of the SCC studied.*

Feature	SCC prior to SRL (n:15)	SCC under SRL (n:11)	*p*-Value
Degree of differentiation			
1	13 (86.7%)	6 (54.5%)	0.16
2	2 (13.3%)	3 (27.3%)	
3	0 (0%)	2 (18.2%)	
Ulceration			
Yes	4 (26.7%)	6 (54.5%)	0.23
Perineural invasion			
Yes	0 (0%)	2 (18%)	0.17
Density of peritumor cell infiltrate			
1	1 (6.6%)	1 (9.1%)	1
2	7 (47.7%)	5 (45.5%)	
3	7 (47.7%)	5 (45.5%)	
Thickness (mm)	2.3	1.02	0.003
Endothelial surface/dermal surface	8.2×10^{-2}	5.35×10^{-2}	0.02
Total vascular surface/dermal surface	9.72×10^{-2}	6.30×10^{-2}	0.03
Treg cells/total infiltrating cells	1.24×10^{-2}	1.85×10^{-2}	0.02
Treg cells/dermal surface (μm^2)	9.2×10^{-5}	1.41×10^{-4}	0.02
Langerhans cells /tumor surface (μm^2)	1.32×10^{-4}	0.52×10^{-4}	0.75
Langerhans cells surface/tumor surface	2.00×10^{-3}	0.42×10^{-3}	0.70
Ki67+ surface/tumor surface	8.02×10^{-4}	18.35×10^{-4}	0.004
Ki67+ cells/tumor surface (μm^2)	4.75×10^{-2}	13.81×10^{-2}	0.01

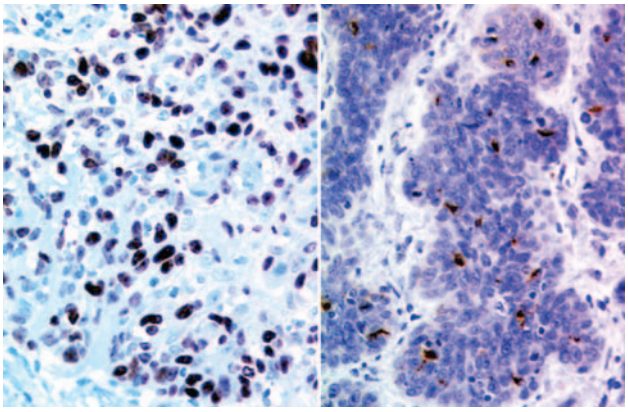

Figure 3. *Squamous cell carcinoma. Left panel: Expression of Ki-67 by tumor cells. Right panel: intratumor CD207/langerin+ Langerhans cells (immunoperoxidase, original magnification ×200).*

Discussion

Several studies have shown that SRL, used at therapeutic doses in organ transplantation, exerts an antitumoral effect both *in vitro* and *in vivo*. *In vivo* studies include namely a murine model of hepatic metastasis from CT-26 colon adenocarcinoma cell lines (12) and a rat model of hepatocarcinoma (15). In these models, SRL was shown to decrease tumoral vascularization and size. In OTR, the use of SRL-based immunosuppression is associated with a decreased incidence of tumors, including cutaneous ones (4, 5, 16). Accordingly, conversion of CSA to SRL, associated or not with withdrawal of other immunosuppressants, usually induces regression of Kaposi's sarcoma, a tumor of endothelial origin (17-19).

The antitumoral effects of SRL seem to be due to inhibition of tumor neovascularization, exerted through a decrease of VEGF synthesis and secretion and inhibition of stimulatory signals induced by the binding of VEGF on endothelial cells (12). VEGF, fibroblast growth factor 2 and angiopoietins secreted by inflammatory cells and tumor cells under hypoxia induce growth of newly-formed vessels from

pre-existing ones; this increase of peritumor vascularization enhances further tumor growth. SRL inhibits the PI3K-Akt-p70S6 signalling pathway, which is necessary for the stimulation of endothelial cells by VEGF (20, 21) and is activated in Kaposi's sarcoma cells (22). The antiangiogenic effects of SRL *via* reduced VEGF production have also been shown in non-tumor cells, including a model of corneal neoangiogenesis in rabbits (23) and in endometriosis (24). Additional mechanisms that likely contribute to the antiangiogenic effects of SRL include inhibition of proliferation and differentiation of endothelial cell progenitors (25) and increasing sensitivity of endothelial cells to apoptotic signals (26). SRL also inhibits cytokine-driven smooth muscle cell proliferation and migration (27-29), an effect likely contributing to its antiangiogenic properties.

On the basis of the aforementioned data, we postulated that SRL-based immunosuppression would also reduce peritumoral angiogenesis in cutaneous SCC developing in OTR receiving this drug in comparison with SCC developing under non SRL-based immunosuppressive treatments. In an animal model, it was recently shown that SRL reduces vascularity of tumors developing in mice previously irradiated with UV (30). The results of our study are in keeping with this finding and show for the first time in humans that peritumoral vascularization is reduced in cutaneous SCC developing under immuno-suppressive doses of SRL. The anti-CD31 antibody, which was used in our study and those of the literature, reveals both vascular and lymphatic vessels, therefore the antiangiogenic effect could be directed against both vascular and lymphatic vessels. This is in keeping with recent results showing that SRL exerts an antilymphangiogenic effect both *in vitro* and *in vivo via* a decrease of synthesis of VEGF-C, the isoform involved in (tumor) lymphangiogenesis and lymphatic metastatic spread (31, 32).

