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Plasma TGF-β1 as a risk factor for gingival overgrowth

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Abstract

Background and Aims: Induction of the pro-fibrotic growth factor TGF-β1 has been suggested as a possible mechanism through which immunosuppressant drugs may induce gingival overgrowth. This study aims to investigate plasma levels of TGF-β1 and relate them to the development and severity of gingival overgrowth in immunosuppressed transplant patients.

Materials and Methods: One hundred and thirty-two ciclosporin-treated and 13 tacrolimus-treated transplant patients and 24 drug-free control subjects underwent a full periodontal examination including a determination of the presence and severity of gingival overgrowth.

Results: Plasma TGF-β1 concentrations were determined by ELISA, and were found to be significantly elevated in samples from the transplant patients (mean = 29.1 ng/ml) as compared with controls (mean = 6.1 ng/ml, $p < 0.0001$). There was no significant difference between the levels of plasma TGF-β1 in the ciclosporin- and tacrolimus-treated patient groups.

Conclusions: Furthermore, concomitant treatment with calcium channel blockers did not influence the levels of plasma TGF-β1 in the patients group. The relationship between gingival overgrowth, independent periodontal variables and TGF-β1 plasma concentrations was examined using univariate and multivariate regression analyses; low TGF-β1 plasma concentrations were found to be a risk factor for gingival overgrowth in immunosuppressed patients concomitantly receiving a calcium channel blocker.

Key words: plasma TGF-β1; overgrowth; transplant

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Drug-induced gingival overgrowth (GO) can occur as an unwanted effect of the patients' immunosuppressive regimen following organ transplantation. Patients are usually immunosuppressed with ciclosporin (Neoral™; Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA) and often concomitantly medicated with a calcium channel blocker to reduce hypertension and ameliorate the nephrotoxocity of ciclosporin; this is commonly nifedipine or amlodipine (Thomason et al. 1993, Thomason et al. 1997, James et al. 2000).

GO occurs in approximately 30% of patients medicated with ciclosporin alone and 45% of patients' additionally using calcium channel blockers (Seymour et al. 1987, Thomason et al. 1993). Overgrowth of gingival tissue is generally characterised by an accumulation of fibrous tissue with an increase in connective tissue components and a large number of proliferating fibroblasts (Savage et al. 1987). Although the pathogenesis of this condition has not been fully elucidated, it is thought to be multi-factorial, due to the synergistic effect of pharmacological and host factors (Seymour et al. 1996).

A number of risk factors for the severity of gingival overgrowth (GO) have been previously identified, including: blood creatinine levels, time since transplant, age, gender, HLA phenotype, plaque levels and gingival inflammation (Thomason et al. 1996, Afonso et al. 2003). There is considerable information supporting a role for TGF-β1 in mediating the dyrsregulated fibroblast proliferation and extra-cellular matrix synthesis that causes fibrosis and thereby excessive levels of TGF-β1 are associated with many diseases of which tissue fibrosis is a feature (Branton & Kopp 1999, Eickelberg 2001, Sime & O'Reilly 2001). Evidence from the study of isolated gingival fibroblasts in culture demonstrates that these cells exhibit a proliferative response to TGF-β1 (Dennison et al. 1994, Anderson et al. 1998, James et al. 1998). Furthermore, hyper-responsiveness of gingival fibroblasts to autocrine effects of TGF-β1, coupled with increased levels of synthesis in these cells, may be important pathogenic elements in hereditary gingival fibromatosis, a condition that has histopathological features in common with drug-induced GO (Tipton & Dabbous 1998, Colletta et al. 1999, de Andrade et al. 2001).

