

Heterogeneity of collagen secreting cells in gingival fibrosis – an immunohistochemical assessment and a review of the literature

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Abstract

Aim: In this work, we compared the histological features of the gingival lesions clinically diagnosed as fibrotic overgrowths due to various etiologic factors as well as an immunohistochemical assessment of fibroblasts phenotypic heterogeneity using the specific labeling for vimentin, α -smooth muscle actin (α -SMA) and fibroblast specific protein-1 (FSP1). **Materials and Methods:** Tissue samples were obtained from 12 patients clinically diagnosed with fibrotic gingival overgrowth, divided in four groups. Fragments of gingiva were processed for paraffin embedding. Serial sections were used for routine staining Hematoxylin–Eosin, trichromic Masson and Goldner–Szekely, and for immunohistochemical reactions to label vimentin, α -SMA and FSP1 using for signal amplification several techniques (EnVision, LSAB, ABC). **Results:** Storage of collagen fibers, increase of fibroblast number and frequent presence of inflammatory infiltrate are histological issues of all fibrotic gingival overgrowth. The incidence of granulation tissue varies but the frequency of its presence point the attention to the involvement in collagen metabolism imbalance. Immunostaining for vimentin showed a difference between its expression in samples from different groups. Except the cases of fibrosis induced by orthodontic devices, cells positive for α -SMA were rare. FSP1-positive fibroblasts were the most frequent in all cases from all the groups selected for this study. **Conclusions:** The phenotype of fibroblasts is different in gingival fibrosis in relation to the risk factor, at present the most common being vimentin-positive and FSP1-positive fibroblasts. Myofibroblasts are rare in gingival fibrosis, the most numerous being in local lesions caused by wearing orthodontic devices and in syndromic fibromatosis. Further studies are required to elucidate the manner in which the active fibroblasts are recruited in relation to the etiologic factor of gingival overgrowth.

Keywords: gingival fibromatosis, fibroblasts, immunohistochemistry, vimentin, α -SMA, FSP1.

Introduction

Gingival overgrowth (GO) is defined clinically as the thickening or increase the volume of the soft tissues covering the alveolar ridge by more than 1 mm, the degree of overgrowing being variable from restriction to the interdentally papilla to covering the entire tooth crown [1–4].

The current recommendation is the use of “gingival overgrowth” to replace the previously terms “gingival hyperplasia” or “gingival hypertrophy” in order to include the two major changes that cause this pathology, *i.e.*, increase of cell number and enhancing the volume of structural elements of gingival mucosa [1].

There are several criteria for the classification of GO, the most common being the use of etiological factors that determine clinically detected lesion, local or general – systemic, factors.

The most common forms of GO are those determined by local irritation: reactive, due to the existence of bacterial plaque called focal reactive gingival overgrowth (FRGO), inflammatory hyperplasia or epulis, or GO caused by root residues, caries or faulty prosthesis [5].

General causes for GO are the treatment with some anti-convulsant drugs such as phenytoin, immunosuppressives – cyclosporine A, antihypertensive calcium channel blockers – nifedipine, GO determined by systemic diseases – diabetes mellitus, leukemia, or GO caused by hormonal imbalance arising during puberty or pregnancy called epulis [6].

Gingival fibromatosis (GF) occupy a special place and is also known as gingival elephantiasis, idiopathic gingival fibromatosis, hereditary gingival hyperplasia, non-bacterial plaque gingival lesion, gingival gigantism or just hypertrophic gum [7, 8].

Gingival fibromatosis, a very rare lesion – 1:750 000 [9], could be: (i) inherited or isolated, called also non-syndromic or type 1 GF, and (ii) syndromic GF.

Type 1 GF seems to be determined by the mutation of SOS-1 (*Sun of sevenless-1*) gene on 2p21–p22 chromosome. For the first time, this mutation was described in a large Brazilian family [10] and named GINGF1 (*Mendelian Inheritance in Man classification MIM135300*) [11]. Recently was described the type 2 inherited GF associated with mutation of chromosome 5 called GINGF2 (MIM 605544) [11–14].

Syndromic GF is associated with several clinical signs in some syndromes (Zimmermann–Laband syndrome, Rutherford syndrome, Jones syndrome, Cross syndrome) [12, 14–16]. In this case, gingival events are caused by chromosomal abnormalities (duplications, deletions) of chromosomes 2p12–16 [17, 18], 4q (MIM252500), 8 (MIM266270), 14q [19], 19p (MIM266200), 19q (MIM248500) and Xq [6, 10, 20–26].

Both genetic and syndromic fibromatosis are sometimes called idiopathic fibromatosis [8, 15, 27–31].

To avoid these confusions of classification, it was recommended to limit the term “idiopathic gingival fibromatosis” to the GO that involve neither a genetic nor an inherited cause [6, 16].

Irrespective the risk factor, all forms of clinically detected gingival overgrowth have as a common feature the coexistence of various degrees of inflammatory and fibrotic lesions. For example, drug-induced gingival overgrowth is often named fibrous gingival hyperplasia [16] even if only phenytoin determines fibrotic lesions meanwhile calcium channel blockers, such nifedipine, and cyclosporine A induce mainly inflammatory hyperplasia [7, 22, 12].

