Elevated Cutaneous Smad Activation Associates with Enhanced Skin Tumor Susceptibility in Organ Transplant Recipients

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Abstract Purpose: Nonmelanoma skin cancer incidence is enhanced >50-fold in patients taking antirejection drugs (ARD) following organ transplantation. Preclinical studies suggest that ARD treatment increases transforming growth factor-β1 (TGF-β1) levels, which contribute to enhanced tumor susceptibility independent of the immunosuppressive effects of ARDs. This study investigates whether TGF-β signaling is elevated in transplant patients.

Experimental Design: Immunohistochemical tissue microarray analysis was used to determine the levels of TGF-β1, TGF-β2, TGF-β3, TβRII, and activated P-Smad2/3 and P-Smad1/5/8, which are phosphorylated directly by distinct TGF-β/BMP receptor complexes. We analyzed >200 cutaneous lesions and adjacent nonlesional skin samples from 87 organ transplant recipients, and 184 cutaneous lesions and adjacent skin samples from 184 individuals who had never received ARDs.

Results: We found significantly higher levels of P-Smad2 in both nonlesional and lesional tissue from transplant recipients compared with those not exposed to ARDs (P ≤ 0.001). In contrast, P-Smad1/5/8, a marker of activation of the bone morphogenetic protein signaling pathway, was generally not expressed at higher levels in patients taking ARDs, including analysis of nonlesional skin, actinic keratoses, carcinoma in situ, or squamous cell carcinoma but was differentially expressed between keratoacanthoma from transplant recipients compared with those from non-transplant recipients (P ≤ 0.005).

Conclusions: Observation of elevated P-Smad2 levels in transplant recipients is consistent with the notion that elevated TGF-β signaling may contribute to malignancy in organ transplant recipients. Disparate P-Smad1/5/8 expression levels between keratoacanthoma from the two patient groups might reflect the distinct BMP-responsive cell of origin for this hair follicle–derived lesion. (Clin Cancer Res 2009;15(16):5101–7)

Organ transplantation, pioneered in the 1960s, is now a routine and widespread procedure for individuals with chronic diseases of the kidney, heart, liver, lung, and other organs. Forty years of experience has revealed the sinister side effects of long-term treatment of organ transplant recipients (OTR) with antirejection drugs (ARD), i.e., a vastly elevated risk of malignancy (1, 2). The most common malignancy of OTRs is nonmelanoma skin cancer (NMSC), with elevated risk factors of 39-fold to 100-fold in patients of European descent. Increased risk is greater for squamous cell carcinoma (SCC) than for basal cell carcinoma, with a shifting of the usual basal cell carcinoma/SCC ratio from 3:1 to 1:2 (3). NMSC in OTRs is often accompanied by increased numbers of warts and premalignant actinic keratoses. OTRs frequently develop multiple skin malignancies, and these have been reported to be more invasive than SCCs of non-OTRs and are more frequently locally recurrent after excision (4, 5). In ~10% of cases, SCCs in OTRs are metastatic, thus representing a significant health burden (1, 2).
Translational Relevance

Organ transplant recipients (OTR) have a >50-fold increased risk of developing skin malignancies, particularly invasive squamous cell carcinoma, which is a major health burden in this population. Preclinical studies have suggested that antirejection drugs increase transforming growth factor-β1 (TGF-β1) and TGF-β signaling levels, which might contribute to this elevated cancer risk. Here, we show that TGF-β signaling via P-Smad2 is indeed elevated in nonlesional skin and squamous carcinoma of OTRs. Several specific TGF-β-inhibitory drugs are now in clinical trials for various applications. Moreover, the established antihypertensive drug, losartan, has been shown to reduce circulating TGF-β levels. It might be useful to treat premalignant cutaneous lesions with topical TGF-β inhibitors in order to reduce skin malignancy, whereas avoiding the possible side effects of systemic drug delivery. It might even be considered worthwhile to administer losartan for difficult to manage OTRs with many squamous cell carcinomas.

