Transglutaminase 2 May Be Associated with Peri-implant Gingival Overgrowth: Preliminary Assessments

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Tissue transglutaminase 2 (TG2) is ubiquitously expressed in normal tissues and plays an important role in the pathophysiology of wound healing. An increase in periodontal tissues has been previously reported in cyclosporine-induced gingival overgrowth. **Purpose:** The aim of this study was to explore associations between TG2 expression and the vascularization and maturation processes of peri-implant soft tissues over time. **Materials and Methods:** Edentulous patients proposed for mandibular implant-retained overdentures were included in the study. Biopsies of the peri-implant mucosa were performed at the first surgical stage and at 4, 8, and 12 months after prosthetic load. A follow-up program was directed to record plaque indexes, bleeding on probing data, and pocket probing depth around implants. An evaluation of the vessels' density was carried out by digital virtual microscopy and using an immunohistochemistry approach (antibodies anti-CD31, anti-TG2). A robust multivariable regression model was implemented. **Results:** According to model results, blood vessel count and probing (as a marker of gingival overgrowth in absence of plaque) significantly decrease over time and are associated with TG2, particularly for values above the median. **Conclusion:** The association of an increased TG2 expression in the extracellular matrix might have a significant impact in the development of gingival overgrowth around a loaded implant. *Int J Prosthodont 2015;28:615–620. doi: 10.11607/ijp.4280*

Angiogenesis is important in inflammation and healing, but its role in the development, progression, and healing of periodontal lesions has not yet been clarified. Vessel density and vascular-related growth factors play a bivalent role, either sustaining the healing processes or participating in inflammation-related tissue damage.[1–4] Several authors have hypothesized that inflammatory infiltrate in peri-implant soft tissue may be important in the evolution of the repairing processes.[5–9] A complex series of events involving inflammation, cell migration and proliferation, extracellular matrix (ECM) stabilization, remodeling, and neovascularization are crucial to peri-implant tissue response to wound healing. Wound healing involves the dynamic interaction of multiple cell types with components of ECM and growth factors. Indeed, abnormal wound healing may also lead to inflammatory and/or sclerotic conditions (such as renal and pulmonary fibrosis).[10,11] Therefore, identification of the molecular events taking place during wound repair is essential to develop new, effective supportive treatment protocols for prosthetic implant patients in relation to wound care.

Tissue transglutaminase 2 (TG2), a multifunctional protein cross-linking enzyme that stabilizes tissues, plays a central role in the pathophysiology of wound healing through catalyzing and mediating protein cross-linkings, which modulate fibroblast activity and

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matrix engineering, thus stabilizing ECM, promoting cell-matrix adhesion[3] and fibroblast proliferation and migration.[12] TG2 is ubiquitously expressed in normal tissues, although some cell types, such as endothelial cells, macrophages, fibroblasts, and smooth muscle cells, consistently show higher protein expression.[13] Moreover, TG2 can be found in the ECM, where it may colocalize with fibronectin and promote the cross-linking of small molecules.[14] However, excessive cross-linking by TG2 has been implicated in the pathogenesis of fibrotic reactions in various sites that participate in the hypertrophic healing processes.[13,15] According to this evidence, an increase has been previously reported in cyclosporine-induced gingival overgrowth.[17,18]

On the basis of these premises, the protocol is intended to assess whether the number of small-sized (probably immature) vessel structures, along with the distribution of TG2 in peri-implant tissue, might play a key role in the development of an abnormal wound healing and maturation of the peri-implant soft tissues, possibly leading to gingival overgrowth or altered repairing processes, evaluated as health of the peri-implant soft tissues (probing depth, bleeding on probing, and plaque index).

Materials and Methods

The angiogenetic changes that take place over time in peri-implant soft tissues were monitored, evaluated as vessel density and expression of vessel/ECM-related molecules (TG2) in patients wearing implant-retained overdentures, from the first surgical step up to 12 months after prosthetic load. The density of small expression of the healing-related molecule TG2 was assessed through objective vessels in the implant-retained overdenture after prosthetic load and ECM identification using an automated analysis with slide digital virtual microscopy in combination with traditional microscopy.