Gingival mucosa has an epithelium and the connective tissue with various cell types, mainly fibroblasts, both being incriminated in GO but the cellular and molecular mechanisms involved are incompletely understood.

Due to the reduced incidence of this pathology, the histological features of fibrotic overgrowth are briefly described in literature, especially as results of isolated case reports. Oral epithelium is enlarged and acanthotic, with deep epithelial ridges [32]. According to some authors, the enlargement of lamina propria is due to the accumulation of thick collagen bundles and numerous fibroblasts [14, 28, 33–35]; in contrary, others claimed a reduced number of fibroblasts [36–38].

Collagen storage is the consequence of the imbalance between collagen synthesis and breakdown. Obviously, fibroblasts are the main cells responsible for collagen synthesis in gingival mucosa. Data regarding the mitotic activity of fibroblasts in gingival fibrotic lesions are still controversial. As in phenytoin-induced GO, GF revealed an increased rate of fibroblasts proliferation besides a diminished fibroblast apoptosis [39]. Other authors reported a normal or even diminished mitotic activity of fibroblasts [40].

A phenotypic heterogeneity of fibroblasts was also claimed for the fibrotic gingiva compared with the normal one [9, 41–43].

In this work, we compared the histological features of the gingival lesions clinically diagnosed as fibrotic overgrowths due to various etiologic factors as well as an immunohistochemical assessment of fibroblasts phenotypic heterogeneity using the specific labeling for vimentin (Vim), α -smooth muscle actin (α -SMA) and fibroblast-specific protein-1 (FSP1).

☐ Materials and Methods

Tissue samples were obtained from 12 cases of fibrotic gingival overgrowth collected during 2008–2012. Patients of both sexes included in the study were aged between 7–59 years and after giving their informed consent were

subjected to therapeutic gingivectomy performed in relation to the initial diagnosis and the clinical evolution after treatment in the Clinic of Oro-Maxillo-Facial Surgery of the Emergency County Hospital of Craiova, Romania.

Study groups were set up according to the criteria proposed by the reference papers [6, 10, 11, 15, 22, 27, 30, 35, 39, 44].

Study groups

We established four groups as follows:

- Ist Group – four cases of focal reactive gingival overgrowth (FRGO): patients with localized lesions of the gingival mucosa induced by the bacterial plaque, caries or root residues and three cases of fibrotic GO developed after the use of an orthodontic device;

- IInd Group – two cases of inherited GF: patients coming from families with GO; as an exclusion criteria we took into account the absence of any other clinical symptom for syndromic GF, as well as the lack of any treatment with drugs inducing fibrotic GO (*i.e.*, phenytoin) in the last six months;

- IIIrd Group – two cases of syndromic GF: patients having syndactyly, hypoplasia of the nails and discrete mental retardation;

- IVth Group – three cases of idiopathic GF: gingival changes aroused as isolated clinical manifestations being not associated with one of the conditions used to select the others groups; family, prenatal, medical and therapeutic history of the patients proved as non-contributor to the onset of gingival changes.

Reagents

The reagents needed for the histological staining were purchased from Merck KGaA, Germany and Bio-Optica, Italy. Normal swine serum, mouse monoclonal anti-human α -smooth muscle actin (α -SMA), mouse monoclonal anti-human vimentin, rabbit polyclonal anti-S100A4 and polyclonal swine ‘Multi Link’ were purchased from Dako. Vectastain (Vector Laboratories, USA), LSAB and EnVision (Dako, USA) kits were used to amplify the immune reactions and 3,3’-diaminobenzidine tetrahydrochloride (Sigma-Aldrich Co., USA) and hydrogen peroxide as developers. The anhydrous mounting medium (Neo-Mount), hydrogen peroxide and buffers were purchased from Merck.

Histological analysis

Tissues were fixed in buffered formalin and processed for paraffin embedding. Blocks of paraffin were cut at 3 μ m thicknesses using a Leica microtome, dewaxed, rehydrated and stained with Hematoxylin–Eosin (HE), trichromic Goldner–Szekely and Masson to evaluate collagen storage.

Immunohistochemistry

Serial sections of 3 μ m from all groups were dewaxed in xylene and rehydrated *via* graded alcohols. Antigen retrieval was performed after microwave incubation of sections in citrate buffer, pH 6. Endogenous peroxidase activity was blocked with methanol and 0.3% hydrogen peroxide solution. Sections were treated with normal swine serum in order to block unspecific binding and

then were incubated over night with one of the primary antibodies mentioned in the Table 1 and performed further the next day according to the protocol for each amplifying method selected.

Table 1 – Characteristics of the antibodies used for the immunohistochemical study

Antibody	Source	Dilution	Method
Monoclonal mouse anti-human α -smooth muscle actin (1A4)	Dako M0851	1:100	EnVision
Monoclonal mouse anti-human vimentin (V9)	Dako M0725	1:50	LSAB
Polyclonal rabbit anti-human S100A4	Dako, A5114	1:200	ABC

3,3'-Diaminobenzidine tetrahydrochloride and hydrogen peroxide were used for color development and Mayer's Hematoxylin for nuclear counterstaining. For each antibody tested was performed a negative control replacing the primary antibody with phosphate-buffered saline (PBS), pH 7.4–7.6.