There has been considerable debate as to the causes of elevated NMSC risk in OTRs. Most SCCs from both non-OTRs and OTRs are initiated by UV irradiation, but exposure to sunlight potentiates risk for SCC in OTRs, with an elevated relative risk of 48-fold in those with high previous sun exposure versus only 2.4-fold in OTRs with low previous sun exposure (6). Immunosuppression may enhance tumorigenesis by reducing resistance to infection by human papilloma virus (7). It has been difficult, however, to study the viral contribution to excess NMSC risk in OTRs because of the generally high incidence of detectable human papilloma virus DNA even in normal skin of non-OTRs. Nevertheless, the theory that viral infection is a major factor in elevated cancer risk in OTRs is supported by the spectrum of tumor types, other than NMSC, that are prevalent in this patient population, i.e., those known or suspected to have a viral etiology (8, 9). Immunosuppression may also act directly to reduce tumor immune surveillance, thus supporting malignant tumor outgrowth independent of viral status (10). Nevertheless, not all immunosuppressive drugs enhance cancer susceptibility in experimental models (11, 12). Indeed, the newer drugs, sirolimus (Rapamycin) and FTY720, have been shown to be tumor-suppressing rather than tumor-promoting (11–15).

It has been suggested that elevated transforming growth factor-β1 (TGF-β1) levels might contribute to the tumor-promoting action of ARDs, independently of the potent immunosuppressive effects of these drugs (16, 17). TGF-β modulate many cellular processes and TGF-β1, in particular, is a critical regulator of tissue homeostasis in the adult. These secreted cytokines exert their biological effects by binding to a cell surface heteromeric complex of type I and type II TGF-β receptors, TβRI and TβRII. Engagement of TGF-β with TβRII results in phosphorylation and activation of TβRI and consequent activation of receptor-associated Smads (R-Smads), Smad2 and Smad3, by phosphorylation of their carboxyl termini. The canonical TGF-β signaling pathway involves the nuclear translocation of P-Smads within a hexameric complex with other R-Smads, together with nuclear shuttling Smad4 (18). TGF-β can also activate non-Smad signaling pathways, such as the mitogen-activated protein kinase and c-Jun-NH₂-kinase pathways, both indirectly and directly via TβRI and/or TβRII (19).

In the current study, we examined endogenous markers of active TGF-β signaling, specifically, levels of phosphorylated R-Smads, in tumor tissue and adjacent normal skin from OTRs and non-OTRs. Our findings support the hypothesis, generated from numerous preclinical studies, that potentiation of TGF-β signaling in response to ARDs might contribute to elevated SCC incidence in human OTRs.

Materials and Methods

Human tissue samples. This study was conducted according to the principles of the World Medical Association Declaration of Helsinki, and was approved by the Institutional Review Board on Human Research at University of California at San Francisco, San Francisco, California. Clinical samples from surgical tumor excisions were obtained from patients after informed consent. All OTRs for whom there were drug histories had been on a drug regimen of cyclosporine and/or azathioprine and/or prednisone and/or mycophenolic acid and/or FK506, as illustrated in Fig. S1. None of the patients had received extensive treatment with sirolimus or with FTY720 alone. Two hundred and twenty-three skin lesions: 15 actinic keratoses, 34 keratoacanthomas, 96 SCC, and 77 carcinoma in situ (CIS) from 87 OTRs were compared with 184 skin lesions: 15 actinic keratoses, 41 keratoacanthomas, 87 SCC, and 41 CIS from 184 non-OTRs. Of these samples, 181 were represented by more than one independent core on the array. One hundred and eighty-four samples were excised with sufficient adjacent nonlesional skin for analysis, consisting of 74 samples from 30 OTRs, and 110 samples from 105 non-OTRs.

Preparation of tissue microarray. Tumor type was confirmed by H&E staining of formalin-fixed, paraffin-embedded sections. Cylindrical cores (0.6 mm) from each region of interest were arranged randomly with respect to tissue type and patient group within the tissue microarray block. Sections (5 μm) harboring 200 to 300 punches per microscope slide were cut onto Super Frost plus slides (Fisher Scientific).

Antibodies. The following commercially available primary antibodies were used for immunohistochemical staining: α-TGF-β1 (TB21, Anogen) at 1:100 dilution, α-P-Smad1/Smad5/Smad8-Ser463/465 (Cell Signaling) at 1:10 dilution, α-P-TβRII (Santa Cruz Biotechnology) at 1:250, α-P-Smad2/3 Ser465 (Santa Cruz Biotechnology), and α-TGF-β2 (Santa Cruz Biotechnology) at 1:125 dilution. A polyclonal antipeptide antibody raised against amino acids 50 to 60 of mature TGF-β3 was kindly provided by Kathy Flanders (20), and used at a 1:450 dilution.

Immunohistochemistry. Tissue sections were deparaffinized in xylene and rehydrated through a series of ethanol solvents. Endogenous peroxidase was blocked by incubation in DAKO dual endogenous enzyme block. Antigen retrieval was done by heating in 0.01 mol/L of citrate buffer (pH 6), for 6 min at 121°C. Primary antibody was diluted in DakoCytomation antibody diluent, and applied to sections for 1 h at room temperature. Slides were then washed twice for 5 min each in TBS with 0.01% Tween 20, and treated with DAKO EnVision+ dual-link peroxidase system for 30 min at room temperature, followed by development using the DAKO chromagen system for 10 min, and counterstaining in hematoxylin for 1 min.

