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Case Report

# Morphological and molecular analysis of idiopathic gingival fibromatosis: a case report

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***Abstract***

**Aim:** We analyse a case of idiopathic gingival overgrowth using morphological and molecular methods. As this overgrowth involves collagen accumulation in the gingival connective tissue, we measured the collagen turnover to clarify the pathogenic mechanisms potentially involved.

**Materials and Methods:** The patient was a 29-year-old Italian woman with enlargement of the gingivae throughout the entire mandible and maxilla. Morphological analyses were carried out on haematoxylin–eosin and Sirius red-stained paraffin-embedded gingival sections. mRNA levels of collagen type I and III, matrix metalloproteinase (MMP)-1, transforming growth factor-$\beta$1 and lysyl hydroxylase (LH)2b were determined by RT-PCR on cultured gingival fibroblasts and compared with healthy control fibroblasts. Interstitial collagen and MMP-1 content in the supernatants were assessed, respectively, by dot blot and SDS zymography.

**Results and Conclusions:** In Sirius red-stained sections of the patient's overgrown gingivae, interstitial collagen content was 29% higher than controls. Her gingival fibroblasts had higher collagen type I, MMP-1 and LH2b gene expression and unmodified interstitial collagen, type I protein levels in the supernatants. These findings would seem to suggest that in this case collagen accumulation in the gingival connective tissue was not associated with increased synthesis and decreased degradation.

Key words: collagen turnover; gingival overgrowth; idiopathic gingival fibromatosis

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Gingival overgrowth (GO) is defined as abnormal growth of maxillary and mandibular gingivae. GO causes aesthetic changes and clinical symptoms such as pain, speech disturbances, abnormal tooth movement, dental occlusion problems, enhanced risk of caries and periodontal disorders (Brunet et al. 1996). GO is associated with multiple factors including inflammation, drug use (Hassell & Hefti 1991, Seymour & Jacobs 1992, Dongari et al. 1993), neoplasia, hormonal disturbances and hereditary factors; however, in some idiopathic cases, its pathogenesis remains unknown (Brunet et al. 1996, Sakamoto et al. 2002).

Drug-induced GO has been extensively characterized in humans and in experimental models, and it would appear that uncontrolled growth of gingival connective tissue occurs because of increased deposition of extracellular matrix (ECM) components, particularly interstitial collagen; altered collagen turnover may be the main switch for GO development (Bolzani et al. 2000; Kataoka et al. 2000, Hyland et al. 2003).

Interstitial collagen, particularly type I (COL-I), is one of the major components of the ECM of gingival connective tissue. Its content is determined by the finely tuned balance between synthesis and degradation by matrix metalloproteinases (MMP) (Woessner 1991, Birkedal-Hansen 1993). If these regulatory mechanisms are deranged, collagen may increase, leading to GO. This process may involve some cytokines, such as transforming growth factor (TGF)-$\beta$1, which is the major mediator influencing collagen turnover (Ignotz & Massague 1986, Sporn et al. 1987, Sporn & Roberts 1990, Border & Ruoslahti 1992).

Collagen biosynthesis and ECM stability is also determined by post-translational hydroxylation of collagen lysine residues, accomplished by lysyl hydroxylase (LH). Therefore, any perturbation of LH levels could contribute to specific connective tissue disorders (Walker et al. 2005).

The aim of this study was to characterize by morphological and molecular approaches a case of idiopathic GO in a 29-year-old woman, to cast some light on the pathogenic mechanisms.

