mTOR Inhibition by Everolimus Does Not Impair Closure of Punch Biopsy Wounds in Renal Transplant Patients

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Background. Mammalian target of rapamycin (mTOR) inhibitors are approved to prevent allograft rejection and control malignancy. Unfortunately, they are associated with adverse effects, such as wound healing complications that detract from more extensive use. There is a lack of prospective wound healing studies to monitor patients treated with mTOR inhibitors, such as everolimus or sirolimus, especially in nondiabetics. Methods. Patients receiving everolimus with standard immunosuppressant therapy or standard immunosuppressant therapy without everolimus were administered 3-mm skin biopsy punch wounds in the left scapular region. Homeostatic gene expression was examined in the skin obtained from the biopsy and wound surface area was examined on day 7. Peripheral blood mononuclear cells were examined for cytokine production. Results. There are no significant changes in autophagy related 13, epidermal growth factor, insulin-like growth factor binding protein 3, IL-2, kruppel-like factor 4, and TGFβ1 gene expression in the skin suggesting that there is little impact of everolimus on these genes within nonwounded skin. Peripheral blood T cells are more sensitive to cell death in everolimus-treated patients, but they retain the ability to produce proinflammatory cytokines required for efficient wound repair. Importantly, there is no delay in the closure of biopsy wounds in patients receiving everolimus as compared to those not receiving mTOR inhibition. Conclusions. Everolimus treatment is not associated with impaired closure of skin biopsy wounds in kidney transplant recipients. These data highlight the importance of exploring whether larger surgical wounds would show a similar result and how other factors, such as diabetes, impact wound healing complications associated with mTOR suppression.

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Unfortunately, less is known about wound healing complications associated with these drugs. In contrast to sirolimus, results from a retrospective analysis of 3 multicenter clinical trials found that de novo everolimus treatment has no statistical increase in adverse wound healing events at doses of 1.5 mg/d and below. Higher everolimus doses of 3 mg/d did show an increase in the adverse wound healing events suggesting the amount of mTOR suppression is correlated with complications. At this point, all of the published studies examining wound healing in patients receiving mTOR targeted therapy have been retrospective and/or rely on patient reported adverse events. As of yet there have been no studies that monitor the closure of comparably sized wounds on patients prescribed mTOR inhibitors. In addition, it is important to initially focus on nondiabetic patients to independently assess the impact of mTOR inhibition on wound closure.

The objective of this study was to determine whether everolimus impairs the closure of biopsy wounds in kidney transplant recipients. Patients receiving everolimus with standard immuno-suppressant therapy (EVR) or standard immuno-suppressant therapy without everolimus (STD) were administered 3-mm skin biopsy punch wounds and wound closure was monitored 7 days later. In addition, complications associated with wound closure were reported. mTOR signaling regulates many key cellular processes including autophagy, growth factor production and proliferation that are important in maintaining skin homeostasis. We examined nonwounded skin for expression of the following genes: kruppel-like factor 4 (KLF4) (keratinocyte differentiation), autophagy-related 13 (ATG13) (autophagy), IGFBP3, epidermal growth factor (EGF), TGF-β (growth factor production) and IL-2 (T-cell function). Last, peripheral T-cell survival, activation and function were assessed to identify the impact of mTOR inhibition on αβ and γδ T-cell populations that play roles in the prevention of skin infection and participate in tissue repair. Together this study examines the local and systemic impact of everolimus treatment on the skin and the healing of biopsy wounds.

### TABLE 1

Demographic characteristics, disease characteristics, and percentage of wound closure of participants by assigned drug group (N = 40)

| Total (N = 40) | Everolimus (N = 20) | Control (N = 20) | P |
|----------------|---------------------|------------------|---|
| Age (mean), y  | 44.5                | 50.8             | 38.3 | 0.02 |
| Male           | 29 (72.5%)          | 16 (80%)         | 13 (65%) | 0.28 |
| Ethnicity      |                     |                  |     | 0.003 |
| Hispanic       | 18 (45%)            | 5 (25%)          | 13 (65%) |     |
| White          | 15 (37.5%)          | 12 (60%)         | 3 (15%) |     |
| Asian          | 4 (10%)             | 3 (15%)          | 1 (5%) |     |
| African American| 2 (5%)             | —                | 2 (10%) |     |
| Other          | 1 (2.5%)            | —                | 1 (5%) |     |
| BMI (%, mean)  | 27.1%               | 26.4%            | 27.7% | 0.38 |
| Cause of Disease |                   |                  |     | 0.67 |
| Hypertension   | 21 (52.5%)          | 12 (60%)         | 9 (45%) |     |
| G.D.           | 15 (37.5%)          | 6 (30%)          | 9 (45%) |     |
| Other          | 4 (10%)             | 2 (10%)          | 2 (10%) |     |

* P value for t test for difference in numeric variables and χ² test for categorical variables to test the difference between drug groups or ANOVA for more than 2 levels in categorical variables.

