

Transglutaminase 2 expression is significantly increased in cyclosporine-induced gingival overgrowth

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Summary. Cyclosporine A is a potent immunosuppressant used to prevent organ transplant rejection and treat various autoimmune diseases. However, cyclosporine A can also induce gingival overgrowth, which is characterized by increased extracellular matrix due to an altered balance between collagen synthesis and degradation.

This study proposed to verify whether transglutaminase 2, an enzyme thought to be responsible for the assembly and remodelling of extracellular matrix, plays any role in the pathogenesis of cyclosporine A-induced gingival overgrowth.

Cyclosporine A-induced gingival overgrowths were collected from 21 liver transplant patients and case-controlled with 20 non-hyperplastic gingival biopsies from healthy patients who had previous periodontal treatment. In both groups, the presence and tissue distribution of transglutaminase 2 were determined by immunohistochemistry and analyzed in comparison with the tissue morphology and expression of lymphocyte-related antigens (CD3 and CD20) and a vessel-related marker (CD34).

Transglutaminase 2 expression showed a significant increase (2.6-fold) in the stromal component of cyclosporine A-treated patients compared with controls ($p<0.001$), which suggested that transglutaminase 2 had a role in the pathogenesis of the disease. Further studies should investigate the therapeutic effect of anti-transglutaminase 2 drugs (putrescine or 1,4-diaminobutane) in these patients.

Key words: Transglutaminase 2, Cyclosporine, Gingiva, Overgrowth

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Introduction

Cyclosporine A (CysA) is a potent immunosuppressant used to prevent organ transplant rejection and treat various autoimmune diseases. Although the pathogenesis is still unknown, CysA-induced gingival overgrowth (GO) has been principally attributed to an altered balance between collagen synthesis and degradation. Indeed, the main feature of GO is an increase in connective tissue extracellular matrix (ECM) accompanied by inflammatory processes within the affected tissues. It is believed that various molecules that participate in the homeostasis of the ECM may contribute to GO. Recently, gingival fibroblasts derived from CysA-treated patients were reported to show a reduced production of matrix metalloproteinase-1 (MMP-1) *in vitro* (Sukkar et al., 2007). Interestingly, another study proposed that MMP-1 and other related enzymes and factors contributed to CysA-induced GO (Tuter et al., 2002). Indeed, transforming growth factor-beta (TGF-beta) and its receptors (which are involved in wound healing processes) have been shown to play a role in CysA-induced GO (Wright et al., 2001). Little is known about other molecules that participate in connective tissue remodelling under specific conditions, such as wound healing. The present study focused on transglutaminase 2 (TG2), which is considered one of the most important enzymes affecting cell-matrix interaction and ECM reorganization (Stephens et al., 2004).

Transglutaminase 2 belongs to the transglutaminase family, which is a group of structurally and functionally related enzymes that can be described as natural biological glues (Griffin et al., 2002). Transglutaminase 2 has been shown to play a central role in the control of fibroblast activity and matrix engineering through a variety of functions. Some of these functions are

catalytic and mediate protein cross-linking, which stabilises ECM and promotes cell-matrix adhesion (Aeschlimann and Paulsson, 1991; Martinez et al., 1994; Aeschlimann et al., 1995; Corbett et al., 1997; Verderio et al., 1998; Gaudry et al., 1999; Aeschlimann and Thomazy, 2000) and fibroblast proliferation and migration (Griffin et al., 2002). Although some cell types, such as endothelial cells, macrophages, fibroblasts and smooth muscle cells, consistently show high TG2 protein expression, TG2 is ubiquitously expressed in normal tissues (Thomazy and Fesus, 1989). Moreover, TG2 can be found in ECM, where it may co-localise with fibronectin and promote cross-linking of small molecules (Esposito et al., 2003). Furthermore, increased TG2-mediated cross-linking has been described in the pathogenesis of fibrotic reactions (Wodzinska, 2005) and hypertrophic healing processes (Linge et al., 2005).