Study Population

A convenience sample of 9 edentulous patients (8 females, 1 male; age range: 54 to 69 years, average: 61.9 years) were included in the study. Exclusion criteria were systemic disorders, signs and symptoms of temporomandibular disorders (TMD), and smoke habits. The source population is composed of patients referred to the Department of Oral and Maxillofacial Rehabilitation and Dental Implants (Torino, Italy) and rehabilitated with complete dentures in this department. The study design was approved by the local Ethics Committee (410/417/70/2008). Written informed consent was obtained from all subjects.

Clinical Procedure and Biopsy

Titanium implants (Nobel Biocare) were placed in the symphyseal area, with the aim of retaining the complete mandibular denture. Surgical treatment was performed according to a standard technique.

Prosthetic abutments were connected 3 months after the surgical stage. The abutments chosen were 2 mm higher than the gingival margin and have been recorded in the clinical chart. The mandibular complete overdenture was retained by 2.25-mm-diameter ball attachments. Each patient was instructed on the oral hygiene procedures for both the denture and the titanium abutment (brushing and flossing). Patients were examined at the first surgical stage and at follow-up visits 4, 8, and 12 months after abutment connection. During the three follow-up visits the following peri-implant parameters were recorded[19]:

- Plaque index: Each of the four surfaces of the implants (buccal, lingual, mesial, and distal) was given a score from 0 to 4 (0 = no plaque; 4 = plaque on all surfaces) based on visual inspection, and at the end, a mean score for each patient was generated.
- Bleeding index (BI): This was assessed by gently running a periodontal probe 1 mm into the gingival sulcus parallel to the wall of the abutment. If bleeding became visible within 20 seconds, a BI score was given.
- Pocket probing depth: Extrasulcular probing was performed and measured between the gingival margin and the implant abutment junction. Six measurements were made for each implant. The subscores for each patient were used to calculate each patient's final score.[19]

Four mucosal biopsies (ranging from 3 to 5 mm^2) were taken. At the first surgical stage (at implant insertion), a mucosal sample was cut from the flap in the area of the future implant site (T_0). This biopsy was used as basal reference point. At 4, 8, and 12 months after prosthetic load, the sample was excised from peri-implant tissues (T_1, T_2, T_3).

Keratinized mucosa was observed around the implants of all patients.

Biopsy Procedures

The biopsy site was anesthetized with 1 cartridge of mepivacaine (Carbocaine 2% without adrenaline, AstraZeneca) by infiltration of the alveolar mucosa at least 1 cm from the biopsy site to prevent any modification of the histomorphometric features of the tissue. An incision was performed around the abutment; the