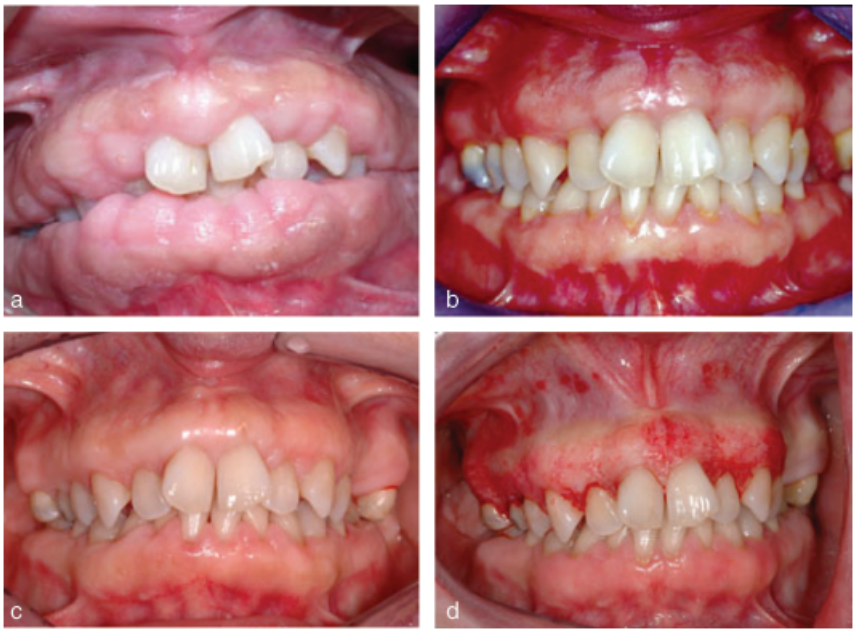


*Fig. 1*. (a) Clinical photograph showing gingival overgrowth. The gingivae are enlarged over the entire mandible and maxilla. The gingivae were normal in colour. (b) Gingival status after the first surgery treatment. (c) Changes in gingival morphology at the end of the first year, showing enlargements in buccal aspects. (d) Gingival tissues after post-treatment adjustments.

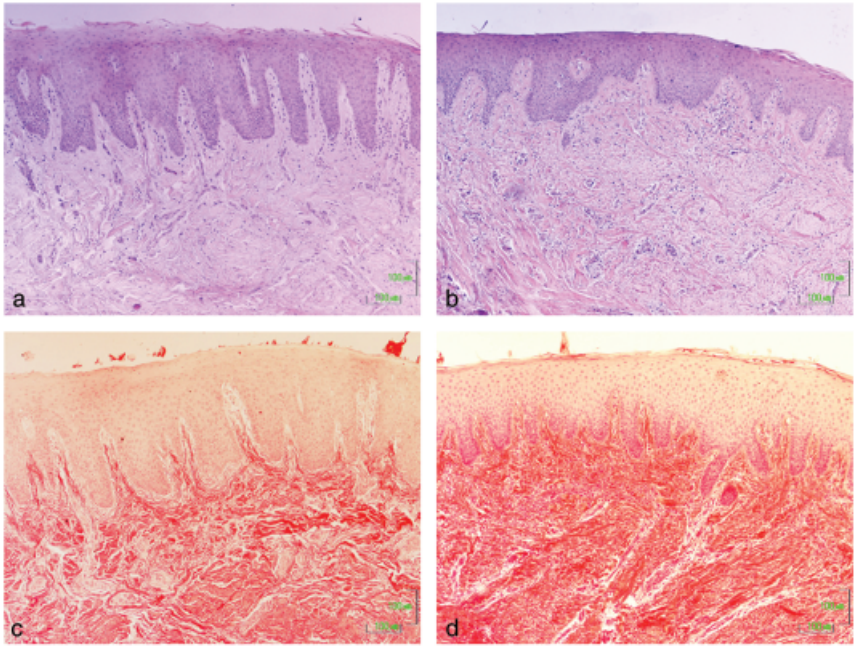


*Fig. 2*. Microphotographs of controls (CT) and gingival overgrowth (GO) gingiva paraffin-embedded sections stained with haematoxylin–eosin (a, b) or with Sirius red (c, d). (a, c): CT; (b, d): GO. Scale bar is 100 $\mu$m.

## Material and Methods

All the experiments were undertaken with the understanding and written consent of each subject and according to the World Medical Association Declaration of Helsinki.

### Clinical history

A 29-year-old woman presented herself at our University Clinic of Milan with generalized gingival swelling that caused speaking, eating and aesthetic problems as well as clinical and radiographic signs of periodontal and dental disease. Her family history was not significant for disease transmission. Her medical history was not indicative of drug-induced gingival enlargement or hormonal changes. Blood and gingival cariotypes were analysed, but no abnormalities were detected.

Intra-oral examination found hyperplastic gingivae at both arches, covering almost all the teeth on their buccal and lingual aspects; soft tissue was normal in colour but the shape and size were abnormal (Fig. 1a).

Gingival enlargement had started at the time of eruption of permanent dentition; this fibromatosis had influenced the position of her teeth so the dentition appeared with diastemas and malpositioning. She had many cavities and poor plaque control. Panoramic radiographic examination revealed destructive cavities, bone loss and periapical lesions.