Evaluation

Slides were observed and registered with a Nikon Eclipse microscope coupled to a digital camera. Images were finally processed using the Microsoft Office Picture Manager. The evaluation of the immunohistochemical reactions was done by two different observers according to the following:

- Negative reaction – absence of the brown deposits in analyzed structures;
- Moderate or strong positive reaction according to the intensity of brown deposits in all the microscopic fields observed for each slide.

For the semiquantitative evaluation and comparison of the results, we observed using the objective $\times 20$ five microscopic fields for each of the three sections randomly selected for each case.

Total number of labeled cells was recorded as follows: (-) less than five positive cells; (+) between 5–10 positive cells; (++) between 10–50 positive cells; (+++) more than 50 positive cells.

Results

Histological staining

Serial sections for each case revealed significant difference between groups. The enlargement of the entire gingival mucosa observed in FRGO was determined as much by the thickening of both epithelium and lamina propria (Figure 1a). Collagen deposition in the chorion was in form of thick bands whose intersection leaves islands of inflammatory infiltrate, predominantly subepithelial, especially at the top of the ridges. The epithelium had many deep and branched papillae, presenting areas of parakeratosis alternating with others of hyperkeratosis (Figure 1b).

Detailed views of cross-sections revealed an increased amount of ECM in the papillae, with few cells and blood vessels, but increased density of connective fibers, which transform the loose in a dense connective tissue.

The interface between epithelium and lamina propria often appears irregular, interrupted by cells similar to those from profound epithelial layers. These cells, which seem located in the superficial chorion, in proximity of the

basal lamina, are easily distinguished from the inflammatory cells located deep in the epithelium (Figure 1c).

In these cases, mucosa lining the gingival sulcus has a much-thickened epithelium, with swollen superficial cells or presenting areas of parakeratosis and intense inflammatory cells infiltration. At this level, we observed frequently areas of acanthosis and acantholysis (Figure 1d).

Detailed examination of slides obtained from the cases with FRGO reveals inflammatory infiltration predominantly located at the tip of epithelial ridges and discrete in the angles between collagen bundles. The accumulation of collagen occurs sometimes in thick hyalinized bundles including a small number of nuclei (Figure 1e). The inflammatory infiltrate is composed of lymphocytes and plasma cells but also macrophages (Figure 1f).

Samples obtained from the cases of fibrotic GO developed after the use of an orthodontic device revealed the morphological features previously described. Epithelial thickness and keratinization of superficial layers were more obvious, with many areas of parakeratosis in the epithelium of the fixed mucosa. We also noticed discrete areas with ulcerative lesions in the sulcular epithelium besides the epithelial and subepithelial important inflammatory infiltration previously described (data not shown).

Microscopic changes revealed after the examination of histological samples with non-syndromic inherited GF (IInd Group) and syndromic (IIIrd Group) were very similar so they will be presented together.

Figure 2 (a and b) show an overview of two different cases of GF from the IInd and IIIrd Groups, respectively. It is noted an overall thickening of the mucosa with acanthosis, deep and branching epithelial ridges sometimes joined by epithelial bridges. Orthokeratosis was constantly observed in the superficial epithelium. Subjacent chorion is full of thick collagen bundles, sometimes intersecting or with tortuous aspect. Collagen bands rarely leave spaces between them may even have the appearance of a dense and homogeneous structure where the limit between the collagen bundles becomes indistinguishable. Another aspect constantly observed is the deleting of microscopic differences between superficial, papillary chorion, normally composed of loose connective tissue, and deep, dense chorion (Figure 2, a and c).

Detailed images of cross-sections through the chorionic papillae showed that the basal epithelial layer is composed of cells with different aspects than the keratinocytes that represent the majority population. The latter often have nuclei with mitotic figures and among them stand cells with smaller and hyperchromic nuclei or cells without adhesion to adjacent cells, showing a clear pericellular halo. Most of these “clear cell” that can be identified in histological stains are lymphocytes that infiltrate the superficial chorion. However, we identified also cells that seem to split from the others, having a different histological appearance from the neighboring keratinocytes and “clear cells” (Figure 2d).

A different situation is shown in Figure 2b, images belonging to a case of non-syndromic fibromatosis. Connective cells are condensed into small islands of loose connective tissue that is creeping through thick collagen bundles sometimes hyalinized. Cell islands include chronic inflammatory infiltrate with numerous lymphocytes, plasma cells and sporadic macrophages and mast cells.

These cell agglomerations are well vascularized, unlike the areas of fibrous condensation where vessels are extremely rare and of small diameter. In these areas, we observed young fibroblasts with rich cytoplasm, numerous extensions and hypochromic nuclei, seemingly cells with an intense synthetic activity (Figure 2, e and f).

