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ORIGINAL ARTICLE

The effects of up to 240 days of tacrolimus therapy on the gingival tissues of rats – a morphological evaluation

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BACKGROUND: Tacrolimus, an immunosuppressive drug used in organ transplantation, has been reported not to induce gingival overgrowth. However, prevalence studies are limited, and the methods used for assessing gingival overgrowth varies among studies.

OBJECTIVE: The purpose of this study was to evaluate the effects of up to 240 days of tacrolimus therapy on gingival tissues of rats.

MATERIALS AND METHODS: Rats were treated for 60, 120, 180 and 240 days with daily subcutaneous injections of 1 mg/kg body weight of tacrolimus. After histological processing, the oral and connective tissue, volume densities of fibroblasts (V_f), collagen fibers (V_{cf}) and other structures (V_o) were assessed in the region of the lower first molar.

RESULTS: After 60 and 120 days of treatment with tacrolimus, gingival overgrowth was not observed. The gingival epithelium, connective tissue, as well as the values for V_f, V_{cf}, and V_o were similar to those of the control rats ($P > 0.05$). After 180 and 240 days of the treatment, gingival overgrowth was associated with a significant increase in the gingival epithelium and connective tissue as well as an increase in the V_f and V_{cf} ($P < 0.05$).

CONCLUSIONS: Within the limits of the experimental study, it may be concluded that the deleterious side effects of tacrolimus on the gingival tissues of rats may be time-related.

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Keywords: tacrolimus; gingival overgrowth; immunosuppression drugs; animal study

Introduction

Introduction of tacrolimus-based immunosuppressive regimens has improved renal transplant survival rates (Scientific Registry of Transplant Recipients, 2003). The pharmacodynamics of tacrolimus are very similar to cyclosporin A (CsA) and the drug has been used successfully as an alternative to CsA to prevent graft rejection and to treatment autoimmune diseases (Faulds *et al*, 1993; Spencer *et al*, 1997), although it may cause side effects such as nephrotoxicity, neurotoxicity, and glucose metabolism disorders. Conversely, hyperlipidemia, hypertension, and hirsutism are less likely with tacrolimus than with CsA (Spencer *et al*, 1997). Unlike CsA, tacrolimus does not appear, however, to induce gingival overgrowth (James *et al*, 2001; McKaig *et al*, 2002; Spolidorio *et al*, 2006), although some authors suggest that the severity of gingival overgrowth seen in patients taking tacrolimus is less than that with CsA (Hernandez *et al*, 2000; James *et al*, 2000; Thorp *et al*, 2000; Radwan-Oczko *et al*, 2004; de Oliveira Costa *et al*, 2006). In contrast, recent reports have described tacrolimus-induced gingival overgrowth in rats (Prabhu and Mehta, 2006), although the dose administered in this case was 1.5 mg/kg, or 50% greater than the clinical dose.

Alterations in tissue metabolism caused by drugs may be dependent on many variables, such as patient gender, dosage and serum level, and concurrent drug therapy (Seymour *et al*, 2000). Another factor that must be considered is the duration of therapy (Montebugnoli *et al*, 2000; Spolidorio *et al*, 2004).

Studies exploring the effects of long-term tacrolimus therapy on gingival tissues are scarce and, certainly, such observations are necessary in experimental models. Therefore, the purpose of this study was to describe the histometry and densities of fibroblasts and collagen fibers in the gingival tissue of rats treated with tacrolimus for different time periods.

Materials and methods

Eighty male Holtzman rats (*Rattus novergicus albinus*), weighing 50 g, were housed under similar conditions in cages with access to food and water *ad libitum*. The animals were randomly distributed into eight groups of 10 animals each. All protocols described below were approved by the Institutional Experimentation

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Committee of the School of Dentistry of Araraquara, Araraquara, São Paulo, Brazil. Four groups were treated with tacrolimus (Prograf®; Janssen Cilag, São Paulo, Brazil), injected subcutaneously in a daily dose of 1 mg/kg body weight (Jiang *et al*, 1991, 1995). Four groups were used as controls and received subcutaneous injections of saline solution during all periods. The experimental periods were 60, 120, 180, and 240 days. According to other authors, this dosage provides plasma peak and trough levels of tacrolimus of approximately 11.2 ng/ml (Li *et al*, 2003; Muramatsu *et al*, 2005; Voggenreiter *et al*, 2005). Control rats from the other groups were daily injected subcutaneously with saline (NaCl 0.9%). All rats were weighed weekly.