* One patient did not get the follow-up measurement done.

ANOVA, analysis of variance.
CoolPix S3300) to monitor wound closure kinetics on days 0 and 7 with the exception of 5 subjects that had images acquired on day 9 because they were unable to return on day 7. Wound surface area was measured and quantified using ImageJ software (NIH). Day 0 represents 100% of the original wound size. The percentage of wound closure was calculated. Since 2 wounds were generated on each patient, data was first analyzed using the mean of the 2 wounds and then data was analyzed using a random number generator to choose a wound from each patient for analysis. Similar results were obtained with either of these statistical analyses. 34 patients were included in these analyses, as 1 patient failed to return for wound imaging and 5 patients delayed their return visit for imaging until day 9 postwounding.

**Quantitative Polymerase Chain Reaction**

Skin taken from the 3-mm punch biopsies at day 0 was stored at −80°C. The biopsies (30 mg) were incubated in RNAlater-ICE (Life Tech, Carlsbad, CA) according to manufacturer’s protocol. Skin biopsies were manually homogenized and RNA purified using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Quality control quantification of RNA was performed using a NanoDrop 1000. RNA from 7 EVR patients and 7 STD patients were selected for their high purity and integrity and stored at −20°C for further examination of gene expression. The RNA samples (13.05 ng) were converted to cDNA using iScript cDNA Synthesis Kit (BioRad, Hercules, CA) as suggested by the manufacturer and stored at −20 °C. SsoAdvanced Universal SYBR Green SuperMix was used in conjunction with PrimePCR Assay Panels for Real-Time polymerase chain reaction (PCR) and Digital PCR (BioRad). Six genes of interest in skin homeostasis were selected: ATG13, EGF, insulin-like growth factor binding protein 3 (IGFBP3), interleukin 2 (IL-2), KLF4, and TGFβ1. Amplification was performed in duplicate. The amplification protocol

![Graphs showing gene expression](image1.png)

**FIGURE 1.** Expression of genes involved in skin homeostasis by EVR and STD patients is similar. RNA was isolated from the skin of STD (squares) and EVR (circles) patients and qPCR analysis performed. The GOI was normalized to HPRT1 expression. The means are depicted with a line. P values were calculated using Pearson’s chi-squared test and unpaired Student’s t test (*p >0.05). GOI, gene of interest; quantitative polymerase chain reaction, qPCR.
follows that outlined by the manufacturer: 95 °C for 2 minutes, followed by 40 cycles of denaturing at 95 °C for 5 seconds and annealing at 60 °C for 30 seconds. All data was normalized with the housekeeping gene hypoxanthine phosphoribosyltransferase1 (HPRT1). Data were acquired by iCycler Optical Module and analyzed by GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla CA). Samples (2 EVR and 1 STD) that did not exhibit amplification with the housekeeping gene were excluded.

**PBMC Isolation and Staining for Flow Cytometry**

Whole blood (10-15 mL) was obtained from EVR- and STD-treated patients. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque (VWR, Radnor, PA) density gradient centrifugation. Sufficient cell numbers for stimulation and flow cytometry analysis were obtained from 18 EVR and 15 STD patients. Cells were incubated in complete RPMI 1640 containing 10% FCS with or without 5 μg/mL phytohemagglutinin (PHA) (Sigma, St. Louis, MO) for 15 hours at 37 °C. To perform flow cytometry, cells were stained with the following antibodies: CD3 (UCHT1), αβ TCR (IP26), Vδ2 (B6), CD27 (U323), IFN-γ (B27), and CD69 (FN50) (Biolegend, San Diego, CA). Briefly, cells were permeabilized and fixed using the Cytofix/Cytoperm Fixation/Permeabilization Kit per manufacturer’s instructions (BD Bioscience, San Diego, CA). Data were acquired on a LSR-II flow cytometer and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

**Enzyme-Linked Immunosorbent Assay**

Supernatant from PHA-stimulated and unstimulated PBMC from each patient was harvested and stored at −80°C. Measurement of the concentration of total TNF-α, IL-22, and IL-17A was performed with LEGEND MAX ELISA kits with precoated plates (Biolegend) according to the manufacturer’s instructions. Samples were performed in triplicate, except 1 patient was performed in duplicate due to a loss of sample. Data from 11 EVR and 13 STD patients were acquired with a Model 680 Microplate Reader (BioRad) at 450 nm and analyzed by GraphPad Prism 5.0d software (GraphPad Software Inc.).