Cases included in the IVth Group were characterized by the most pronounced polymorphism of lesions. They also show thickening of the fixed gingival mucosa, with an enlarged epithelium and varying degrees of keratosis. Epithelial papillae are deep and highly branched with a papilloma appearance, sometimes with connecting epithelial bridges (Figure 3, a and b). It also maintains individualized cells through the basal keratinocytes, mainly located at the top of epithelial ridges (Figure 3c).

Lamina propria is characterized by the simultaneous

presence of fibrosis which predominates in terms of occupied area but which is invariably accompanied by inflammation. In some cases, the inflammatory infiltrate is concentrated in certain areas, usually in the superficial, subepithelial chorion (Figure 3b) and sometimes it is disseminated among collagen bundles (Figure 3a). In other cases the fibrillar and inflammatory component are more balanced as extent and distribution in the gingival mucosa. Because of more extensive inflammatory lesions, the vascular supply is better represented with numerous de novo small vessels among the nodules of inflammatory infiltrate. In chorion, inflammatory cells are lymphocytes, plasma cells, few macrophages and mast cells and the few cells are observed between the collagen fibers, which look like secreting fibroblasts with hypochromic nucleus, with nucleoli, and clear vacuolar cytoplasm (Figure 3, c and d).

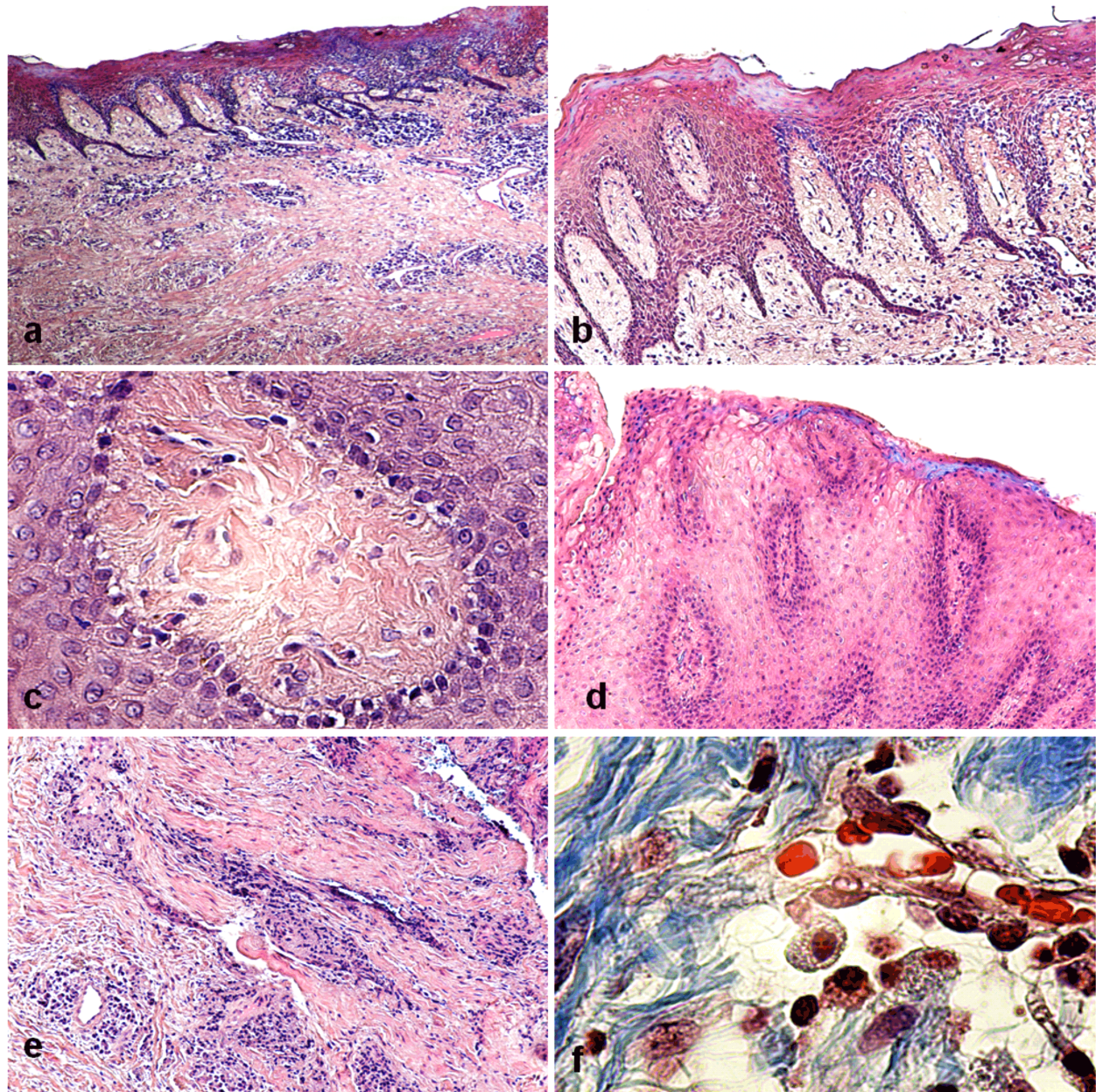


Figure 1 – Focal reactive gingival fibrosis. (a) Overview, HE staining, $\times 40$; (b) Detailed image – elongated and branched epithelial ridges, subepithelial clusters of inflammatory infiltrate, HE staining, $\times 200$; (c) Cross-section of a papilla in the dense papillary chorion, HE staining, $\times 400$; (d) Epithelium with parakeratosis, spongy appearance of keratinocytes from the prickle cell layer, HE staining, $\times 200$; (e) Deep chorion with thick collagen bundles and discrete inflammatory infiltrate, HE staining, $\times 200$; (f) Detail for an area of perivascular inflammatory infiltrate, trichromic Masson staining, $\times 1000$.