Histology techniques
The rats were killed by an overdose of anesthesia (ketamine, Francotar®; Virbac do Brazil Ind. e Com. Ltda, São Paulo, São Paulo, Brazil) at the end of the experimental periods, and the mandibles were carefully removed and soaked in 10% formalin. Decalcification was carried out in Morse solution (50 ml of 50% formic acid and 50 ml of 20% sodium citrate). Serial paraffin sections (5 μm) were made from the bucco-lingual aspects of the whole first left and right lower molar and stained with hematoxylin and eosin. Each first lower molar has a mesial–distal diameter of approximately 1 mm, producing sections of 5 μm each. Histometric and stereological studies were made on the buccal gingiva.

Histometry
Gingival epithelium and connective tissue area measurements were made with the help of a Zeiss microscope at a magnification of 125x using a Sigma computer program (Mocha; Jandel Scientific, San Rafael, CA, USA). From each tooth, 10 measurements were made in sections of 50-μm intervals each. For statistical analysis, the mean from each animal was used, calculated from the 20 measurements obtained from the first right and left molars (Figure 1).

Stereology
Volume densities of fibroblasts (V_f), collagen fibers (V_{cf}) and other structures (V_o), i.e., blood vessels, nerves, and unidentified structures, were estimated according to the principles established by Delesse (1848), which were applied to Weibel's (1974) histology. The count was performed with the help of a Zeiss microscope, using oil immersion at a magnification of 1000X. A square lattice of 25 test points was projected into the microscope ocular, with the use of a microvid system to connect the microscope to a computer. For each animal, 16 sections were selected (eight from the left molar and eight from the right) and 25 points were counted in each section. V_f, V_{cf} and V_o were expressed as percentages of the total points counted.

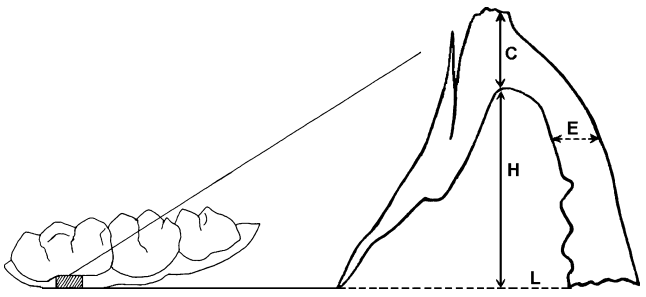

Figure 1 Schematic illustration showing the regions where linear measurements were made. E, epithelium thickness; C, epithelium crest; H, gingival connective tissue height; L, connective tissue width

Statistical analysis
Measurements were expressed as mean and standard deviation. Statistical analyses were made by one-way analysis of variance (ANOVA) and Tukey test.

Results

Histological findings
The gingival tissues of the control rats in all analyzed periods, as well as those of the rats treated with tacrolimus for 60 and 120 days demonstrated normal morphology with keratinized stratified squamous epithelium. The interface between the epithelium and the connective tissue was strongly interdigitated with numerous tall, narrow connective tissue papillae projecting into the epithelium (Figure 2). The connective tissue was dense, and presented fine collagen fibers that were interspersed with delicate vessels and fibroblasts. After 180 and 240 days, gingival overgrowth was observed in all gingival areas, but was more evident on the buccal gingival tissue of the lower molar teeth. The gingival epithelium was hyperplastic, with deep papilla interdigitations. The connective tissue was dense, and demonstrated thick collagen fibers that were interspersed with delicate vessels and fibroblasts. Few inflammatory cells were observed (Figure 3).

Histometric findings
Table 1 shows the linear measurements (μm; mean $\pm$ SD) of the epithelium and connective tissue of the buccal gingiva of the lower first molars of control and treated rats. The gingiva of the control rats, as well as those of rats treated with tacrolimus for 60 and 120 days were similar ($P > 0.05$). On the other hand, the values of the tacrolimus-treated rats were significantly increased at 180 and 240 days of treatment, compared with those of the control, as well as at 60 and 120 days ($P < 0.05$).

Stereometric findings
The stereometric findings of the control groups and of the groups treated with tacrolimus are demonstrated in Table 2. In the control groups, V_f, V_{cf}, and V_o were 11.87%, 66.66%, and 21.47%, respectively, in the buccal gingiva and remained constant in all the periods studied. After 60 and 120 days of treatment, the values of V_f, V_{cf}, and V_o were similar to those of the control groups ($P > 0.05$). There was a significant increase in V_f and V_{cf} at 180 and 240 days ($P < 0.05$), while V_o decreased when compared with those of the control groups ($P < 0.05$).