The immunosuppressive action of ciclosporin centres on its action on T-cells

although it is increasingly appreciated that the effects of this drug are not restricted to the cells of the immune system (Nabel 1999). It is well established that ciclosporin upregulates TGF-β1 synthesis, a fact that might explain the fibrogenic effect of this drug in a variety of cells and tissues (Li et al. 1991, Khanna et al. 1994, Shehata et al. 1995, Wolf et al. 1995, Khanna et al. 1998). Significantly, in vivo studies in animal models have shown that ciclosporin increases kidney TGF-β1 levels. This is associated with concomitant increase in circulating TGF-β1 and that these effects are abrogated by administration of anti-TGF-β1 antibodies (Khanna et al. 1999). In previous clinical studies, circulating levels and gingival crevicular fluid levels of TGF-β1 have been reported to be increased in ciclosporin-treated transplant patients (El-Garnel et al. 1998, Shin et al. 1998, Buduneli et al. 2001) although one study did not replicate these findings (Hughes et al. 1999). Tacrolimus also increases the expression of extracellular matrix proteins and enhances fibrosis in an animal model (Frizell et al. 1994). Tacrolimus seems to have effects similar to ciclosporin in terms of stimulating TGF-β1 synthesis and secretion in lymphoid and non-lymphoid tissues albeit through different biochemical mechanisms (Han et al. 1995, Khanna et al. 1999). Interestingly, intra-renal expression of TGF-β1 was enhanced with both ciclosporin and tacrolimus therapy (Bicknell et al. 2000, Jain et al. 2002, Khanna et al. 2002). However, quantitative analysis of the comparative levels of TGF-β1 in biopsies from ciclosporin and tacrolimus-treated patients have not revealed consistent results (Bicknell 2000, Mohammed et al. 2000, Jain 2002, Khanna et al. 2002). Furthermore, there are differences between the expression of latent TGF-β1 and active TGF-β1 which might be pathologically important (Mohammed et al. 2000).

There is accumulating evidence that gingival overgrowth is associated with increased levels of TGF-β1 although there are no reports relating circulating levels of TGF-β1 to gingival overgrowth and other candidate risk factors for this condition (James et al. 1998, Wright et al. 2001).

Aims

The aim of this study was to determine the plasma levels of TGF-β1 in ciclosporin- and tacrolimus-treated transplant patients and to investigate its role as a possible risk factor for the development and severity of gingival overgrowth.

Material and Methods

The study received ethical approval from the Joint Ethical Committee of the University of Newcastle upon Tyne and the Northern and Yorkshire Regional Health Authority.

Transplant patients were recruited from the Periodontology clinic at Newcastle Dental Hospital and the cardiac and renal transplant clinics at the Freeman Hospital, Newcastle upon Tyne. Patients were immunosuppressed with either ciclosporin or tacrolimus. Control subjects ($n = 24$) with no pharmacological history of ciclosporin, calcium channel blockers or any other medication known to induce gingival overgrowth were recruited from staff in the Dental Hospital.

Demographic, medical and periodontal data were collected from all patients at the time of examination. The periodontal examination was undertaken by one clinician (J. S. E.) and was confined to the six most anterior teeth in each arch. Plaque was assessed using the system described by Silness & Loe (1964). Probing depths were also determined at each of four sites (mid-buccal, mesial, distal and mid-lingual/palatal) and the percentage of sites exhibiting probing depths of 4 or more mm was calculated. Finally, a papillary bleeding index (PBI) was recorded (Saxer & Muhlemann 1975).

Upper and lower alginate impressions of each patient were taken and from these plaster models were prepared. GO was scored on the models as described previously (Seymour et al. 1985). In this method, each buccal and lingual/palatal interdental papilla of the six upper and lower anterior teeth is given a score of between 0 and 5 depending on the amount of horizontal and vertical enlargement. A total of 20 interdental papillae are, therefore, examined giving a maximum overgrowth score of 100. The GO score was calculated by a second clinician (J. M. T.) who was blinded to the patients' identity and medical history.