### Surgery

The patient was first treated with an external bevel gingivectomy under general anaesthesia; during surgery, a diode laser was used to recontour gingival tissue and control bleeding. Nine fragments were removed, the largest being 13 cm long (Fig. 1b).

### Morphological and image analysis

Immediately after surgery, each gingival biopsy fragment (1 $cm^2$) was fixed in 4% formalin in 0.1 M phosphate buffer saline (PBS), pH 7.4, for 5 h at room temperature, routinely dehydrated, paraffin embedded and serially sectioned (thickness 5 $\mu$m). Sections were haematoxylin–eosin and Sirius red stained. For Sirius red staining, slides were deparaffinized and immersed for 15 min. in saturated aqueous picric acid containing 0.1% Sirius red F3BA (Sigma, Milan, Italy), a stain specific for collagen that stains collagenous proteins distinctly red.

The images were captured and digitized using an image analysis system with specific software (Isole, ICH, Italy) (Dioguardi et al. 2003). The software automatically selects the collagenous portion on the basis of similarities in the colour of adjacent pixels.

### Cell culture

Human gingival fibroblasts were obtained from the GO patient, and

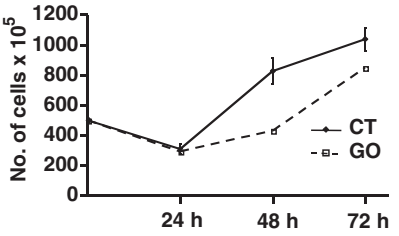


*Fig. 3*. Proliferation of cultured controls (CT) and gingival overgrowth (GO) gingival fibroblasts at the indicated intervals of time. Cells were plated in T-75 flasks (500,000 cells/flask) and allowed to attach.

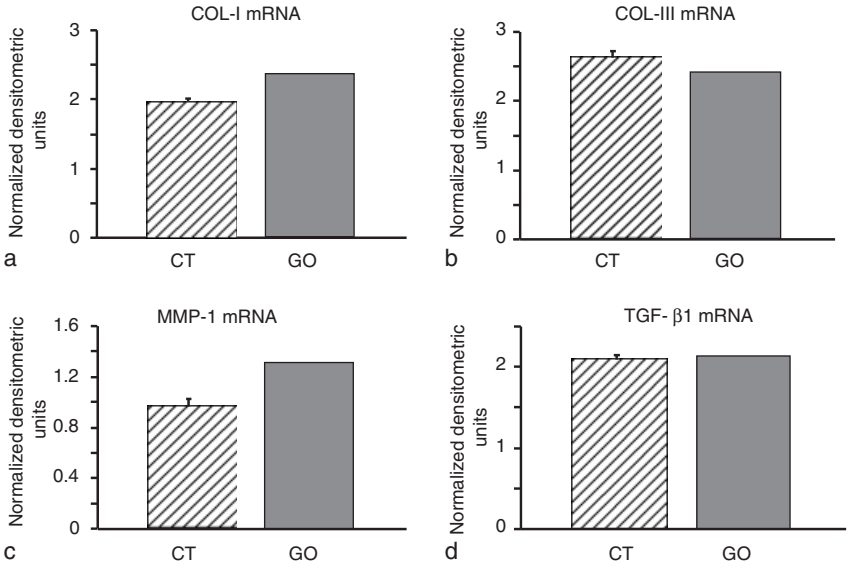


*Fig. 4*. Bar graphs showing interstitial collagen type I (COL-I) (a), COL-III (b), matrix metalloproteinase (MMP-1) (c) and transforming growth factor (TGF)-$\beta$1 (d) mRNA levels in controls (CT) and gingival overgrowth (GO) cultured fibroblasts. Changes in mRNA are expressed as normalized optical densities relative to GAPDH mRNA. Values are means ± SEM for duplicate samples.

from five healthy non-smoking volunteers (two males and three females, aged 20–32 years) as controls (CT). All CT had clinically normal gums with no signs of inflammation, hyperplasia and no history of use of drugs associated with GO.