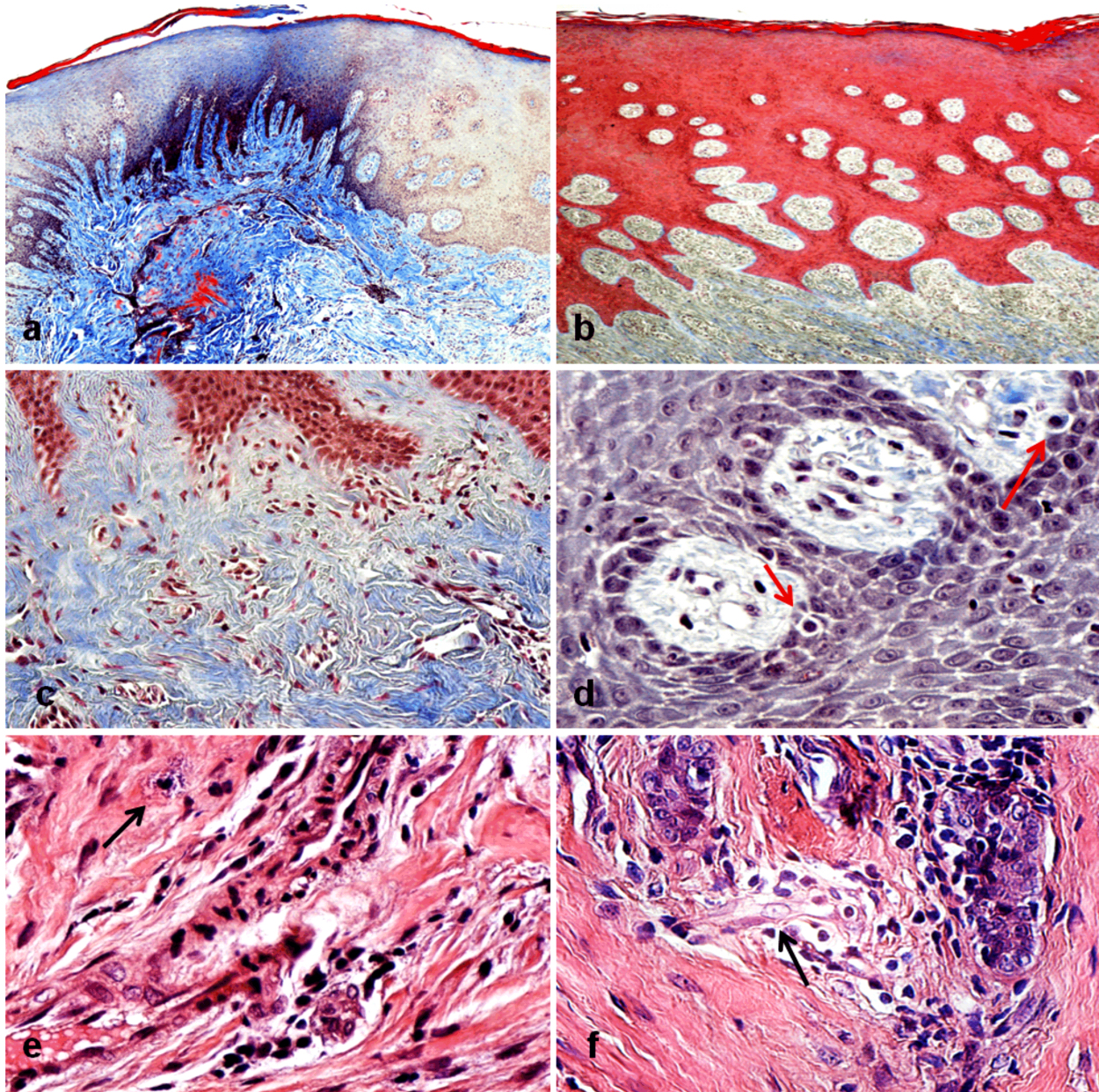


Figure 2 – Gingival fibromatosis (GF). (a) Syndromic GF, trichromatic Masson staining, $\times 100$; (b) Non-syndromic GF, trichromatic Masson staining, $\times 100$; (c) Lamina propria with thick and tortuous collagen bundles, with few fibroblasts and blood vessels, trichromatic Masson staining, $\times 200$; (d) Cross-section of connective papillae – at the arrowheads cells without junctions which seem to individualize, trichromatic Masson staining, $\times 400$; (e) Detailed image, inherited fibromatosis – the arrowhead indicates a big mast cell, HE staining, $\times 400$; (f) Detailed image, inherited fibromatosis – at the arrowhead, a myofibroblast active cell, HE staining, $\times 400$.