Peripheral blood (10 ml) was obtained by venepuncture and collected into sterile lithium heparin vacutainers to prevent coagulation. This was centrifuged at room temperature at 800 $\times$ g for 15 min. The top 2/3 of plasma was removed to avoid extracting platelets from the cell pellet and re-centrifuged for a further 15 min. Clarified plasma was then stored in a -70°C freezer.

Plasma TGF-β1 was determined by sandwich ELISA. A 96-well ELISA plate (Immulon 2, Dynex, Worthing, UK) was coated overnight at room temperature with 100 μl mouse anti-human TGF-β mAb (2 μg/ml) (R & D Systems, Abingdon, UK) diluted with coating buffer ($NaHCO_3$ 0.05 M pH 9.6). Wells were aspirated, washed three times with wash buffer (PBS/0.5% Tween 20 pH 7.3) and blocked with 200 μl of 5% BSA for 2 h at room temperature. The plates were aspirated and washed with washing buffer three times thereafter. Prior to TGF-β1 assay, plasma samples (25 μl) were diluted (1 in 4) in a diluent buffer (1.4% delipidised bovine serum/0.05% Tween 20 in 0.05 M Tris-buffered saline) and activated by addition of 100 μl of 2.5 N acetic acid/10 M urea followed by incubation for 10 min at room temperature. Activated plasma samples were neutralised by the addition of 100 μl of 2.7 N NaOH/1 M HEPES.

Binding of TGF-β1 was performed by incubation of 100 μl of activated plasma samples further diluted (1 in 4) with diluent buffer for 2 h at room temperature. Plates were subsequently washed three times more and then incubated with 100 μl of biotinylated anti-human TGF-β1 mAb (50 ng/ml in diluent buffer) (R & D Systems) for 2 h at room temperature. 100 μl of avidin–horseradish peroxide conjugate (1/1500 in diluent buffer) (Sigma, Poole, UK) was added, incubated for 30 min at room temperature and washed five times. Hundred microlitres of TMB substrate solution (Kirkgaarde & Perry, Middlesex, UK) was added to each well and after a 15–30 min incubation at room temperature, the colour development reactions were stopped by the addition of 50 μl 1 M H_2SO_4 to each well and the OD was measured at 450 nM using a microtitre plate reader (Titretek, Labsystems, Finland). The TGF-β1 content was determined from a standard curve of recombinant human TGF-β1 (rTGF-β1) (R & D Systems).

Statistical methods

Comparison of plasma TGF-β1 concentrations between patients and controls and within the transplant group were

made using the unpaired *t*-test. The relationships between previously identified demographic, periodontal, and pharmacokinetic risk factors for GO score (Thomason et al. 1996) were investigated in the patient groups as described below. The variables included in the analyses were age, gender, creatinine level, time since transplant, PBI, when appropriate, the use of a calcium channel blocker together with the plasma TGF-β1 concentrations, and the plaque score. The patient cohort was classed into two groups for statistical analysis based upon their drug regime:

1. All immunosuppressed transplant patients including those concomitantly medicated with a calcium channel blocker.
2. Immunosuppressed transplant patients not medicated with a calcium channel blocker.

Individual patient data, including demographic, pharmacokinetic and periodontal variables, were collected and recorded on a spreadsheet in a statistical database. All statistical analysis and modelling were undertaken using commercially available software packages (Minitab 2000; Stata 1993). Data were plotted as a histogram and visually assessed for normal distribution.

The relationship between GO score and the listed independent variables were assessed using both univariate and stepwise multivariate regression modelling techniques. Both backward and forward stepwise regression analysis were used and in all cases the same results were obtained. Results of the regression analyses are presented in the form of regression coefficients, their 95% confidence intervals, and *p*-values. The adjusted R^2 statistic is reported for the multiple regression modelling.

Results

Plasma TGF-β1 concentrations in all transplant patients ($n = 145$) ranged from 0.02 to 94.1 ng/ml (mean = 29.1 ng/ml) and in control subjects ($n = 24$), from 0.07 to 22.9 (mean = 6.102 ng/ml). TGF-β1 was found to be significantly elevated in samples from the transplant subjects compared to normal controls ($p < 0.0001$).