Gingival biopsies were washed with sterile PBS, plated in T-25 flasks, incubated in DMEM supplemented with 10% heat-inactivated foetal bovine serum and antibiotics (10 U/ml penicillin, 10 mg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% $CO_2$. When fibroblasts grew out from the explant, they were trypsinized (0.1% trypsin–0.02% EDTA) for secondary cultures. Viability was assessed by the Trypan blue exclusion method. Human gingival fibroblasts were used between the fourth and fifth passage.

Molecular evaluations were performed on fibroblasts cultured for 72 h, using duplicate cultures for each sample.

#### RT-PCR

Total RNA was extracted from fibroblasts using a modification of the guanidine isothiocyanate method (Tri-Reagent, Sigma). One microgram of total RNA was DNase I digested and then reverse transcribed in 20 $\mu$l final volume of reaction mix (Promega, Milan, Italy).

The following primers were used for RT-PCR: GAPDH 5′-ATTCCATGGCACCGTCAAGGCT, 3′-TCAGGTCCACCACTGACACGTT (571 bp); COL-I 5′-GGCGGCCAGGGCTCCGAC, 3′-AATTCCTGGTCTGGGGCACC (347 bp); COL-III 5′-TGG TGT TGG AGC CGC TGC CA, 3′-CTC AGC ACT AGA ATC TGT CC (376 bp); MMP-1 5′-GGT GAT GAA GCA GCC CAG, 3′-CAG TAG AAT GGG AGA GTC (437 bp); TGF-$\beta$1 5′-CAGAAATACAG CAACAATTCCTGG, 3′-TTGCAGTG TGTTATCCCTGCTGTC (186 bp); and LH2b 5′-AGC TGT GGT CCA ATT TCT GG, 3′-GCA ATG AAC TCC CG G ATA AA (688 bp).

Amplification reactions were conducted in a final volume of 25 $\mu$l containing 2.5 $\mu$l of cDNA, 200 $\mu$M of the four dNTPs, 100 pmol of each primer and 2.5 U of Taq DNA polymerase (Euro-Taq, Euroclone, Milan, Italy). The RT-PCR protocols used are the following: COL-I: denaturation, 94°C (1 min.); annealing, 61°C (90 s); elongation, 72°C (2 min.); 35 cycles. COL-III: denaturation, 94°C (1 min.); annealing, 55°C (90 s); elongation, 72°C (2 min.); 32 cycles. MMP-1: denaturation, 94°C (30 s); annealing, 53°C (1 min.); elongation, 72°C (1 min.) +72°C (10 min.) to finalize extension; 32 cycles. TGF-$\beta$1: denaturation, 94°C (1 min.); annealing, 60°C (90 s); elongation, 72°C (2 min.); cycles 35. GAPDH: denaturation, 94°C (30 s); annealing, 62°C (1 min.); elongation, 72°C (1 min.) +72°C (10 min.) to finalize extension; cycles 25. LH2b: denaturation, 94°C (1 min.); annealing, 55°C (90 s); elongation, 72°C (1 min.); 35 cycles.

The RT-PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide and quantified by densitometric analysis (Image Pro-Plus, Silver Spring, MD, USA). The results were normalized on GAPDH gene expression.

#### Dot blot

Cell culture media from CT and GO fibroblasts were concentrated 20-fold with Centricon 10 columns (Amicon, Millipore, Milan, Italy). Protein content was determined by a standardized colorimetric assay (DC Protein Assay, Bio Rad, Milan, Italy); 20 $\mu$g of total protein per sample in a final volume of 200 $\mu$l of Tris buffer saline (TBS) was spotted onto a nitrocellulose membrane in a Bio-Dot SF apparatus (Bio Rad). Membranes were blocked for 1 h with 5% skimmed milk in TBST (TBS containing 0.05% tween-20), pH 8, and incubated overnight at 4°C in monoclonal antibody to COL-I (1:1000 in TBST) or to COL-III (1:1000). After washing, membranes were incubated in HRP-conjugated rabbit anti-mouse serum (1:40,000) for 1 h. Immunoreactive bands revealed by the Opti-4CN substrate (Amplified Opti-4CN, Bio Rad) were scanned densitometrically.