**Statistical Analysis**

Statistical analyses were performed using SAS version 9.4 (SAS Institute, Inc., Cary, NC), Microsoft Excel (Seattle, WA) and Prism 5.0d software (GraphPad Software Inc.). Pearson’s chi-squared test, unpaired Student’s t test and analysis of variance for polynomial data were performed. Findings were considered significant with a P value less than 0.05 and highly significant with a P value less than 0.001.

**RESULTS**

**Patient Demographics**

A total of 40 patients were enrolled from July 2012 to February 2014. As shown in Table 1, patients that received the assigned drug, everolimus are older than patients in the control group (50.8 and 38.3 years, respectively; P < 0.02). About one third of patients receiving everolimus are white, whereas 32.5% are Hispanic in the control group (P = 0.003). There is no difference in sex, body mass index (BMI), and cause of disease between patients receiving everolimus and control groups.

![FIGURE 2](image)

**FIGURE 2.** Gating strategy for the analysis of PBMC from patients enrolled in the study. PBMC were isolated from STD and EVR patients, cells were either stimulated with PHA for 18 hours (Stim) or left unstimulated (No Stim), stained with antibodies specific for activation markers and T-cell markers, and analyzed using flow cytometry. A, Live cell gate, CD3+ gate, αβ or γδ T-cell gate. B, Live, CD3+, αβ TCR+ cells were analyzed for CD27 and CD69 expression. C, Live, CD3+, γδ TCR+ cells were analyzed for CD27 and CD69 expression.
Patients Treated With Everolimus Do Not Exhibit Altered Expression of KLF4, ATG-13, IGFBP3, EGF, TGF-β, or IL-2 in Nonwounded Skin

RNA was extracted from patient skin samples and examined for quality and quantity. Samples with high RNA quality and quantity were further studied using quantitative PCR. RNA obtained from patients treated with EVR was compared to STD patients. No significant difference in gene expression was observed in everolimus-treated patients when the gene of interest was normalized with HPRT1 (Figure 1). Patients administered everolimus do not exhibit significantly altered KLF4, ATG13, EGF, IGFBP3, or IL-2 gene expression in nonwounded skin. In addition, constitutive TGF-β1 gene expression in the skin is unchanged in everolimus-treated patients. Although these pathways are not impacted, it is possible that other key pathways in skin homeostasis such as adhesion molecule expression or extracellular matrix deposition are modulated.

PBMC Viability and T-Cell Survival After Activation Are Reduced in Everolimus-Treated Patients

Wound healing is a complex process that involves the infiltration of a variety of cell types from the periphery to the site of damage. T lymphocytes from the peripheral blood are recruited to the wound to eradicate pathogens and produce cytokines to skew the immune response toward Th1, Th2, or Th17.12,13 To determine how everolimus impacts T lymphocytes in the peripheral blood of kidney transplant recipients, PBMC were isolated from the blood and examined using flow cytometry. The gating strategy is depicted in Figure 2.

PBMC from patients treated with EVR exhibit reduced cellular viability in the peripheral blood as compared to STD patients (Figure 3). The mean percent of live cells represented 76.1% of cells in control as compared to 66.68% of cells in everolimus-treated patients (P < 0.05). This did not impact the proportion of CD3+ T cells in the PBMC (M = 57.67 control, M = 54.58 everolimus) or the CD3+ αβ/γδ ratios (αβ T cells M = 82.32 control, M = 80.86 everolimus; γδ T cells M = 1.458 control, M = 1.633 everolimus). Upon stimulation of the PBMC with PHA, cellular viability was reduced in both groups likely due to activation induced cell death (Figure 4A). The percentage of PBMC in the live gate from the everolimus-treated patients was more severely impacted upon stimulation (M = 46.46) as compared with the control group (M = 68.56) (P = 0.003).