When transplant patients were classified according to immunosuppressant medication, plasma TGF-β1 in samples from both ciclosporin and tacrolimus groups were found to be significantly elevated compared to controls ($p = 0.008$ and 0.002, respectively). No significant difference was found for plasma TGF-β1 between ciclosporin ($n = 132$, mean = 27.21 ng/ml) and tacrolimus medicated groups ($n = 13$, mean = 35.4 ng/ml).

Plasma TGF-β1 concentrations from transplant patients medicated with calcium channel blockers and those with no calcium channel blockers were then compared to determine if they were related to concomitant medication. Patients medicated with calcium channel blockers ($n = 79$) had plasma TGF-β1 concentrations ranging from 0.04 to 78.5 ng/ml (mean 28.31 ng/ml) compared with those without calcium channel blockers ($n = 66$) where plasma TGF-β1 values ranged from 0.02 to 69.5 ng/ml (mean = 26.6 ng/ml) (Fig. 1). There was no significant difference between these groups at the 5% level. When compared to normal controls (see above) the differences were highly significant ($p < 0.0001$).

The results of the univariate and multivariate regression analyses for Group 1 patients (i.e. all immunosuppressed transplant patients including those concomitantly medicated with calcium channel blocker) are reported in Tables 1 and 2. When examined, univariately significant relationships were noted for the variables: gender, duration of therapy, creatinine concentration, papillary bleeding index, medication with calcium channel blockers and plasma concentration of TGF-β1 (Table 1). The results of the stepwise regression analysis are reported in Table 2. Of the variables investigated, age, gender (maleness), medication with a calcium channel blocker, plasma concentration of TGF-β1 and periodontal bleeding index were identified as risk factors for the condition. Creatinine concentration and

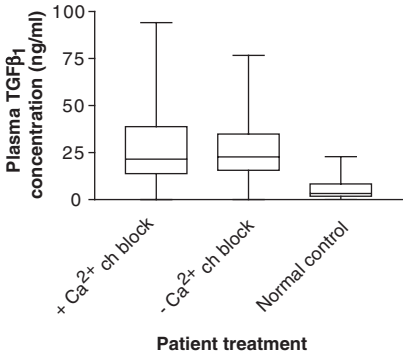

Fig. 1. Mean values of TGF-β1 levels in plasma from immunosuppressed patients medicated with ($n = 79$) and without ($n = 76$) concurrent calcium channel blockers. Control $n = 24$.

Table 1. Univariate regression analysis investigating relationship between gingival overgrowth score in patient group 1 (immunosuppressed including those concurrently medicated with Ca^{2+} blocker) and clinical variables

	Coefficient	*p*-Values	95% CI for regression coefficient
age	− 0.13	0.195	− 3.1, 0.07
gender	− 6.62	0.021	− 12.22, − 1.01
duration	0.07	0.019	0.01, 13
creatinine	− 0.05	0.018	− 0.99, − 0.01
PBI	2.08	0.001	0.88, 3.27
PI	0.18	0.026	− 0.43, 0.8
medicated with calcium channel blocker	5.85	0.024	0.78, 10.9
plasma TGF-β1	− 0.16	0.028	− 0.297, − 00.02

PBI, papillary bleeding index; PI, plaque index.

Table 2. Backward stepwise regression analysis investigating relationship between gingival overgrowth score in patient Group 1 (immunosuppressed including those concurrently medicated with Ca^{2+} blocker) and clinical variables

	Coefficient	*p*-Value	95% CI
age	− 0.33	< 0.0001	− 0.50, − 0.15
gender	− 5.59	0.032	− 10.7, − 0.50
medicated with calcium channel blocker	8.44	< 0.0001	4.06, 12.8
plasma TGF-β1	− 0.14	0.023	− 0.26, − 0.02
PBI	3.34	< 0.0001	1.96, 4.73
constant	32.9	< 0.0001	19.33, 46.5

PBI, papillary bleeding index.
Adjusted R^2 30.5%. Creatinine and duration of therapy dropped from model.