Immunohistochemical study

To identify the phenotype of fibroblasts involved in collagen synthesis we incubated serial sections of each case belonging to the four groups with the following antibodies: anti-vimentin, anti- α -SMA and anti-S100-A4 (FSP1).

Immunostaining for vimentin showed a difference between the degrees of expression of this molecule specific for cells of mesenchymal origin between samples from different groups, determined by the number of positive cells and also by their location.

As can be seen in Figure 4, cases with FRGO included in the Ist Group have a lamina propria with many vimentin-positive cells located mainly in the superficial chorion of connective papillae and somewhat rare in the deep

chorion (Figure 4a). In some cases, we noticed several pro-inflammatory cells among vimentin-positive fibroblasts (data not shown). In cases belonging to Ist Group, in which the GO was due to orthodontic devices, the number of vimentin-positive cells was significantly lower than that reported for FRGO. Despite the fact that detailed images show numerous fibroblast-like cells, some of them are negative for vimentin (Figure 4c).

Immunoreaction for α -SMA show “mirror” results for the two categories of fibrotic GO considered in the Ist Group. FRGO demonstrates a weak positivity in the mesenchymal cells, while cells of blood vessels are constantly labeled (Figure 4b). We used this as an internal control for the immunohistochemical reaction but in the same time, we noted the relatively small number of blood vessels in the chorionic papillae and the deep chorion (Figure 4, b and d).

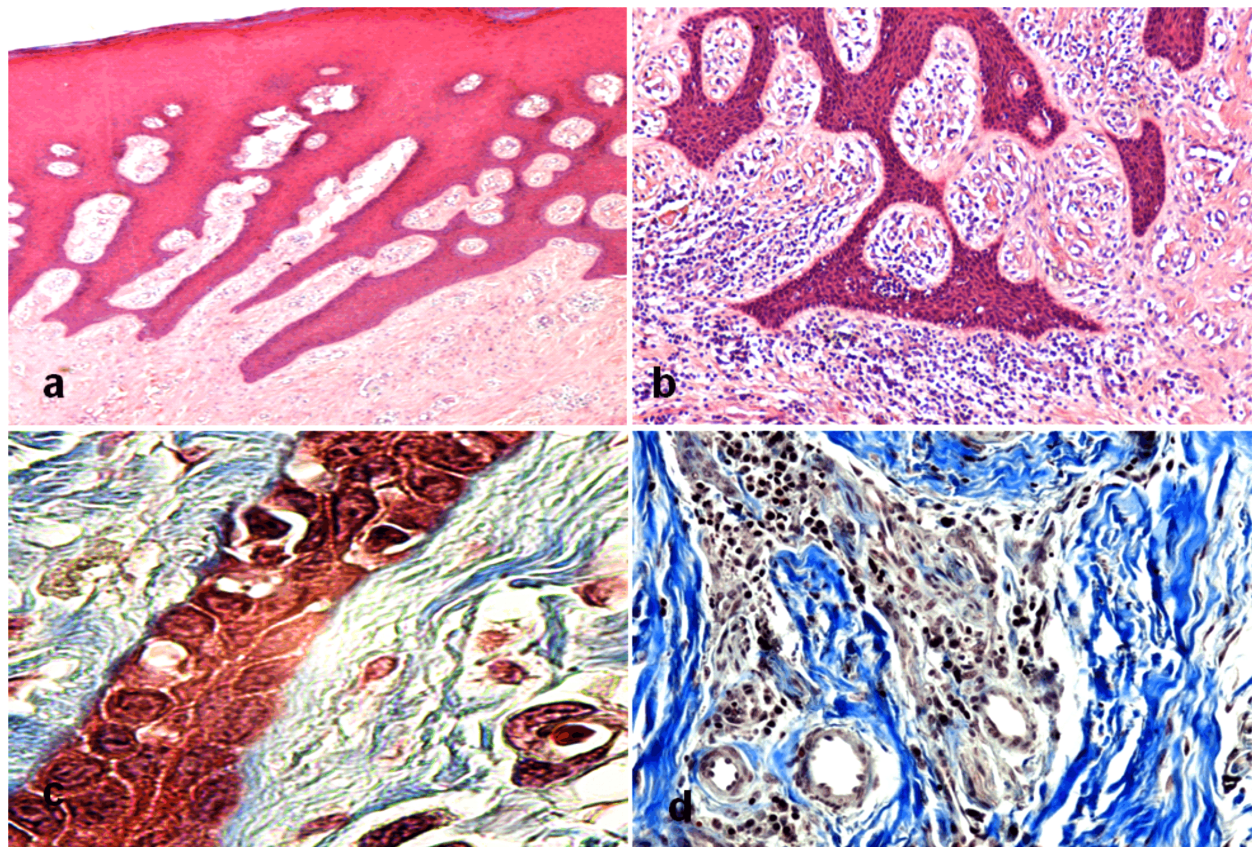


Figure 3 – Idiopathic gingival fibromatosis. (a) Overview – deep epithelial ridges and massive collagen storage, HE staining, $\times 200$; (b) Granulation tissue in the superficial chorion, HE staining, $\times 200$; (c) Deep epithelial ridge – among keratinocytes stands clear cells, but also individual elements that seem detached from neighboring cells, trichromic Masson staining, $\times 1000$; (d) Detailed image of the inflammatory infiltration of deep chorion, trichromic Masson staining, $\times 200$.