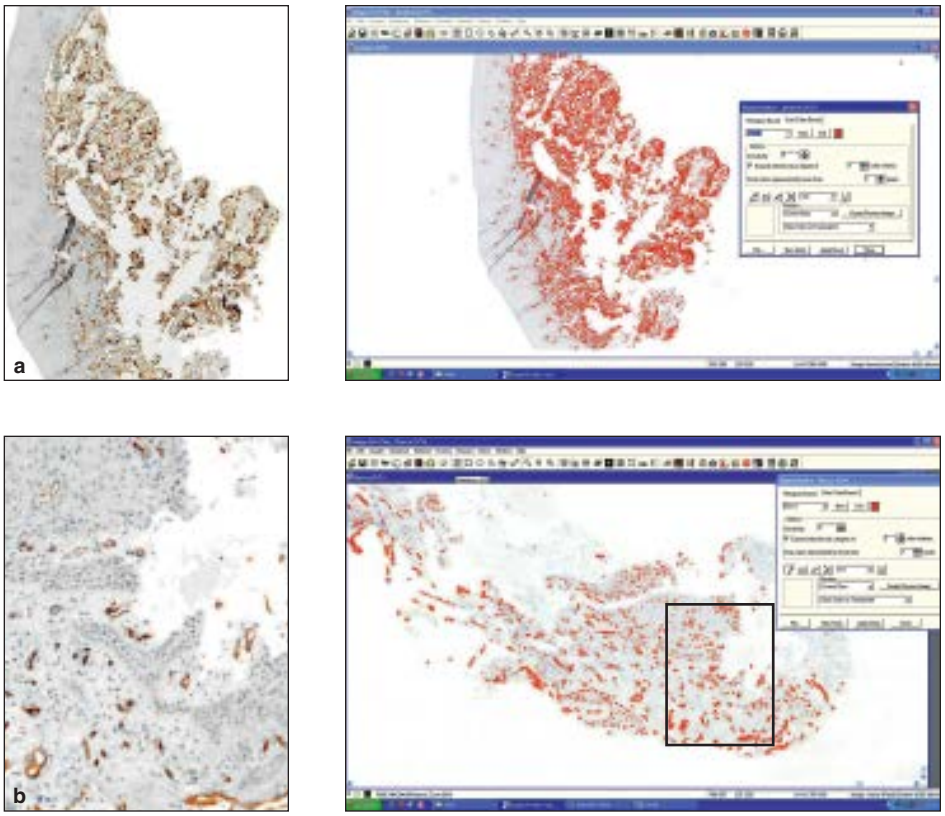

Fig 1 **(a)** Blood vessel staining using anti-CD31 in mucosal sample in the area of the future implant site at T_0, as seen by traditional optic microscopy (10×). **(b)** Sequential biopsy 4 months after prosthetic load (T_1) shows a reduction in the number of blood vessels (immunohistochemistry using an anti-CD31 antibody) compared to T_0.

peri-implant surgical wound healed by second intention. The tissue samples were embedded in a 4% formalin solution immediately after excision and before immunohistochemical and histological examination. The 3- to 5-µm-thick sections were cut and collected on either glass slides for the histological procedures or 0.1% poly-L-lysine-coated slides for immunohistochemical analysis.

Histological Procedures

The histological sections were stained with hematoxylin-eosin for light microscopic examination. Immunohistochemical reactions were performed on vessel structures (CD31 monoclonal antibody, clone 1A10, Diapath) and TG2 (monoclonal antibody, clone AB-1 CUB 740, Neomarkers) tissue distribution in an automated immunostainer (Ventana BenchMark AutoStainer, Ventana Medical Systems). Appropriate positive and negative controls were used in each staining run. Furthermore, to avoid nonspecific positive staining, some slides were incubated without the primary antibodies or with nonimmune serums; these tests showed negligible immunoreactions.

The presence and tissue distribution of TG2 were evaluated as previously described.[16] An automated microscope (Slide Digital Virtual Microscopy, Olympus) and software (dotSlide, Olympus) were used to scan all the CD31 immunohistochemical slides, which highlighted the vascular structures, at 10× magnification to obtain virtual slide image (VSI) files.

Statistical Analysis

Robust linear regression models were developed using SAS software (SAS Institute) to explore the relationships between TG2 and probing score, blood vessel count, and dental plaque over time. The models included time of assessment as a covariate and TG2 as a linear spline with a knot at its median value to allow for nonlinear effects.

Results

At 12 months after prosthetic load, no implant had been lost. Plaque was found in some of these patients, but decreased with strict control of the oral hygiene procedures.

All patients showed a decrease in the number of blood vessels when the first and last samples were compared (12 months postload) using digital virtual microscopy (Fig 1). Biopsies harvested 12 months after

Table 1 Multivariate Regression Model Results

	Probing			Blood vessel count			Plaque index		
	Coefficient	95% CI	*P* value	Coefficient	95% CI	*P* value	Coefficient	95% CI	*P* value
Time	−0.46	−0.83, −0.08	.02	−21.3	−33.9, −8.6	< .01	−0.01	−0.06, 0.05	.059
TG2 below the median	0.08	03.00, 0	< .01	−0.81	−2.14, 0.52	.20	−0.01	−0.02, 0.01	.55
TG2 above the median	0.03	01.00, 0	< .01	0.42	01.00, 0	.04	0.00	−0.01, 0.01	.18