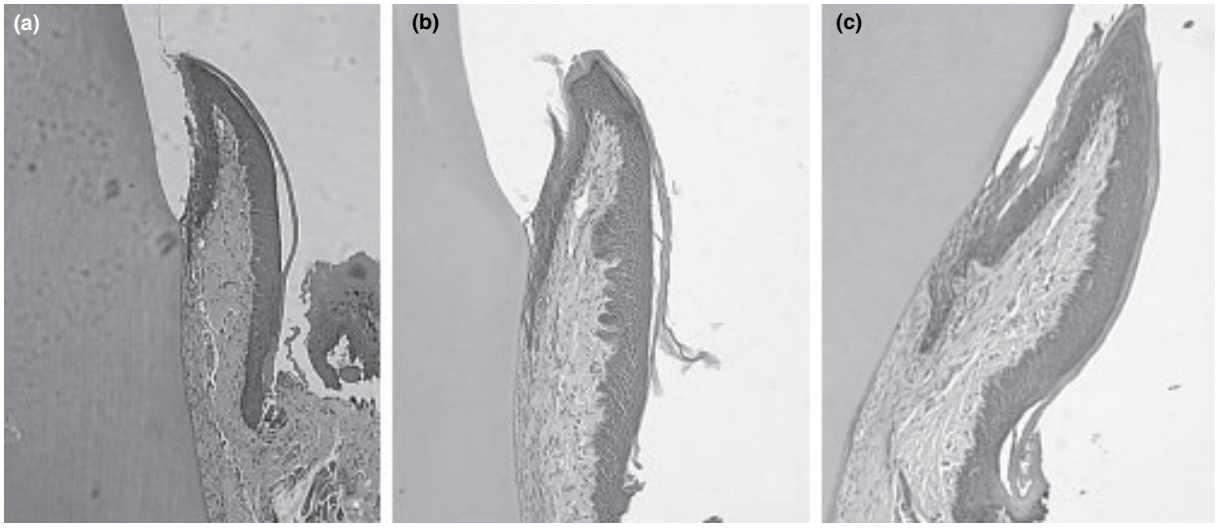

Figure 2 Gingiva of the control rats (**a**) as well as of the rats treated with FK506 for 60 days (**b**) and 120 days (**c**) showing normal morphology (H&E, 200×)

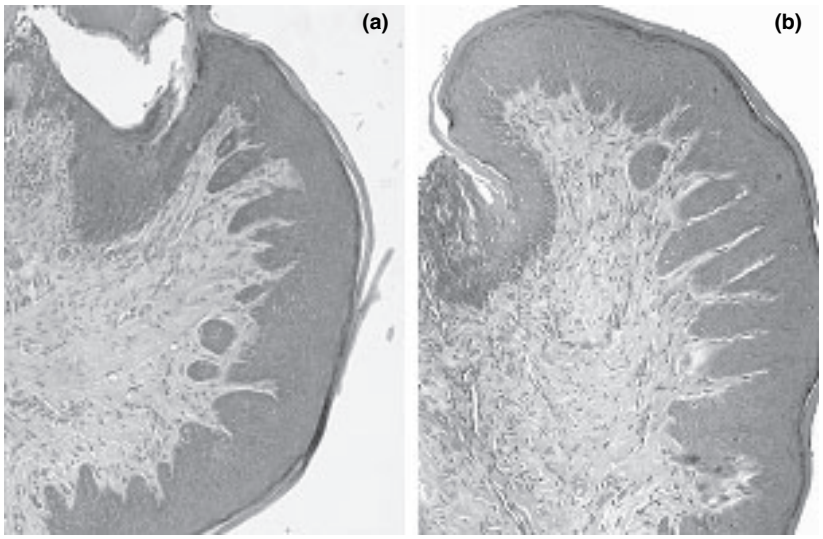

Figure 3 Gingival overgrowth in rats treated with FK506 for 180 days (**a**) and 240 days (**b**) (H&E, 200×)

Table 1 Linear measurements (μm; mean ± SD) of epithelium thickness (E), epithelium crest (C), gingival connective tissue height (H) and connective tissue width (L) of the buccal gingival of first lower molars of normal rats and treated with FK506 in various periods of treatment