#### SDS zymography

Concentrated culture media were mixed 3:1 with sample buffer (containing 10% SDS). Samples (5 $\mu$g total protein per sample) were run under non-reducing conditions without heat denaturation onto 7.5% polyacrylamide gel (SDS-PAGE) co-polymerized with 1 mg/ml of type I gelatin. The gels were run at 4°C. After SDS-PAGE, the gels were

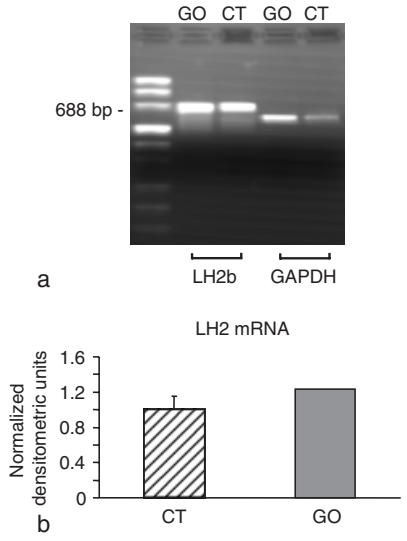


*Fig. 5.* (a) Representative RT-PCR for lysyl hydroxylase (LH)2b. The PCR products were electrophoresed on a 2.5% agarose gel. (b) Bar graphs showing LH2b mRNA levels in controls (CT) and gingival overgrowth (GO) cultured fibroblasts. Changes in mRNA are expressed as normalized optical densities relative to GAPDH mRNA. Values are means ± SEM for duplicate samples.

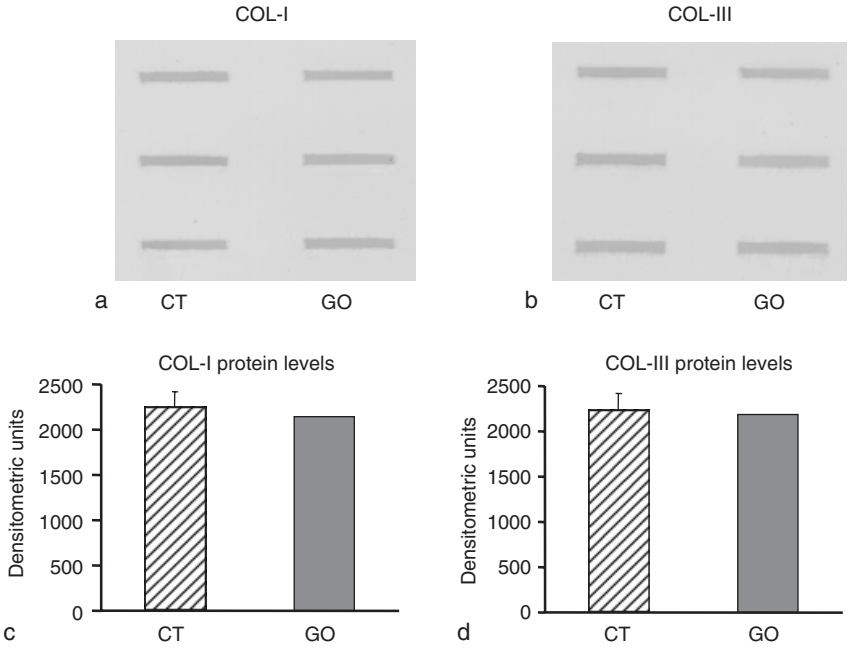


*Fig. 6.* Dot blot showing interstitial collagen type I (COL-I) (a) and COL-III (b) immunoreactive bands in fibroblast supernatants. Bar graph presenting COL-I (c) and COL-III (d) protein levels assessed by dot blot analysis. Data are reported as densitometric units after scanning of the immunoreactive bands. Values are means ± SEM for duplicate samples.

washed twice in 2.5% Triton X-100 for 30 min. each and incubated overnight in a substrate buffer at 37°C (Tris-HCl 50 mM, $CaCl_2$ 5 mM, $NaN_3$ 0.02%, pH 7.5). The MMP gelatinolytic activity was detected after staining the gels with Coomassie brilliant blue R250, as clear bands on a blue background (Kleiner & Stetler-Stevenson 1994, Gagliano et al. 2005). To confirm the identity of this activity, purified MMP-1 and MMP-2 (100 ng, Calbiochem, San Diego, CA, USA) were run as standards.

## Results

### Clinical results

The post-surgical period was uneventful and when we saw the patient 15 days later tissue healing was good, although a little enlargement persisted at the VI sextant lingual. All hyperplastic tissue around the teeth was removed 2 months after the first surgery, with no apparent re-growth for several months.