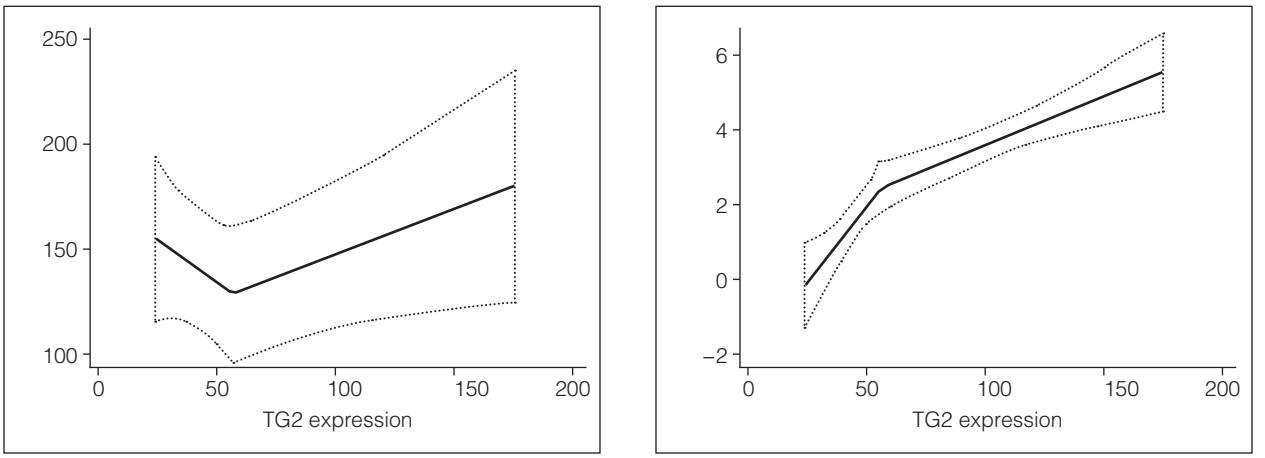

Fig 2 Multivariable regression model results. Data are reported graphically for blood vessel count **(a)** and probing score **(b)**.

load showed a significantly lower average number of blood vessels (100.15 ± 28 vessels/mm^2) compared to the average number found before implant installation (179.2 ± 55.01 vessels/mm^2) and at both the 4- and 8-month controls. The immunohistochemical results of TG2 expression of peri-implant soft tissues from the sequential biopsies highlighted a TG2 positivity in the ECM on all specimens. All patients showed a progressive and continuous increase in the number of stromal cells when the samples were compared at all follow-up sessions.

As shown in Table 1, both blood vessel count and probing significantly decreased at each assessment.

In the absence of a linear relationship between predicted blood vessel count and TG2 expression ($r = -0.17$, $P = .32$) and between predicted probing score and TG2 expression ($r = 0.05$, $P = .78$), a linear regression analysis cannot be performed. However, splitting data values over and under the median, which is a rather common choice in biomedical statistics, resulted in a suitable analysis.

An association between probing score and TG2 was demonstrated (Fig 2). Each unit increase of TG2 corresponded to an average probing score increase of 0.03 to 0.08, depending on whether TG2 was above or below its median value. For example, a TG2 increase from 40 to 52.5 corresponded to a mean probing increase of 1, while a TG2 increase from 70 to 82.5 corresponded to a mean probing increase of less than half a point. The association between blood vessel count and TG2 was present only for TG2 values above the median. No association could be found between plaque index and TG2.

Discussion

In this study population, neoangiogenesis in peri-implant soft tissues was demonstrated using automated digital image analysis on histological sections of peri-implant soft tissues from the sequential biopsies. The present study monitored the time course of the angiogenetic changes in the peri-implant soft tissues, evaluated as capillary density and expression of vessel-related molecules (TG2) in implant-retained overdentures from the first surgical step until 12 months postload. A decrease in the number of blood vessels was observed in all cases, in comparison to T_0.

The vascular system in the periodontal tissues differs both structurally and functionally from the vascular system in the peri-implant tissues. Indeed, there is usually poor vascularization of the connective tissue around the implant, especially close to the titanium abutment. Little is known about the changes that take place over time in peri-implant tissues after implant loading.[3]

The volumetric increase of the blood vessel compartment might be due to the increase in both the diameter and number of blood vessels; however, only

the number of small (immature) vessel structures can be considered an indicator of angiogenesis and may bear prognostic value in the evolution of tissue remodeling.[20] Preti et al[21] observed a greater number of vessels in loaded peri-implant tissues. This increase in blood vessel density was subsequently justified by the presence of platelet-activating factor, a potent inflammation mediator in the soft tissue that surrounds failed implants.[22]

To date, to the best of our knowledge, all the available studies deal with only a few characteristics of the peri-implant vascular system and none have taken this aspect as the main object of the research. In addition, no data are reported on the evolution of the vascularization of the peri-implant soft tissues by means of an analysis of sequential histological specimens.

To address this issue, the present study analyzed the histological changes and vessel density in the mucosa loaded by a denture and the peri-implant soft tissue after the prosthetic load of the implant overdenture. The resulting data suggest that the density and quality of vessels with the evaluation of TG2 expression in the ECM (which participates in supporting the mucosal tropism) in peri-implant soft tissues is associated with peri-implant soft tissue maturation processes.