Table 3. Univariate regression analysis investigating relationship between gingival overgrowth score in patient Group 2 (immunosuppressed not medicated with Ca^{2+} blocker) and clinical variables

	Coefficient	*p*-Values	95% CI for regression coefficient
age	−0.40	0.002	−0.66, −0.15
gender	−3.70	0.43	−13.0, 5.62
duration	0.05	0.208	−0.03, 0.13
creatinine	0.01	0.73	−0.05, 0.07
PBI	14.2	<0.0001	7.29, 21.2
PI	5.16	0.016	0.99, 9.33
plasma TGF-β1	−0.25	0.021	−0.45, −0.04

PBI, papillary bleeding index; PI, plaque index.

Table 4. Backward stepwise regression analysis investigating relationship between gingival overgrowth score in patient Group 2 (immunosuppressed not medicated with Ca^{2+} blocker) and clinical variables

	Coefficient	*p*-Value	95% CI
age	−0.38	0.001	−0.60, −0.16
plasma TGF-β1	−0.29	0.001	−0.46, −0.13
PBI	14.6	<0.0001	8.41, 20.7
constant	46.4	<0.0001	33.9, 58.8

PBI, papillary bleeding index.
Adjusted R^2 43.9% creatinine, plaque score, duration of therapy and gender dropped from the model.

duration of therapy were dropped from the final model. The adjusted R^2 value for the model was 30.5%. Reworking the model omitting plasma concentration of TGF-β1 resulted in the introduction of duration of therapy into the model, but although more subjects were included in the model ($N = 145$), the adjusted R^2 value was only 16.8%, illustrating the importance of plasma concentration of TGF-β1 (data not shown).

The results of the univariate and multivariate regression analyses for Group 2 patients (immunosuppressed transplant patients not medicated with calcium channel blocker) are reported in Tables 3 and 4. When analysed univariately, plasma TGF-β1, age, papillary bleeding index and plaque score were identified as risk factors for gingival overgrowth score at the 5% level (Table 3). However, when analysed using multivariate modelling, only age, papillary bleeding index and plasma concentration of TGF-β1 were identified as risk factors once adjusted for the effect of other variables in the model (Table 4). The adjusted R^2 value for the model was 46.8%.

Discussion

The rationale of the study was to determine if elevations of TGF-β1 found in other fibrotic conditions were also present in GO and to relate this to the severity of the gingival changes both univariately and after adjustment for the effect of other known risk factors for this condition.

Evidence from histological studies suggests that TGF-β1 (and other isoforms) are elevated in the gingival tissues of patients suffering from drug-induced GO (James et al. 1998, Wright et al. 2001). Although one study failed to find any increased expression of TGF-β1 in gingival tissues from (ciclosporin-A) (CsA)-treated patients with clinically significant GO (Uzel et al. 2001), there are some subtle differences in the tissue localization of TGF-β1 in GO and it has been suggested that this may be due to the source and/or epitope specificity of the antibodies employed in the various studies (Wright et al. 2001). To date there have been no studies directly comparing circulating TGF-β1 levels in GO with TGF-β1 in gingival tissues from the same patients.

Concern has been expressed that variation in the analytical techniques used and in the sample preparation might influence the accuracy of TGF-β1 measurement in studies of circulating TGF (Kropf et al. 1997, Fredericks & Holt 1999, Grainger et al. 2000). However, there are a number of studies which report that CsA therapy increases the circulating levels of TGF-β1, and the findings of the present report are consistent with these findings (Coupes 1994, El-Garnel et al. 1998, Shin et al. 1998). Nevertheless, other reports suggest that neither tacrolimus nor ciclosporin influences circulating TGF-β1 (Hughes et al. 1999, Coupes et al. 2001).