Peripheral Blood T Cells Produce IFN-γ, TNF-α, and IL-17A to a Similar Degree in Everolimus-Treated Patients and Controls

Activated T cells downregulate the T-cell costimulatory molecule CD27, a Traf-linked TNF receptor family member important for the generation of T-cell memory.14 αβ T cells from patients treated with everolimus exhibit downregulated CD27 expression similar to STD patients (Figure 4, M = 51.86 to 43.86 control, M = 56.74 to 39.99 everolimus). In contrast, γδ T cells in everolimus-treated patients did not exhibit a significant CD27 reduction (Figure 4, M = 44.13 to 24.00 control, M = 35.15 to 29.03 everolimus) suggesting a reduced ability to become fully activated via this costimulatory molecule. Both αβ and γδ T cells are able to upregulate CD69 in response to activation in both STD and EVR patients (Figure 4) suggesting that early activation proceeds normally. It is interesting to note that 4 patients in the EVR group showed constitutive CD69 expression on both αβ and γδ T-cell populations before stimulation, while no STD patients exhibited elevated CD69 levels before activation (Figure 4). This indicates recent T-cell activation in the everolimus-treated patient population.

Th1 αβ and γδ T cells produce IFN-γ upon stimulation to promote cell-mediated immune response and intracellular

FIGURE 3. PBMC from EVR treated patients are less viable than STD, but the proportion of αβ and γδ T cells remains unchanged. Compilation of flow cytometric data gated on (A) Live, (B) Live, CD3+ cells, (C) Live, CD3+, αβ TCR+ cells, and (D) Live, CD3+, γδ TCR+ cells. Each circle represents 1 patient. The mean values are depicted with a line. P values were calculated using Pearson’s chi-squared test and unpaired Student’s t test (P > 0.05).
defense against bacterial and viral pathogen within the host. T lymphocytes from EVR patients upregulated intracellular IFN-γ production upon stimulation with PHA similar to STD patients (Figure 4). The upregulation did not reach significance in the αβ T-cell population, although the percent of IFN-γ+ T cells increased upon activation in each patient. Cytokine secretion was further examined by performing enzyme-linked immunosorbent assay on the supernatants obtained from the stimulated PBMC samples. Both IL-17A and TNF-α were produced upon stimulation, suggesting normal function and differentiation of T_{H1} and T_{H17} populations in EVR patients when compared to STD (Figure 5). Elevated IL-17A production was observed in the EVR patients, but this did not reach significance between the EVR and STD patient populations (Figure 5, M = 45.22 control, M = 64.18 everolimus). IL-22 was produced constitutively by PBMC from both treatment groups similarly regardless of stimulation (Figure 5). Together the

**FIGURE 4.** Peripheral blood T cells from EVR-treated patients are fewer in number upon activation, but still able to express activation markers and produce cytokines. PBMC isolated from EVR or STD patients were stimulated (●) with PHA or left unstimulated (○) and analyzed by flow cytometry. Comparison of the percentage of Live, CD3+, αβ or γδ TCR+ cells before and after stimulation in EVR versus STD groups were analyzed for (B) CD27, (C) CD69, and (D) IFN-γ production. The mean value is depicted with a line. P-values were calculated using Pearson's chi-squared test and unpaired Student's t-test (*P > 0.05, **P > 0.001).
data show that PBMC from EVR patients retain the ability to produce IFN-\(\gamma\), TNF-\(\alpha\) and IL-17A, which are important for clearing infection and healing wounds.

**EVR and STD Patients Exhibit Similar Biopsy Punch Wound Closure on Day 7**

Three-millimeter biopsy punch wounds were performed on renal transplant recipients receiving a standard immunosuppressant regimen with or without everolimus. Digital images were acquired at the time of wounding and at a follow-up appointment on day 7 postwounding as this is a critical juncture in wound repair when the inflammatory phase has ended, the proliferative phase is ongoing, and the maturation phase is initiating. EVR patients (M = 33.3%) exhibited a similar percentage of their wound closing after 7 days as STD patients (M = 33.5%) (Figure 6). These results suggest that patients treated with everolimus exhibit normal closure of biopsy wounds during the first week of healing. This does not exclude the possibility that later stages of wound repair are delayed; however, none of the patients in our study exhibited adverse or severe adverse wound healing events. In addition, BMI was not a significant predictor of poor wound closure during the first 7 days (Figure 6) because the percentage of wound closure at day 7 was similar in low and high BMI patients regardless of everolimus administration. It is interesting to note that the patient with the highest BMI (43.7) and the oldest patient (age 73 years old) had larger wounds at day 7 as compared to the group average regardless of everolimus treatment. Additionally, there was no significant correlation between everolimus levels and wound closure in patients that received the drug.