Because connective tissue homeostasis has been shown to be altered in gingival overgrowth, we decided to investigate whether TG2 expression increased in gingival overgrowth. To test this hypothesis, both the expression and the distribution of TG2 were compared in a series of surgical specimens taken from gingival overgrowth and non-hyperplastic gingival tissues of patients who had undergone periodontal therapy.

Materials and methods

Patients and clinical procedures

CysA-induced GO tissues were collected from 21 liver transplant patients (5 females and 16 males) aged 25–66 years (the average age was 51.95 ± 13.19). The gingival specimens were excised from papillary areas in the anterior sextants where overgrowth lesions were more pronounced. The patients had been on a CysA immunosuppression regimen for an average of 5.95 years (range 3–10), with dosages between 125 and 300 mg/day (the mean dose was 185.96 ± 33.45 mg/day). None of the patients had a history of any other drugs known to affect periodontal tissues.

Control gingival biopsies were obtained from 20 healthy adults (5 females and 15 males) aged 29–64 years (the average age was 52.17 ± 10.29) with clinically healthy periodontia. These patients had not taken any medication likely to influence periodontal health in the 6 months before enrolment.

The study design was approved by the local ethics committee of the University of Torino.

The clinical periodontal parameters, including gingival index (GI) (Loe and Silness, 1963) and plaque index (PI) (Silness and Loe, 1964), were recorded on the upper and lower anterior teeth (canine to canine) at the sampling sites of both groups. The degree of GO (hyperplastic index, HI) was graded numerically on plaster study models using a method previously described by Seymour et al. (1985). A score of 30% was considered the critical value to distinguish CysA responders from non-responders (Seymour and Smith,

1991). Based on the results, CysA-treated patients who had severe overgrowth (>30%) without alveolar bone loss were selected for the study. To reduce or eliminate gingival inflammation, transplant patients underwent instruction on daily plaque control and supra- and sub-gingival scaling procedures with ultrasonic and hand instruments. After completion of the etiologic periodontal therapy, patients were placed on a strict recall maintenance program, which included reinforcement of oral hygiene instructions and full mouth scaling every 2 months.

Gingival sampling

In CysA-treated patients, inter-dental sites in single rooted teeth were selected as sampling sites if they exhibited an HI score of 4 or 5 and showed clinically detectable inflammation. None of the patients had any restorations, crowns or bridge construction around the GO. Using a method described by Vardar et al. (Vardar et al., 2004), gingival specimens were collected before the etiologic phase of periodontal treatment. In the control group, clinically healthy gingival tissue specimens were obtained during crown lengthening procedures. At least three samples from each patient were analyzed to obtain representative data.

Histological and immunohistochemical procedures

The tissue samples were embedded in a 10% formalin solution immediately after excision and before immunohistochemical and histological examination. The specimens were fixed in a 4% formalin solution overnight, dehydrated in graded alcohols (80, 90, 100%) and xylol, and embedded in paraffin at 55–60°C. The 3–5- μm -thick sections were cut and collected on either glass slides for the histological procedures or on 0.1% poly-L-lysine-coated slides for immunohistochemical analysis.

The histological sections were stained with haematoxylin-eosin for light microscopic examination, and ten fields were selected at random within each tissue specimen for histological and immunohistochemical analysis. Hyperplasia and acanthosis of the oral epithelium were evaluated at 40x magnification and measured in μm using a micrometric ocular. Hyperplasia was measured from the outer epithelial surface to the epithelial-connective tissue border, and acanthosis was measured from the deepest point in the epithelial-connective tissue interface to the more coronal area in the dermal papillae inserting into the epithelium.

A manual counter (40x magnification) was used to quantify the inflammatory infiltrate, which was expressed as the lymphocyte, macrophage and plasma cell count in the connective tissue beneath the gingival epithelium. The data analysis was based on the average of the counts.