At the end of the first year, there were some changes in gingival morphology, first in its superficial texture, and then enlargements in both the buccal and palatal-lingual aspects (Fig. 1c); a gingivectomy was performed again (Fig. 1d). During the post-treatment controls, the patient was checked for plaque and bleeding index and, if necessary, locally enlarged gingival tissues were adjusted under local anaesthesia with or without a laser surgical procedure.

### Morphological and image analysis

Under light microscopy haematoxylin–eosin-stained sections of CT and GO samples appeared similar (Fig. 2a, b). In the epithelium of GO specimens, there was no acanthosis, i.e. an epithelium with an increased number of the living cells layers; the connective tissue included an increased amount of collagen fibre bundles running in all directions, with some inflammatory cell infiltration.

Sirius red stained the collagen specifically, and computerized analysis of sections of gingiva indicated the fibrotic area had a 29% higher collagen content in the connective tissue of GO than CT (Fig. 2c, d).

### Cell viability and proliferation

Phase-contrast microscopy analysis of CT and GO fibroblasts in culture did not indicate any real difference in their morphology. However, there were fewer GO fibroblasts at all the intervals considered: after 24, 48 and 72 h these reductions amounted to 6%, 49% and 15% compared with CT (Fig. 3).

### Molecular analysis

The molecular analysis results are shown in Fig. 4. COL-I gene expression tended to be higher in GO fibroblasts than CT (20%) (Fig. 4a) but COL-III mRNA levels were almost unchanged in both (Fig. 4b). MMP-1 mRNA levels tended to be expressed more in GO than CT (37%) (Fig. 4c), while TGF-$\beta$1 gene expression was virtually the same (Fig. 4d). LH2b mRNA levels in GO fibroblasts were higher, compared with the mean LH2b gene expression determined in CT (Fig. 5a, b).

### Dot blot

COL-I and COL-III content was similar in CT and GO fibroblast supernatants (Fig. 6).

### SDS zymography

MMP-1 expression in CT and GO fibroblast supernatants is presented in Fig. 7. The densitometric analysis of lytic bands identified as proMMP-1 revealed higher latent interstitial collagenase levels in the GO sample compared with the mean levels observed in CT (32.5%). Two other lysis band are evident, consistent with proMMP-2 and proMMP-9; the densitometric analysis

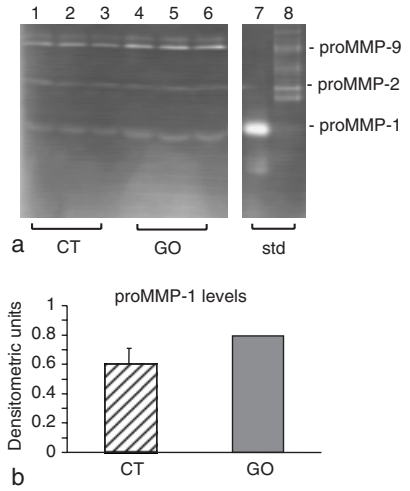


*Fig. 7.* Representative gelatin zymogram of matrix metalloproteinases (MMPs) in conditioned human gingival fibroblast supernatants from controls (CT) and gingival overgrowth (GO) cultured for 72 h. The lytic bands weighing 66 kDa and in the 60/50 kDa region correspond to proMMP-1 and proMMP-2, respectively, as confirmed by purified MMP-1 (lane 7) and MMP-2 (lane 8) electrophoretic patterns. The lytic bands with higher molecular weight are consistent with proMMP-9. Lanes 1–3: representative CT supernatant run in triplicate. Lanes 4–6: GO supernatant run in triplicate. STD: purified MMP-1 and MMP-2. (b) proMMP-1 amounts in fibroblast conditioned media after densitometric analysis of lytic bands following SDS zymography. Data are mean SEM of duplicate samples.

of lysis band revealed a similar expression of proMMP-2 and increased proMMP-9 levels (34%) in GO, compared with CT.

## Discussion

Drug therapy and hereditary factors are two of the main causes of GO. Both forms involve increased ECM accumulation. We analysed the cellular and molecular processes in the case of idiopathic GO, to explore the possible biological mechanisms underlying its pathogenesis, with particular attention to the collagen component of ECM. The patient was diagnosed as having ''idiopathic'' gingival fibromatosis because her medical and family histories were unremarkable, her medical history was not indicative of drug-induced gingival enlargement or hormonal changes and there was no evidence of genetic transmission. Moreover, blood and gingival cariotypes were analysed, but no abnormalities were detected.