**DISCUSSION**

The major goal of immunosuppressive therapy is to alleviate graft rejection while retaining immunity to pathogens and the capacity to repair tissue. Sirolimus is an mTOR inhibitor, which has been shown to cause adverse effects including wound healing complications when compared to patients who are not taking mTOR inhibitors.\(^{16-18}\) We have performed a study to precisely monitor wound healing real time in patient-administered everolimus, a derivative of sirolimus. Our study suggests that punch biopsies can be safely

![Graphs showing wound closure](image)

**FIGURE 5.** PBMC from patients treated with EVR produce Th1 or Th17 cytokines upon PHA stimulation. (A) IL17A (B) IL 22 and (C) TNF-\(\alpha\) were analyzed from the supernatant of PHA stimulated PBMC from EVR or STD groups. The mean is depicted with a line. \(P\)-values were calculated using Pearson’s chi-squared test and unpaired Student’s \(t\) test (*\(P > 0.05\)).

**FIGURE 6.** Closure of biopsy wounds is similar between EVR and STD groups. A, Images of the wounds were acquired on days 0 and 7. Image J was used to determine the change in surface area defined as the % wound closure. B, The percent of wound closure was compared between EVR and STD and lean (<30) BMI versus high (>30) BMI. The mean is depicted with a line. \(P\) values were calculated using (A) Pearson’s chi-squared test and unpaired Student’s \(t\) test and (B) ANOVA for polynomial data (*\(P > 0.05\)). ANOVA, analysis of variance.
performed in renal transplant recipients treated with everolimus at 2.5 mg. It is unclear whether these findings can be correlated to surgical wounds which would be larger and deeper. Early studies using sirolimus doses in the 5 to 15 mg range showed surgical wound complications. However, a recent post hoc analysis of patients in the SCHEDULE trial found no difference in adverse wound healing events between patients on everolimus as compared to cyclosporine. Similarly, an analysis that pooled the data from 3 clinical trials found no increase in wound healing events in patients with everolimus at 1.5 mg compared with MPA, whereas patients treated with everolimus at 3 mg did have more wound healing events than MPA. At this point, it is important to differentiate the roles that inhibitor dose and derivative use play in wound healing complications.

Patient reporting and retrospective studies are heavily used to examine associations between medications and wound repair complications. Although there are advantages to these methods including the ability to analyze data from a large number of patients, there are key disadvantages that can lead to misleading interpretations. First of all, the patient may not report all adverse events. Second, if the study is run through a particular clinic, the patient may go elsewhere for wound care. Third, complicating factors such as wound size, time of wound acquisition, and treatment received are not controlled. To avoid these disadvantages, our study carefully monitored the association of drug with wound closure over a particular window of time, for a particular size of wound, at a particular site on the body.

T lymphocytes use mTOR signaling for functions associated with allograft rejection, but also for normal homeostasis, tissue repair and pathogen eradication. mTOR inhibition alters T helper cell differentiation, lymphocyte migration, and morphology change. These changes in T helper cell behavior can impact the peripheral and local T-cell populations. In this study we show that peripheral blood T lymphocytes within everolimus-treated patients are present and have a similar ability to produce cytokines as in STD patients. T cells from EVR patients were able to produce the proinflammatory cytokines IFN-γ, TNF-α, and IL-17A suggesting mTORC1 function is preserved. However, in everolimus-treated patients the T lymphocytes are significantly less viable upon PHA mitogen stimulation, suggesting T cells are becoming sensitive to activation induced cell death. This provides further evidence that mTOR regulates cell survival and reception of growth factor signals.

Chronic nonhealing wounds represent a major clinical problem with a prevalence of 2% in the United States. Open wounds can be even more dangerous for immuno-suppressed patients as such transplant recipients who are more susceptible to infection. Although the sample size in this study is small, wound closure was analyzed in all patients, whereas retrospective studies examine many patients with only a few exhibiting wound healing complications. Future studies should provide a direct comparison of wound healing in patients treated with sirolimus to patients treated with everolimus at varying doses. In addition, the future inclusion of diabetic patients would be important as they comprise a major group of transplant recipients and are particularly prone to nonhealing wounds.

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