For the patients with fibrotic GO secondary to wearing orthodontic devices, we observed a much higher incidence of α -SMA-positive cells compared with patients of the same Ist Group but with GO due to local inflammatory conditions. As can be observed in Figure 4d, numerous but not all fibroblast-like cells from the deep chorion have a positive reaction for α -SMA. Lymphatic vessels react negatively.

The third antibody that we used to identify fibroblasts was S100A4, also called FSP1. Immunohistochemical reactions showed an intense positivity in all cases included in the Ist Group, both for GO determined by local factors and that caused by wearing orthodontic devices. In all cases, positive reactions were observed in many cells of the lamina propria and in a various degree in cells from the epithelium. In the epithelium, one can distinguish some cells with extremely positive reaction similar to those from the lamina propria and others with faint positive reaction (Figure 4, e and f). Both fibroblast-like and some pro-inflammatory cells in the lamina propria have positive reaction for FSP1.

As for the general histological features, the immunohistochemical reactions for vimentin were similar for cases included in the IInd Group – inherited GF and IIIrd Group – syndromic fibromatosis.

The antibody labeled many cells in the lamina propria, as can be observed in Figure 5, a and b. As in the Ist Group, positive cells were present in greater number in the superficial chorion and less in the deep chorion. We

also noticed cells with cytoplasmic positivity present in the basal epithelial layer.

Immunohistochemical reactions for α -SMA were, however, different in the IInd and IIIrd Groups. In some cases from both groups, the positive reaction was observed almost exclusively in cells that circumscribe blood vessels (Figure 5c), but there were also cases belonging to the IIIrd Group in which we noted a significant number of α -SMA-positive mesenchymal cells in the lamina propria (Figure 5d). Immunohistochemical reactions for S100A4 (FSP1) for all cases from the IInd and IIIrd Groups also showed a high incidence of positive cells. Many of them were located in the lamina propria but also in the epithelium, particularly in the deep and prickly cell layers (Figure 5, e and f). As can be observed in Figure 5 (b and e), serial sections obtained from the IIIrd Group showed almost identical positivity for vimentin and FSP1, the only difference being that the latter antibody labeled cells in the epithelium more than vimentin, which is absent in keratinocytes.

The IVth Group included cases diagnosed as idiopathic fibrotic GO in the absence of any relevant family history or association with another clinical syndrome. The group emphasized a heterogeneous histological appearance with varying degrees of gingival inflammation as granulation tissue. Immunoreaction for vimentin showed few positive cells in the lamina propria (Figure 6a) and the α -SMA positivity was restricted to the cells that circumscribe blood vessels, which were relatively in a great number compared to the IIIrd Group but mesenchymal cells (*i.e.*,

myofibroblasts) in the lamina propria were extremely rare (Figure 6b). The reaction to FSP1 for cases from the IVth Group showed two kinds of response in the epithelium: one similar to that reported in the IInd and IIIrd Groups, with the presence of various degrees of positivity in the keratinocytes from all layers and other when only isolated

cells close to the basement membrane were positive, the rest of keratinocytes showing negative reaction. In both conditions, many fibroblast-like and pro-inflammatory cells showed positive reaction (Figure 6, c and d).

Table 2 summarizes the results of the immunohistochemical reactions.