Consistently, Cornelini et al[20] demonstrated that the difference in microvessel density between healthy sites and peri-implantitis was statistically significant and that the vascular endothelial growth factor (VEGF) expression in the vessels was lower in peri-implantitis samples as well. They concluded that VEGF is most likely involved in both the maintenance of periodontal physiology and the progression of peri-implant inflammatory disease. Also, VEGF expression in the stromal cells of peri-implantitis samples seemed to be a predictive factor for implant failure.[20,23] To recapitulate, literature evidenced that neovascularization and the expression of vessel-related molecules are involved in the maintenance of the health of peri-implant tissues.[24]

It is well known that vessel morphology is important to distinguish small (probably immature) vessel structures, which would probably display altered wall permeability, from mature ones. The present study found no relationship between small vessels and an increase in the inflammatory cell component (ie, mononucleated inflammatory cells) as assessed by the pathologists, but did find a relationship between small vessels and the externalization of TG2 from endothelial cells to ECM. Since TG2 is involved in the tissue remodeling processes as well as in its abnormalities (ie, cheloid and hypertrophic scars), in the absence of inflammation due to plaque accumulation it could conceivably play a role in determining gingival overgrowth in these patients. Indeed, the variation in TG2 expression should be considered not as a sign or as a response to an inflammatory event (eg, plaque-induced), but as a characteristic pattern of healing, detectable in immunohistochemical analysis as clinically corresponding with gingival overgrowth.[24]

Conclusions

We propose that an increased neovascularization in the remodelling process in peri-implant tissues poor in mature blood vessels and an increased TG2 expression within the ECM are prognostic factors predicting gingival overgrowth. Gingival peri-implant overgrowth may be a negative prognostic factor for dental implant patients. A natural predisposition to gingival overgrowth, besides being undesired per se, could easily affect the hygienic maintenance of any prosthetic rehabilitation, especially when involving dental implants. Indeed, such a condition could initiate further overgrowth, both plaque-induced, if plaque control becomes difficult to manage, and trauma-induced, if the intaglio surface of a removable denture bears or contacts the overgrown tissues. This scenario might be better managed if it is known in advance. Patients should be taught correct hygienic practices, and hygienic prosthetic designs should be adopted to ensure the health of peri-implant tissues.[25,26]

Elevated TG2 in the peri-implant tissue might be worth investigating as an early predictor of the development of gingival overgrowth and may open new, attractive therapeutic targets, even for future targeted pharmacological interventions.[27]

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Literature Abstract

Systematic Review on Noninvasive Treatment of Root Caries Lesions

Several approaches to prevent the initiation of or to inactivate root caries lesions have been proposed. Thirty-four clinical studies investigating the efficacy of various chemical agents in the form of dentifrices, mouth rinses, and varnishes were systematically reviewed in this paper to investigate their efficacy on the prevention or inactivation of root caries lesions. Most studies reviewed assessed root caries lesions on the basis of surface texture. It was found that the use of dentifrices containing 5,000 ppm fluoride, or 1.5% arginine plus 1,450 ppm fluoride, significantly inactivated more root caries lesions than the use of dentifrices containing 1,100 to 1,450 ppm fluoride. However, evidence level for the efficacy of 1.5% arginine plus 1,450 ppm fluoride dentifrice was graded as very low, so further studies are recommended. No significant differences were found in the efficacy of amine fluoride/stannous fluoride dentifrice plus rinse compared to sodium fluoride dentifrice plus rinse. Professionally applied quarterly chlorhexidine varnish (1% or 10%) or silver diamine fluoride varnish were found to significantly reduce the initiation of root caries lesions compared to placebos. The authors concluded that based on meta-analysis of available data, dentifrices containing 5,000 ppm fluoride and professionally applied quarterly chlorhexidine or silver diamine fluoride varnish may prevent or arrest root caries lesions, though these results should still be interpreted with caution as differing criteria for the assessment of root caries lesions led to possible biases in this review, compounded by low numbers of clinical trials available for review and limiting grade of evidence levels.

Wierichs RJ, Meyer-Lueckel H.J Dent Res. 2014;14,1–11. **References:** 61. **Reprints:** R.J. Wierichs, Department of Operative Dentistry, Periodontology and Preventive Dentistry, RWTH Aachen University, Pauwelsstrasse 30, 52074 Aachen, Germany. Email: rwierichs@ukaachen.de
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