Immunohistochemical reactions were performed to identify mature T (CD3, polyclonal antibody, Dako

Glostrup, Denmark) and B (CD20 monoclonal antibody, clone L26, Dako) lymphocytes, vessel structures (CD34 monoclonal antibody, clone RB END/10, Ventana-Diopath) and TG2 (monoclonal antibody, clone AB-1 CUB740, Neomarkers) tissue distribution. All reactions were performed in an automated immunostainer (Ventana BenchMark AutoStainer, Ventana Medical Systems, Tucson, AZ).

Appropriate positive and negative controls were used in each staining run. Reactive human lymph nodes were used as positive controls for T and B lymphocytes. Vascular structures served as an internal positive control for TG2 in each specimen. Furthermore, to avoid the possibility of nonspecific positive staining, some slides were incubated without the primary antibodies or with non-immune serums; these tests showed negligible immunoreactions.

The CD3, CD34, CD20 and TG2 labelling were all expressed as the percentage of immuno-positive cells compared with the total number of nucleated cells in a selected area (the magnification was 40x). The data analysis was based on the average of the percentages.

Statistical analysis

The differences between CysA-induced GO sites and healthy control samples were tested by unpaired Student's *t* tests (clinical features of patients, clinical periodontal parameters, acanthosis, hyperplasia, inflammatory cells, CD34, CD3, and TG2) or the Mann-Whitney rank test (CD20). Significance level was set at $p<0.05$.

Results

Clinical aspects

There was no statistically significant difference between the two study groups in terms of gender and age composition ($p>0.05$) (Table 1). When the scoring method described by Seymour et al. (Seymour et al., 1985) was applied, the mean overgrowth score for the CysA-treated group was $43.52\pm13.81\%$. All patients responded to immunosuppressive therapy and had clinically significant overgrowth ($HI>30\%$).

Plaque index, gingival index and hyperplastic index values of the transplant group were all significantly higher than those of the healthy control group ($p<0.001$) (Table 1).

Histological features

The histological features observed in the specimens are summarized in Table 2. Although the degree of acanthosis and epithelial thickness was elevated in the CysA-induced GO group compared with control patients, no significant difference was observed in the epithelial layer in terms of hyperplasia and acanthosis at baseline ($p>0.05$). All of the specimens from the CysA-induced GO group showed a moderate to severe inflammatory infiltrate made up of mature plasma cells, lymphocytes, cells with a macrophage-like aspect and multiple vessels in the lamina propria. At baseline, the total number of tissue inflammatory cells in the transplant group was significantly higher than the

Table 1. Clinical features of the patients and periodontal parameters at sampling sites of the study groups. Values are expressed as mean standard deviation (SD).

	AGE mean \pm SD (yr)	Female/Male	PI mean \pm SD	GI mean \pm SD	HI mean \pm SD
CysA (n=21)	51.95 \pm 13.19*	5/16*	1.85 \pm 0.46**	2.47 \pm 0.39**	4.16 \pm 0.32**
Control (n=20)	52.17 \pm 10.29	5/15	0.15 \pm 0.12	0.11 \pm 0.08	0

PI: plaque index; GI: gingival index; HI: hyperplastic index; CysA: Cyclosporine A; yr: year; *: No statistically significant difference between the two study groups ($p>0.05$); **: Significant difference from the healthy control group ($p<0.001$).

Table 2. Histological features of CysA and healthy gingiva (stromal and mucosal counterparts) and statistical comparison. Values are expressed as mean standard deviation (SD).

	Number of stromal cells at a magnification of 40x.	Number of inflammatory cells at a magnification of 40x.	Hyperplasia (μ m)	Acanthosis (μ m)
CysA Mean \pm SD	276.7 \pm 41.5	345.5 \pm 222.3	482.5 \pm 73.1	238.5 \pm 81.5
Control Mean \pm SD	42.7 \pm 12.4	79.3 \pm 17.4	445.3 \pm 76.5	198 \pm 82.5
P CysA /control	< 0.001	< 0.001	0.14	0.13

CysA: Cyclosporine A.