Scoring of tissue cores. Microarrays were examined using an Olympus BX60 microscope with UPlanFl 200/0.50 ocular, and images were captured using an Olympus DP71 camera with DP controller software version 3.1.1.267 (Olympus, Japan). Scoring of each immunohistochemical stain...
was done independently by two investigators who were blinded to patient category. Tissue cores were scored by staining intensity within the epithelial tissue. If expression was heterogeneous, then the assigned score was that observed in ≥75% of the epithelium. Within each immunohistochemical staining experiment ~80% to 90% of tissue cores were informative, with 10% to 20% being lost from the slide during processing. P-Smad2, P-Smad2/3, and P-Smad1/5/8 staining were scored for nuclear intensity, and TGF-β1, TGF-β2, TGF-β3, and TβRII were scored for cytoplasmic/membrane intensity, with numerical scores of 0, 1, 2, and 3 representing negative, low, medium, and high staining intensity, respectively.

**Statistical analyses.** Statistical analyses were undertaken using Stata v10.0 or R.6 Numerical scores from replicate cores were shown to be fairly concordant using a weighted κ test (weighted κ values: P-Smad2 = 0.33, P-Smad2/3 = 0.33, P-Smad1/5/8 = 0.36, TGF-β1 = 0.17, TβRII = 0.25, TGF-β2 = 0.28, and TGF-β3 = 0.32). This is considered highly significant (all P < 10^-5), especially considering tumor heterogeneity. Scores from these paired cores were averaged such that each tumor or skin sample only contributed one observation for subsequent statistical analysis.

Scores from at least two independent investigators blinded to sample identities were shown to be concordant using a weighted κ test. A score of 0.21 to 0.4 indicates fair concordance, 0.41 to 0.6 indicates moderate concordance, and 0.61 to 0.8 indicates substantial concordance.

Age distributions between the non-OTR and OTR groups were compared by standard t test. The difference in staining intensities between non-OTR and OTR groups was assessed using Kruskal-Wallis rank sum test. Statistical significance was set at P < 0.05. The effect of age on P-Smad2 intensity in SCC tumors was assessed by locally weighted linear regression (LOWESS).

**Results**

**Elevated-activation of P-Smad2 in cutaneous lesions of OTRs.** Levels of nuclear localized R-Smads have been directly correlated with the magnitude of transcriptional responses to TGF-β signaling (21). Therefore, to determine whether elevated TGF-β signaling might be associated with enhanced rates of NMSC in OTRs, we examined nuclear phosphorylated R-Smads within skin lesions from OTRs and non-OTRs (Fig. 1). First, we used an anti–P-Smad2/3 antibody raised against the phosphorylated carboxyl-terminal peptide of Smad3 that recognizes the activated, phosphorylated forms of both Smad2 and Smad3. Within all the lesions examined, there was a trend towards higher levels of P-Smad2/3 staining in cores from OTRs compared with those from non-OTRs which was significant for keratoacanthoma and SCC (Fig. 2A).

To validate and extend these findings, a second antibody specific to P-Smad2 was used to assess P-Smad2 nuclear expression levels (Fig. 1). P-Smad2 staining showed the same trend as that seen with the anti–P-Smad2/3 antibody, i.e., higher staining in OTR compared with non-OTRs lesions. However, the differential was even greater than that for P-Smad2/3 in CIS, keratoacanthoma, and SCC (Fig. 2).

**Elevated activation of P-Smad2 in nonlesional skin from OTRs.** To determine whether the increased expression of P-Smad2 was specific to lesional tissue, or was a generalized cutaneous effect, nonlesional skin adjacent to keratoacanthoma, CIS, and SCC were examined for levels of staining for these markers. Indeed, the nonlesional skin from OTRs had higher staining than that of non-OTRs for both P-Smad2/3 and P-Smad2 (Fig. 2), suggesting a systemic effect of ARDs.

Importantly, elevated P-Smad2 and P-Smad2/3 levels in both lesional and nonlesional tissues of OTRs compared with non-OTRs was in striking contrast to data obtained from the same tissue microarrays stained with >20 other antibodies,7 including those for TGF-β1, TGF-β2, TGF-β3, TβRII, and P-Smad1/5/8 (see Figs. 3 and 4; Fig. S4).