	Period (days)			
Treatment	*60*	*120*	*180*	*240*
E				
Control	45.8 ± 0.6	43.3 ± 0.4	48.5 ± 1.4	44.6 ± 0.8
FK506	45.7 ± 1.9 a	43.4 ± 2.0 a	67.5 ± 0.2* b	87.0 ± 0.3* c
C				
Control	46.9 ± 0.4	41.3 ± 0.7	45.7 ± 1.8	47.7 ± 0.7
FK506	46.4 ± 2.1* a	41.2 ± 1.5 b	69.8 ± 0.2* c	69.0 ± 0.2* c
H				
Control	415.9 ± 1.1	414.9 ± 1.2	418.8 ± 0.7	409.7 ± 1.8
FK506	415.7 ± 0.9 a	413.8 ± 0.5* b	493.8 ± 0.7* c	570.8 ± 0.8* d
L				
Control	141.9 ± 1.5	147.4 ± 0.9	166.1 ± 2.3	139.0 ± 0.5
FK506	139.7 ± 0.5* a	145.5 ± 0.7* b	218.5 ± 0.5* c	260.5 ± 0.6* d

Different letters represent statistically significant difference among means in the same group. *$P < 0.05$, statistical significance *vs* control rats in the same period.

Table 2 Volumetric densities of fibroblasts (V_f), collagen fibers (V_{cf}) and other structures (V_o) from the buccal gingival region of the first lower molar in control and FK506 rats

	Period (days)			
Treatment	*60*	*120*	*180*	*240*
V_f				
Control	11.87 ± 0.3	11.01 ± 0.2	10.93 ± 0.4	10.81 ± 0.2
FK506	11.80 ± 1.0 a	11.40 ± 1.1* b	12.40 ± 1.1* c	12.80 ± 1.2* d
V_{cf}				
Control	66.66 ± 0.2	66.70 ± 0.3	65.90 ± 0.2	68.68 ± 0.3
FK506	66.76 ± 1.8 a	66.18 ± 2.3 a	69.00 ± 1.8* b	73.2 ± 1.9* c
V_o				
Control	21.47 ± 0.6	22.29 ± 0.3	20.17 ± 0.3	20.51 ± 0.2
FK506	21.44 ± 1.5 a	21.80 ± 1.9 a	18.60 ± 1.4* b	14.00 ± 1.5* c

Values (%) are presented as mean ± SEM. Different letters represent statistically significant difference among means in the same group. *$P < 0.05$, statistical significance *vs* control rats in the same period.

Discussion

The present study evaluated the effects of up to 240 days of tacrolimus therapy on the gingival tissues of rats. To our knowledge, no previously published study has described the morphometry and the stereology of the gingival tissues of rats treated with tacrolimus for 240 days.

Clinical data have demonstrated that tacrolimus does not induce gingival overgrowth (James *et al*, 2001; McKaig *et al*, 2002; Spolidorio *et al*, 2006) or have suggested that the severity of gingival overgrowth seen in patients taking tacrolimus is less than that with CsA (Bader *et al*, 1998; James *et al*, 2000). Prevalence studies are limited and the methods used for assessing overgrowth and severity vary from study to study (Oettinger-Barak *et al*, 2001; Wondimu *et al*, 2001). On the other hand, there is agreement in the literature with regard to the 'risk factors' of gingival overgrowth. In humans and in animals, various 'risk factors', associated with both the development and expression of drug-induced gingival changes, have been identified and quantified. These risk factors include age, sex, drug variables, concomitant medications, periodontal variables, and genetic factors (Seymour *et al*, 2000). There is evidence that drug-induced gingival overgrowth is more prevalent in young individuals (Allman *et al*, 1994; Bökenkamp *et al*, 1994; Seymour *et al*, 2000). We have recently shown that adult rats do not demonstrate significant gingival alterations when treated with CsA and nifedipine alone (Spolidorio *et al*, 2003). In the present investigation, gingival alterations were not observed in young rats and were seen only in older rats. Another factor that must be considered is the duration of therapy (Montebugnoli *et al*, 2000; Spolidorio *et al*, 2004).

The rat has been extensively used to study the effects of these drugs in the gingiva, and the gingival overgrowth is similar to that in humans. The model is very convenient as rats are small, not expensive, and are easy to handle. The use of a rat model has allowed the strict control of some important variables, such as genetic predisposition, age, dose, and routes of administration of tacrolimus. In fact, the response in rats is more uniform than in humans.

The routes of administration and dosages of tacrolimus that were used in this study gave consistent responses. The dose of 1 mg/kg body weight is sufficient to achieve therapeutic tacrolimus serum levels (Jiang *et al*, 1991, 1995; Akahane *et al*, 1999).This dose is clinically relevant and within the range of doses (0.6–1.0 mg/kg body weight) used in studies of organ and limb transplantation (Kaihara *et al*, 2002; Li *et al*, 2003; Fukunaga *et al*, 2004; Muramatsu *et al*, 2005; Voggenreiter *et al*, 2005).