Table 3. Immunohistochemical features of stromal cellular component in CysA patients and controls and statistical comparison. Values are expressed as means standard deviation (SD).

	TG2 (% of positive cells)	CD3 (% of positive cells)	CD20 (% of positive cells)	CD34 (% of positive cells)
CysA Mean ± SD	73.5±21.4	32.2±15.5	4.2±3.1	32.5±10.3
Control Mean ± SD	28.1±9.8	30.1±14	4±3	14.2±8.8
P CysA /control	< 0.001	0.58	0.97	< 0.001

CysA: Cyclosporine A; TG2: Transglutaminase 2.

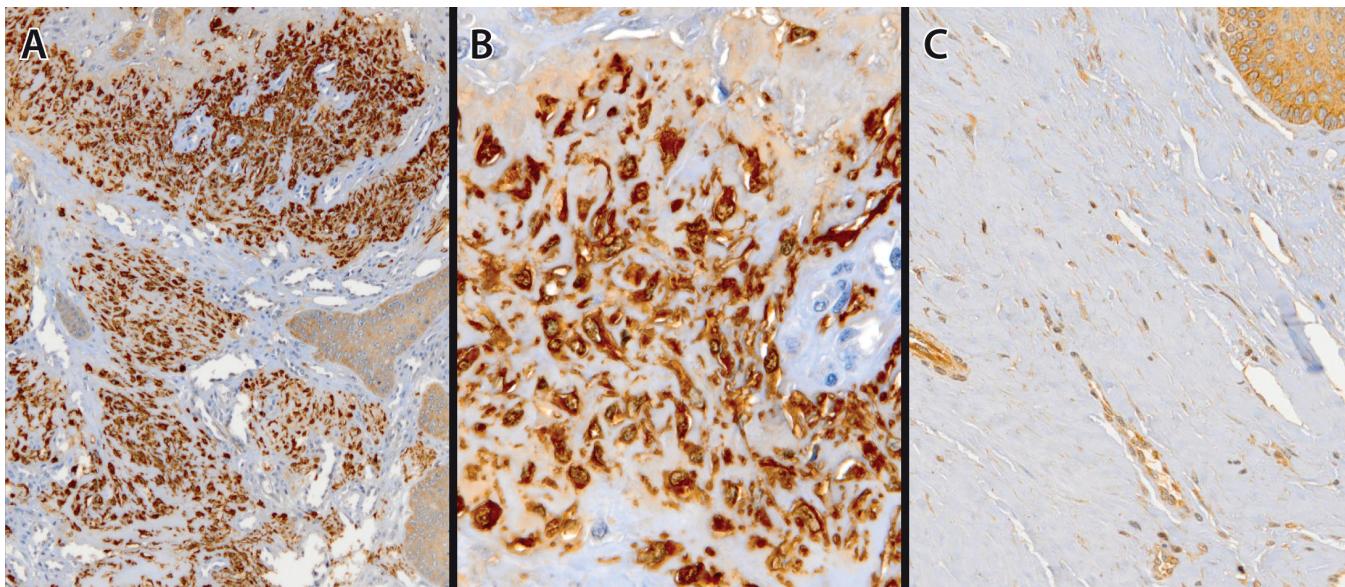


Fig. 1. Transglutaminase 2 expression in CysA-induced GO and controls. The large majority of spindle-like cells in the connective tissue beneath the gingival mucosa in CysA-induced GO showed intense TG2-positive staining at low (**A**, 5x) and high magnification **B**, 40x). In contrast, only a faint positive staining of TG2 (mainly localised in endothelial cells) was observed in normal gingival tissue (**C**, 10x).

healthy control group ($p<0.001$).