**P-Smad2 expression levels in SCC do not correlate with age, gender, or sun exposure.** Studies have shown that Smad2 expression is altered by several variables, including patient gender and age, or body site of the cutaneous lesion (22–24). In this study, we found no differential expression of P-Smad2 in SCCs excised from sun-exposed sites compared with those from sun-protected sites, no correlation between gender and P-Smad2 intensity and P-Smad2 staining did not correlate with patient age in either of the two patient groups (Fig. S2). In conclusion, the only significant factor correlating with P-Smad2 activation was transplant status (Table S1).

**TGF-β1 and TβRII immunostaining are not enhanced in response to ARD therapy.** Given the increased activation of Smad2 in cutaneous samples from the OTR population and the previous observations of enhanced circulating plasma TGF-β1 levels in response to ARDs (25–27), we investigated whether OTRs had increased immunostaining for the ligand, TGF-β1, and its receptor, TβRII (Fig. 1). In contrast to the observed increase in Smad2 activation in OTRs versus non-OTRs, we found no increase in TGF-β1 levels between these groups, if anything, there was a trend for decreased staining, which was not significant (Fig. 3A). We then examined the expression levels of the other TGF-β ligands, TGF-β2 and TGF-β3 (Fig. S4), to determine whether these might account for increased pSmad2 in SCC from OTRs. However, both ligands showed the same staining intensities in OTRs and non-OTRs. TβRII showed a slight but significant reduction in expression in SCCs of OTRs compared with non-OTRs (P = 0.03; Fig. 3B), but in no other lesional type, nor in nonlesional skin.

**The BMP/Smad pathway is activated by ARDs in keratoacanthoma.** The BMP-Smad1/5/8 pathway represents another arm of intracellular signaling of the TGF-β superfamily, initiated by BMPs and acting via specific BMP type I and type II receptors (21). Because the TGF-β and BMP signaling pathways are intimately linked, particularly through Smad4, and these two signaling pathways often inhibit one another (21), we investigated the possibility that ARD-stimulated skin tumors show alterations in BMP signaling.

α-P-Smad1/5/8 staining in tissue arrays (Fig. 1) was very similar between OTRs and non-OTRs when comparing actinic keratoses, CIS, SCC, and nonlesional skin (Fig. 4), suggesting that ARD-induced NMSC involves the induction of the TGF-β/Smad2 pathway, but not the BMP-Smad1/5/8 pathway. In contrast, keratoacanthoma from non-OTRs had a notably lower level of nuclear P-Smad1/5/8 staining compared with other lesional types and with nonlesional skin (P = 0.0006), although this effect was overridden by ARDs in OTRs (Fig. 4).

**Discussion**

The major conclusion from this study is that patients on long-term ARD therapy have increased P-Smad2 signaling

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6 http://www.r-project.org

7 K. Ridd and B. Bastian, personal communication.
within benign keratoacanthoma, CIS, malignant SCC, as well as in adjacent nonlesional skin. Although a tumor-suppressive role for this enhanced signaling pathway in OTRs cannot be excluded, this observation, together with a wealth of preclinical data on TGF-β activities in skin tumorigenesis, would support a role for activation of the TGF-β/Smad2 pathway in outgrowth and progression of SCC in OTRs (16). In mouse skin, overexpression of TGF-β1 acts as a suppressor of the early outgrowth
of benign tumors (28). As carcinomas progress, oncogene activation blunts this negative growth response and tumor cells acquire enhanced cellular plasticity in response to increased TGF-β, resulting in an elevated incidence of NMSC and a more invasive phenotype (28, 29). TGF-β also acts on the tumor microenvironment to stimulate tumor progression (30). The hypothesis that ARDs might enhance tumorigenesis by increasing TGF-β1 levels is therefore plausible.

In a severe combined immunodeficient-beige mouse model, injection of anti–TGF-β blocking antibodies inhibited a CsA-stimulated increase in the number of metastases resulting from injection of A549 adenocarcinoma cells (16). Because the host mice lacked both T cells and natural killer cells, it was concluded that this was a direct effect of TGF-β acting on the tumor cell per se rather than on the immune system. This interpretation was supported by the fact that A549 cells treated with CsA in vitro undergo morphologic changes characteristic of invasive cells, and that these changes are completely prevented by the use of TGF-β blocking antibodies (16). Similar findings have been made in a rat model of metastatic colon cancer using a small molecule inhibitor of TβRI (31).