Every 2–3 months after the first surgery and after 1 year of supportive periodontal therapy, we examined the patient and decided for surgery again, with a new external-bevel gingivectomy. We are now following-up the patient's periodontal status after this treatment and maintain periodontal health with periodic surgical correction of gingival form and architecture.

Morphological and quantitative analysis of the GO biopsies showed a higher collagen content in the gingival connective compartment in GO than CT. This finding may account for the enlargement of the gingivae in the patient. However, the absence of acanthotic gingival epithelium, typical of the gingival lesions in hereditary gingival fibromatosis (Tipton et al. 1997, Hart et al. 1998), may also be a characterizing feature of this case of idiopathic GO.

As interstitial collagen accumulation is the main feature of GO, we investigated the overall mechanisms of collagen turnover, related to both its synthesis and degradation in the gingival fibroblasts from the GO and CT subjects.

Interstitial collagen accumulation in gingival connective tissue may be the result of an imbalance between synthesis and breakdown. Our in vitro investigations show that, although there was an increase of COL-I mRNA levels in GO fibroblasts, COL-I protein levels and COL-III gene and protein were similarly expressed in CT and GO samples, as also TGF-$\beta$1 mRNA. The TGF-$\beta$1 gene expression suggests that the situation in this patient was different from hereditary gingival fibromatosis, as cultured gingival fibroblasts from affected individuals generally produce higher levels of TGF-$\beta$1 (Tipton et al. 1997, Tipton & Dabbous. 1998, Wright et al. 2001). However, on the basis of our molecular findings, together with the less proliferation of GO fibroblasts than CT, we can speculate that in this case the fibroblasts do not display an activated-synthesis phenotype, as expected and previously described in hereditary gingival fibromatosis (Fries et al. 1994, Tipton et al. 1997).

With respect to collagen degradation, interstitial collagenase seemed to be upregulated at the mRNA level (MMP-1) and at the protein level (MMP-1, MMP-9) in GO fibroblasts. This pattern is different from that characterizing cyclosporin A-induced GO (Gagliano et al. 2004). Therefore, decreased breakdown of interstitial collagen does not seem to be the main molecular mechanism responsible in this case of idiopathic fibromatosis.

As the in vitro evaluations suggest that fibroblasts from both CT and GO subjects have a similar phenotype in relation to the pathways involved in collagen synthesis and degradation, we can hypothesize that the increased collagen deposition in this patient may be a consequence of post-translational mechanisms, quite likely because of increased collagen cross-links that render the molecule less susceptible to MMP degradation (Kagan 1986), thus favouring its accumulation in the gingival connective compartment. This suggestion is confirmed by the higher long form of LH2 mRNA levels observed in the GO fibroblasts. LH2b, in fact, exists as two alternatively spliced forms, the long and the short ones (Walker et al. 2005). LH2b is the major transcript expressed in all tissues and recent reports suggest that increases in the long LH2 transcript are associated with changes in the pattern of collagen cross-linking (Mercer et al. 2003), and that LH2b is responsible for overhydroxylation of the collagen telopeptides and the concomitant formation of pyridinoline cross-links in the ECM (Van der Slot et al. 2004).

The overall picture suggests that the biological origin of gingival fibromatosis is complex and not all forms of GO are the same, and that there is likely to be more than one biological mechanism in GO development. In fact, gingival fibromatosis may exist as an isolated abnormality or as a part of a syndrome, and recent genetic studies identified a specific gene mutation that segregates with the hereditary gingival fibromatosis phenotype (Hart et al. 2002). The results of our study, although obtained from a single particular case, may contribute to identification of the molecular and cellular processes leading to the understanding of the biological mechanisms underlying the overgrowth.

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**Clinical Relevance**

*Scientific rationale*: If the finely tuned mechanisms of collagen turnover are deranged, an increase of collagen may occur in the gingival connective tissue, leading to GO.

*Principal findings*: In this case of idiopathic GO, collagen accumulation in the gingival connective tissue was not associated with increased synthesis and decreased degradation.

*Practical implications*: Shedding light on the molecular mechanisms leading to GO could offer new therapeutic approaches to replace the repeated surgical reductions that constitute the current management of this pathology.