Moreover, the number of stromal cells within the connective tissue beneath the gingival mucosa was significantly higher in CysA specimens compared with controls ($p<0.001$) (Fig. 1A).

Immunohistochemistry

The immunohistochemical data are summarised in the Table 3. When evaluated in a high-power field, the T (CD3) and B (CD20) lymphocyte components did not show any significant variation between the CysA-induced GO and control groups. In contrast, a statistically significant difference was observed in the number of CD34-positive endothelial cells. The overgrown gingival specimens showed a much higher expression than the healthy control group ($p<0.001$). Interestingly, the percentage of fibroblasts and mesenchymal cells stained with the anti-TG2 antibody was significantly higher (showing a 2.6-fold increase) in

the overgrown gingival specimens compared with the controls ($p<0.001$). In particular, intense TG2 staining was observed in the ECM and spindle-like cells in the overgrown specimens (Fig. 1A,B), especially when compared to the faint positive staining in the normal gingival tissues (Fig. 1C).

Discussion

To the best of our knowledge, this is the first evidence of significantly increased TG2 expression in stromal cells and ECM of CysA-induced GO, which is mainly characterized by an increase in collagen metabolism and a reduction in matrix-degrading enzymes (Trackman and Kantarci, 2004). Although researchers still debate whether CysA alters the collagen balance by up-regulating collagen synthesis or down-regulating its degradation (Trackman and Kantarci, 2004), recent data have supported the latter hypothesis because of the findings that CysA significantly reduced

the level of MMP-1, which affected the ability of fibroblasts to remove the increased collagen (Sukkar et al., 2007). In addition, CysA has been shown to increase the level of tissue inhibitor of metalloproteinase-1 (TIMP-1). Interestingly, the identification of drug-dependent changes in the expression of fibroblast related growth factors, such as platelet-derived growth factor-B (PDGF-B), fibroblast growth factor-2 (FGF-2), TGF-beta, and connective tissue growth factor (CTGF), has been controversial (Trackman and Kantarci, 2004; Wright et al., 2006). Expression levels of TGF-beta isoforms and receptors, however, have been shown to be different between GOs and normal gingival tissues, and some data have implicated TGF-beta in the pathogenesis of drug-induced GO (Wright et al., 2001).

Transglutaminase 2 could contribute to overgrowth processes through the formation of specific bonds, which would confer stability to protein agglomerates (Aeschlimann and Paulsson, 1994). In fact, excessive TG2 cross-linking is considered responsible for uncontrolled healing processes, such as the formation of keloids (Meier and Nanney, 2006).

An increased TG2 immuno-staining within the rich stromal cell component was observed in the series of GO reported herein, whereas normal gingival specimens only showed staining in the endothelial cells and a minority of spindle-like cells. This different expression of TG2 between the two groups was also associated with a greater expansion of the vascular component in the connective tissue of inflamed CysA-induced GO compared with healthy control tissue. Increased TG2 expression and distribution could be involved in the increased level of fibroblast proliferation and the intensified resistance of ECM to degrading enzymes. These TG2-mediated effects could lead to alterations in connective tissue homeostasis, which would participate in the pathogenesis of GO.

A variety of small TG2 molecules and peptide-mimetic inhibitors have recently been identified (Siegel and Khosla, 2007) as possible therapeutic tools to treat patients affected by diseases involving TG2 (e.g., neurodegenerative diseases and celiac sprue) (Griffin et al., 2002). In addition, they have also been used to correct and limit keloids and fibrosis (Wodzinska, 2005; Meier and Nanney, 2006; Telci and Griffin, 2006). The CysA-associated TG2 increase in fibroblasts and ECM of GOs reported in the present study suggested the possibility of clinical management of gingival hypertrophy using specific anti-TG2 molecules (such as putrescine or 1,4-diaminobutane). Indeed, the findings of the present study warrant further investigation and/or confirmation.

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