Elevated levels of TGF-β1 were found in plasma from all immunosuppressed patients, irrespective of their GO status. The quantitative relationship between circulating TGF-β1 and GO is not clear, although, circulating levels are higher in patient's immunosuppressed with tacrolimus and ciclosporin. The adjusted R^2 for the models were greater when TGF-β1 was included, illustrating the importance of this variable. The unexpected finding, however, was the nature of the relationship, as our statistical modelling data indicate a significant inverse correlation between TGF-β1 levels and GO, suggesting that patients are at greater risk of more severe overgrowth changes with a lower rather than a higher TGF concentration. Although this finding is counter-intuitive, there are a number of potential explanations for this.

Immunosuppressive agents given to transplant recipients may increase TGF-β1 levels, hence the raised levels compared to controls. However, the effect of these drugs on TGF-β1 production may reach a steady state and then start to decline as immunosuppression fails. If this were the case then a relationship might be expected between duration of therapy and or creatinine levels. In initial univariate analysis of subjects concurrently medicated with calcium channel blockers, both of these variables are seen to have a statistically significant relationship with GO scores. The positive correlation with duration would support this theory although the inverse relationship with creatinine levels does not. A longitudinal study examining circulating TGF-β1 levels from the initiation of immunosuppressive therapy and relating these not only to creatinine levels but also to the gingival status may establish whether this is the case.

Local levels of TGF-β1 are likely to be more informative and plasma levels may not necessarily mirror local tissue levels of TGF-β1. It is important to note that, associated with its role in fibrogenesis and repair, TGF-β1 has a major role in many aspects of the immune response and is particularly associated with immunosuppression (Letterio & Roberts 1998). TGF-β1 secreting T-cells form an important subset of T-cells with a

critical role in immune regulation and T-cell homeostasis (Goerlick & Flavell 2002). It is interesting to note that our model identified papillary bleeding index (as a measure of tissue inflammation) was a risk factor for GO, supporting previously published findings (Thomason et al. 1995, Thomason et al. 1996). It is possible that TGF-β1, induced by immunosuppressive drug therapy, has a systemic and/or local effect on immunoregulation which, through interaction with other risk factors, influences the development and severity of GO. Thus, the assumption that circulating levels are indicative of the local tissue and gingival crevicular fluid concentration of TGF-β1 may be inappropriate, and it may be that the local levels are more significant in the development of overgrowth.

An obvious interpretation of the inverse correlation of plasma TGF-β1 and GO in individual patients is that increasing circulating levels of TGF-β1 are protective against GO. Although this is not consistent with the known profibrotic effects of TGF-β1, any mechanism for this relationship would at this stage be purely speculative.

Taken together, published data support the hypothesis that both ciclosporin and tacrolimus increase plasma TGF-β1 and that TGF-β1 may have a role in mediating pathological tissue changes induced by both these drugs. Increased circulating levels of TGF-β1 observed in our study could reflect a systemic effect of these drugs but the role of TGF-β1 in the pathogenesis of gingival overgrowth remains obscure.

The pathogenesis of this disorder is a complex process and numerous aspects at molecular, cellular, tissue and systemic level must be further investigated to gain a complete understanding of a condition. TGF-β1 may to play a role in GO, but in isolation, this growth factor is not the sole factor affecting development and severity of the condition although the inverse relationship between circulating TGF-β1 and GO, and the relationship between circulating TGF-β1 tissue and gingival crevicular fluid levels of TGF-β1 requires further investigation in the form of longitudinal studies.

Conclusion

Immunosuppressed transplant patients are at risk of GO. Risk factors for the severity of changes include: (1) Low plasma TGF-β1 concentrations, (2) reduced age, and (3) gingival inflammation. The mechanisms controlling susceptibility and severity of drug-induced gingival overgrowth warrant further investigation.

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