In contrast to earlier reports on SCC from non-OTRs (32–34), we found that the majority of SCC from both non-OTRs and OTRs showed active P-Smad2 signaling. The reason for this discrepancy between previous studies and ours could be multifold. Guasch et al. (34) restricted their studies to genital SCC, which might have a very different etiology from that of cutaneous SCC. Other studies used antibodies against total Smads rather than P-Smads (33), or used smaller sample sizes (32). Nonetheless, consistent with findings by Hoot et al. (33), P-Smad2 staining was slightly but significantly (P = 0.005) decreased in SCC compared with normal adjacent skins of non-OTRs (Fig. 2; Fig. S3), an effect that was overridden in OTRs.

In contrast to P-Smad2, the staining intensities of TGF-β1 and TβRII were relatively unaffected by OTR status, if anything, they showed a tendency for decreased levels in SCC from OTRs. This observation, regarding ligand level, is surprising in light of several reports of increased systemic TGF-β1 levels in response...
to ARDs in humans (25–27, 35) as well as in mice (16, 17, 31). It might be that examination of steady-state levels of the ligand by immunohistochemistry is misleading. In vivo, once activated, TGF-β1 is very rapidly cleared (36), as such, TGF-β1 protein levels might seem depleted at the sites of activation. Similarly, with respect to decreased TpRII staining in OTRs, the activation of TGF-β receptors results in their degradation via the endosomal pathway (37). It is therefore conceivable that rapid receptor turnover during chronic activation might lead to lower immunohistologically detectable levels of TpRII in OTRs, despite enhanced signaling.

It is possible that other TGF-β superfamily ligands that activate P-Smad2 may be elevated in OTRs. TGF-β2 and TGF-β3, which act through the same TpRI/TpRII complex, were ruled out by immunostaining. However, activins, nodal, and myostatin can also activate P-Smad2 via their own distinct serine threonine receptor kinase complexes (38). Another possibility is that increased nuclear localization of P-Smad2 may be caused by changes in nuclear shuttling and turnover of this signaling molecule. P-Smad2 shuttling and stability is regulated by interaction with other transcription factors and by ubiquitylation (18, 21), as well as by phosphorylation of the centrally located Smad “linker region”, for example by the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway (39, 40). Activation by TGF-β1/TpRII is therefore not required in order to explain the elevation of P-Smad2 levels. Nevertheless, reports of elevated systemic TGF-β1 levels in response to ARD treatment (25–27, 41) would suggest that this ligand contributes some component to enhanced P-Smad2 activation.

The observation of reduced levels of the activated BMP pathway components in keratoacanthoma compared with adjacent skin of non-OTRs, and the increased activation of BMP-Smads in keratoacanthoma in response to long-term ARD therapy, is particularly intriguing. Keratoacanthoma are benign lesions that are thought to arise from the hair follicle (42). It is well established that the BMP signaling pathway plays a major role in follicular stem cell maintenance and the adult hair follicle cycle (43–45). BMP ligands, BMP2, BMP4, and BMP6, in particular, are known to be elevated in the stem/progenitor niche of the hair follicle, in which they signal through the BMPR1A receptor to limit stem cell expansion and to induce hair cell differentiation (43, 44, 46). Horsley et al. (44) recently showed that BMPs maintain bulge stem cells through the transcriptional activation of NFATc1, which acts to limit stem cell proliferation. NFATc1 activation and nuclear localization is regulated by the phosphatase, Calcineurin, which in turn is inhibited by the most commonly used ARD, CsA. Thus, ARDs would release stem cells from the BMP-NFAT1c-mediated proliferative constraint. The activation of BMP signaling seen within keratoacanthoma from OTRs compared with non-OTRs may therefore result from preferential expansion of a BMP-responsive follicular stem/progenitor compartment that occurs when the consequent downstream negative growth response is short-circuited by CsA inhibition of NFATc1 activity.

In conclusion, this study, together with a wealth of published preclinical data, suggests that ARD-enhanced TGF-β signaling might contribute to enhanced skin malignancy in the OTR patient population. Several specific TGF-β–inhibitory drugs are now in clinical trials for various applications (47), and the established antihypertensive drug, losartan, has been shown to reduce circulating TGF-β1 levels (48, 49). Such drugs should be used with caution due to possible inhibition of the tumor-suppressive arm of TGF-β signaling that could potentially result in more benign lesions, such as keratoacanthoma. However, moderate down-regulation of excessive P-Smad2 levels in OTRs by the use of topical TGF-β inhibitors (50) might be useful to reduce cutaneous malignancy and further tumor progression. It might even be considered worthwhile to administer losartan for difficult to manage OTRs with many SCCs.

Disclosure of Potential Conflicts of Interest

R.J. Akhurst has received a commercial research grant from Eli Lilly and Company.

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