In agreement with previous studies (Hernandez *et al*, 2000; James *et al*, 2000), the results of the present investigation show that tacrolimus administration for brief periods of treatment (60 and 120 days) does not induce gingival overgrowth. It has been suggested that *in vitro* tacrolimus does not affect the collagen type I gene and protein expression, transforming growth factor (TGF-β1) and tissue inhibitor of matrix metalloproteinase (MMPs) (TIMP-1) mRNA levels, although it significantly increases MMP-1 gene and protein expression and MMP-2 mRNA levels (Gagliano *et al*, 2005b). The involvement of TGF-β1, TIMP-1, and MMP may be important for the mechanism of drug-induced gingival overgrowth. Bolzani *et al* (2000) showed that CsA reduces the production of MMP-1 and -3, as well as the activity of MMP-2 in both rat CsA-induced gingival overgrowth tissue and cultured human gingival fibroblasts. Hempelmann *et al* (1991) reported that CsA reduces MMP-8 activity by human polymorphonuclear leukocytes. CsA inhibits the transcription of MMP-1 by cultured human gingival fibroblasts (Sugano *et al*, 1998) and reduces the amount of the same enzyme in CsA-induced human gingival overgrowth (Thomason *et al*, 1998). Furthermore, chronic CsA-induced nephropathy is characterized by focal interstitial fibrosis, which has been related to increased production of the tissue inhibitor of MMP-1 (Esposito *et al*, 2000). Inhibition of MMP-2 activity has also been associated with this side effect (Fornoni *et al*, 2000). It was recently demonstrated that the secreted protein, acidic and rich in cysteine (SPARC), a glycoprotein that mediates

cell–matrix interactions, seems be involved in CsA-induced gingival overgrowth. In contrast, SPARC mRNA levels are not detected in FK506-treated fibroblasts, suggesting that FK506 may be associated with a role for extracellular matrix stabilization and, consequently, not for inducing gingival overgrowth (Gagliano *et al*, 2005a).

Surprisingly, in the present study, gingival overgrowth was observed during longer periods of treatment (180 and 240 days) in all rats. The gingival overgrowth was more evident in the buccal gingival tissue of the lower molar teeth. The gingival epithelium was hyperplastic, with deep papilla interdigitations. The connective tissue was greater and presented more collagen fibers than the respective control groups, suggesting increased collagen synthesis.

The time of treatment appears to be extremely important. In contrast to the present findings, it has been recently demonstrated that after brief periods of treatment with CsA, gingival overgrowth was associated with a significant increase in epithelium and connective tissue. After 180 and 240 days of treatment, there was a reduction in gingival overgrowth with significant decreases in the epithelium and the connective tissue (Spolidorio *et al*, 2005). These results are in agreement with a prospective longitudinal study (Montebugnoli *et al*, 2000) that showed a relevant role of time in reducing gingival overgrowth in heart transplanted patients undergoing CsA therapy from 6 to 18 months after transplantation. A time-dependent pattern of gingival overgrowth in nifedipine-treated animals was also demonstrated by Fu *et al* (1998).

The exact mechanism of tacrolimus-induced gingival overgrowth during long periods of treatment is not known. We speculate that gingival overgrowth could result from a gradual sensibilization of the gingival fibroblasts, as well as of the gingival epithelium. Long-term tacrolimus therapy may have a direct or indirect action on fibroblasts or in determined populations of gingival fibroblasts, as well as on collagen metabolism via cytokines, growth factors, and the consequent activity of MMPs.

Several *in vivo* and *in vitro* studies have investigated the changes in tissue composition and cellular function that accompany drug-induced gingival overgrowth, mainly those induced by CsA or calcium channel blockers. Most of the attention to date has focused on cytokine expression alterations (Coletta *et al*, 2002; Araújo, Graner, Almeida *et al*., 2003; Ruhl *et al*, 2004).

In the present study, special attention was given to the clarification of the mechanisms of action of tacrolimus on the increased fibroblast proliferation and collagen fibers *in vitro* and *in vivo*, as well as on the protein synthesis and collagenolytic activity, particularly during longer periods of treatment with tacrolimus. Nevertheless, detailed studies are needed to clarify the possible cellular and molecular mechanisms involved in the effects of long-term tacrolimus therapy on the gingiva.

Within the limits of this experimental study, it may be concluded that tacrolimus induces increases in fibroblast and collagen tissue, in parallel with increased severity of overgrowth during long-term treatment.

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