Since CNI have angiogenic properties, it could be speculated that their discontinuation may have contributed to the reduction of vascularization observed in our study in SCC2. However, in one of our patients CSA was withdrawn precociously: SCC1 appeared after CSA discontinuation and azathioprine was replaced by SRL. This finding is in favor of the direct antiangiogenic effect of SRL. On the other hand, the different localizations of tumors could introduce a bias regarding peritumor vascularization, since the dermis in some body areas (such as the face) contains a denser vascular network. In this respect, the two groups of tumors we studied were comparable since the proportion of tumors located on the face or the limbs was very similar between SCC1 and SCC2.

Regarding the histological features of aggressiveness, SCC2 were significantly thinner as compared with SCC1. This finding is not due to bias in patient follow-up, since all OTR included in this study were followed on a regular three-month interval basis after the occurrence of the first SCC, *i.e.* before conversion to SRL, and were therefore followed regularly before and after conversion to SRL. The antiangiogenic effect of SRL could account for the reduced tumor thickness after SRL introduction, since tumor growth largely depends on blood supply. This result is in accordance with an experimental study performed on mice, showing that tumors developing under SRL had a smaller size than those developing under CSA (30).

The remaining pathological features studied (degree of differentiation, density of the peritumoral inflammatory cell infiltrate, ulceration and perineural invasion) did not show statistically significant differences between SCC1 and SCC2. Concerning the density of peritumoral inflammation, our findings are in keeping with those of the murine study, reporting no significant effect of SRL on peritumoral inflammation (30). Our finding of increased tumor growth fraction in SCC2 *vs.* SCC1 is somewhat unexpected and suggests that at therapeutic doses SRL does not inhibit the proliferation of SCC keratinocytes. This is at variance with results reported for other cell lines, but in those studies SRL was used at doses unsuitable for therapeutic use (12). The increased growth fraction in SCC2 *vs.* SCC1 may seem contradictory to their lower thickness and vascularization, but this discrepancy could be due to an increased apoptotic rate of SCC2 *vs.* SCC1. This is also consistent with the fact that the expression of Ki-67 does not seem to be correlated with the clinical aggressiveness of SCC developing in OTR (33).

Although in our study we did not find obvious differences in the overall density of the peritumoral cell infiltrate, we found an increased density of infiltrating $FoxP3^+$ T_{reg} cells in SCC2 *vs.* SCC1. $FoxP3^+$ are natural T_{reg} cells exerting a regulatory/suppressive function on immune reactions; they play a key role in the maintenance of peripheral tolerance toward self-antigens and in the control of inflammatory immune responses to maintain homeostasis. In the setting of organ transplantation, an increase of T_{reg} cells could favor allograft tolerance, (34, 35) although it was recently reported that FoxP3 expression in kidney transplant biopsies is associated with rejection and not favorable outcomes (36). Compared with CNI, SRL has been shown to up-regulate circulating T_{reg} cells in renal graft patients (37, 38) and in mice (14), and to expand human T_{reg} cells *in vitro* (39). FoxP3 cells could function in some cancer cell lines as a transcriptional repressor of cancer *via* S-phase kinase-associated proteins 2 (SKP2) and 27 (40). Conversely, however, tissue-infiltrating T_{reg} cells could inhibit natural killer cells (41), thereby facilitating escape of the tumor from immune surveillance. Therefore the significance of increased numbers of T_{reg} cells in SCC under SRL remains to be further studied.

Langerhans cells are dendritic epidermal immunocompetent cells that are able to elicit immune responses by presenting antigens to naive T-cells, and to induce peripheral immune tolerance. In the setting of organ transplantation, their role

seems equivocal since these cells are able to induce both allograft tolerance and rejection. The effect of SRL on dendritic cells is debated. One study (42) reported a stimulatory effect of SRL on dendritic cells, *via* an increase of CCR7 expression and migration to lymph nodes. However, other studies claimed that, both *in vitro* and *in vivo*, SRL inhibits the maturation (43), the secretion of IL10 and IL12, endocytosis and the capacity for T-cell stimulation of dendritic cells (44). Recently in a mouse model of contact dermatitis, SRL was reported to increase the density of epidermal Langerhans cells by inhibiting their migration to lymph nodes (13). In our study, we did not find statistically significant differences in the numbers of intratumoral Langerhans cells between SCC1 and SCC2. Further studies are needed in order to better assess the effects that SRL exerts *in vivo* on these cells.

In conclusion, our findings show that conversion from CNI to SRL at clinically relevant (immunosuppressive) doses is associated *in vivo* with an antiangiogenic effect on human SCC developing in OTR that could account, at least partly, for reduced tumor thickness. These results confirm relevant animal data from the literature and provide a pathological basis for understanding the favorable effect of SRL on skin carcinogenesis in OTR.

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