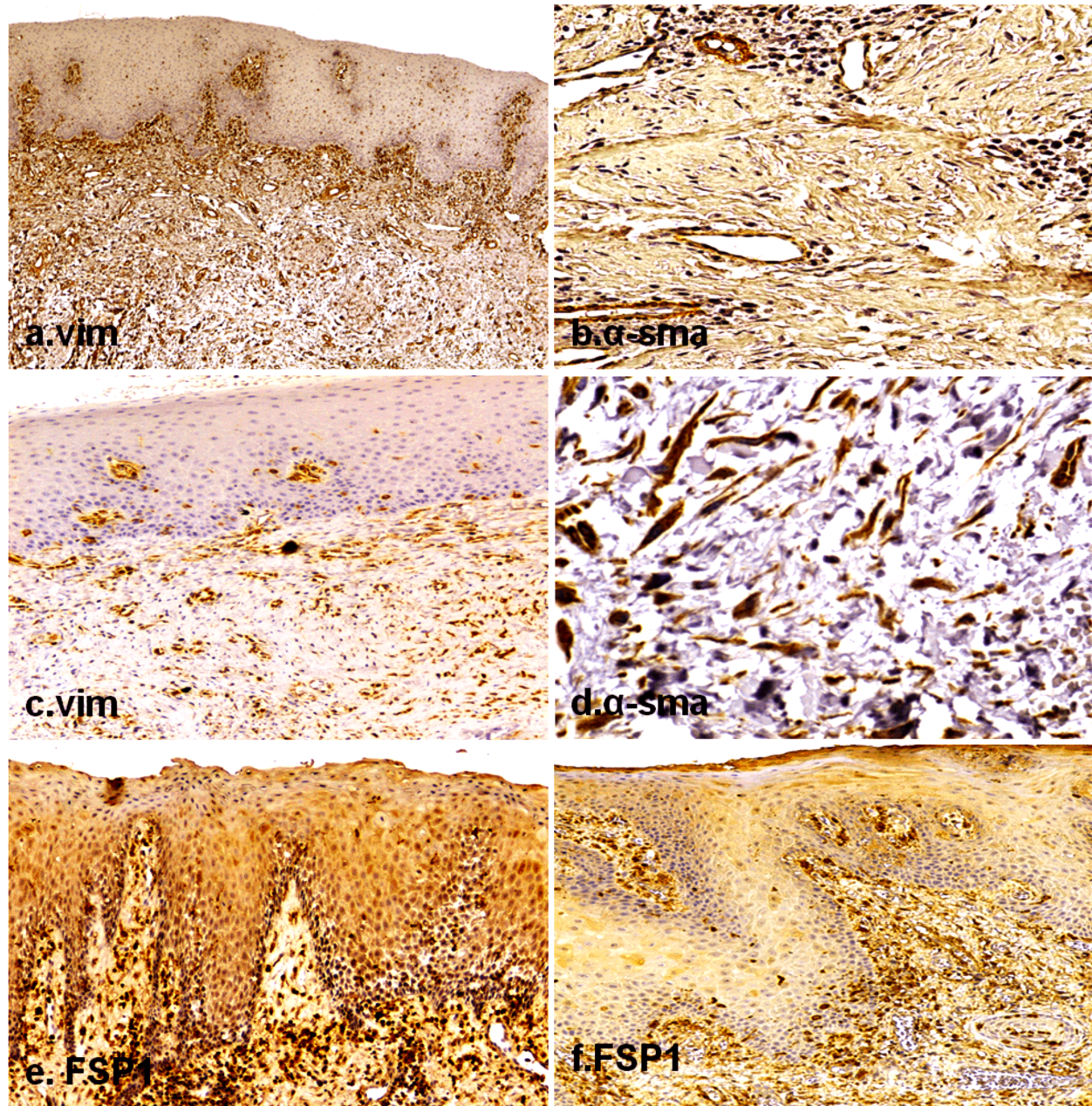


Figure 4 – Immunohistochemical reactions to identify fibroblast phenotypes in the Ist Group. (a) Focal reactive gingival overgrowth (FRGO) – many vimentin-positive cells concentrated mainly subepithelial, ×100; (b) FRGO – few blood vessels and very few cells positive for α-SMA, ×200; (c) GF due to orthodontic devices – faint immunoreaction for vimentin, ×100; (d) GF due to orthodontic devices – many α-SMA-positive cells, ×400; (e) FRGO – positive reaction for FSP1 in epithelium and connective tissue, ×200; (f) GF due to orthodontic devices – few FSP1-positive cells in the epithelium and many in the lamina propria, ×200.

Discussion

Gingival overgrowth cases studied were collected from patients clinically diagnosed with fibrotic GO determined by local factors, those in the Ist Group, while the IInd and IIIrd Groups were constituted of patients with inherited GF and syndromic fibromatosis respectively; the IVth Group included patients with clinically-diagnosed fibrotic

overgrowth whose etiology could not be established – idiopathic fibrotic overgrowth, which we treated as idiopathic GF after patient anamnesis. In the literature, consulted references to the histological aspects of these types of fibrotic accumulations are extremely brief, most often treated only as clinical presentation cases.

All cases studied were characterized by a steady enlargement of the gingival epithelium, which occurs due

to the accumulation of cell rows in the prickle cell layer. Acanthosis was sometimes accompanied by acantholysis with a spongy appearance of the epithelium caused by the loss of adhesion between epithelial cells. This observation was found especially where the epithelium was highly infiltrated with pro-inflammatory cells. Epithelial changes described do not seem to be specific to a particular risk factor because they are described both in the gingival drug-induced overgrowth [45, 46] as well as in the inherited or idiopathic type [12].

Hyperkeratosis and parakeratosis that have been observed in most cases seem to be constant epithelial changes. There is no explanation for these alterations than just those who might be determined by the rise in mitotic index of keratinocytes, observation that was also

reported by several authors [39, 46] and which in turn is determined by the presence of pro-inflammatory cytokines or growth factors [46, 47]. The increase of keratinocytes capacity of replication explain also the constant presence of epithelial papillae, called also epithelial ridges or rete pegs deep, sharp and sometimes branched [46, 48] representing epithelial cells that clog deep in the subjacent chorion that in turn penetrates between these epithelial ridges by chorionic papillae. The results we have presented also show an increase in the number and depth of these epithelial ridges occurring sometimes branched and joined by epithelial bridges. The role of these deep epithelial ridges with many epithelial cell divisions could be to provide cells able to synthesize collagen by epithelial-mesenchymal transition (EMT) [49–51].

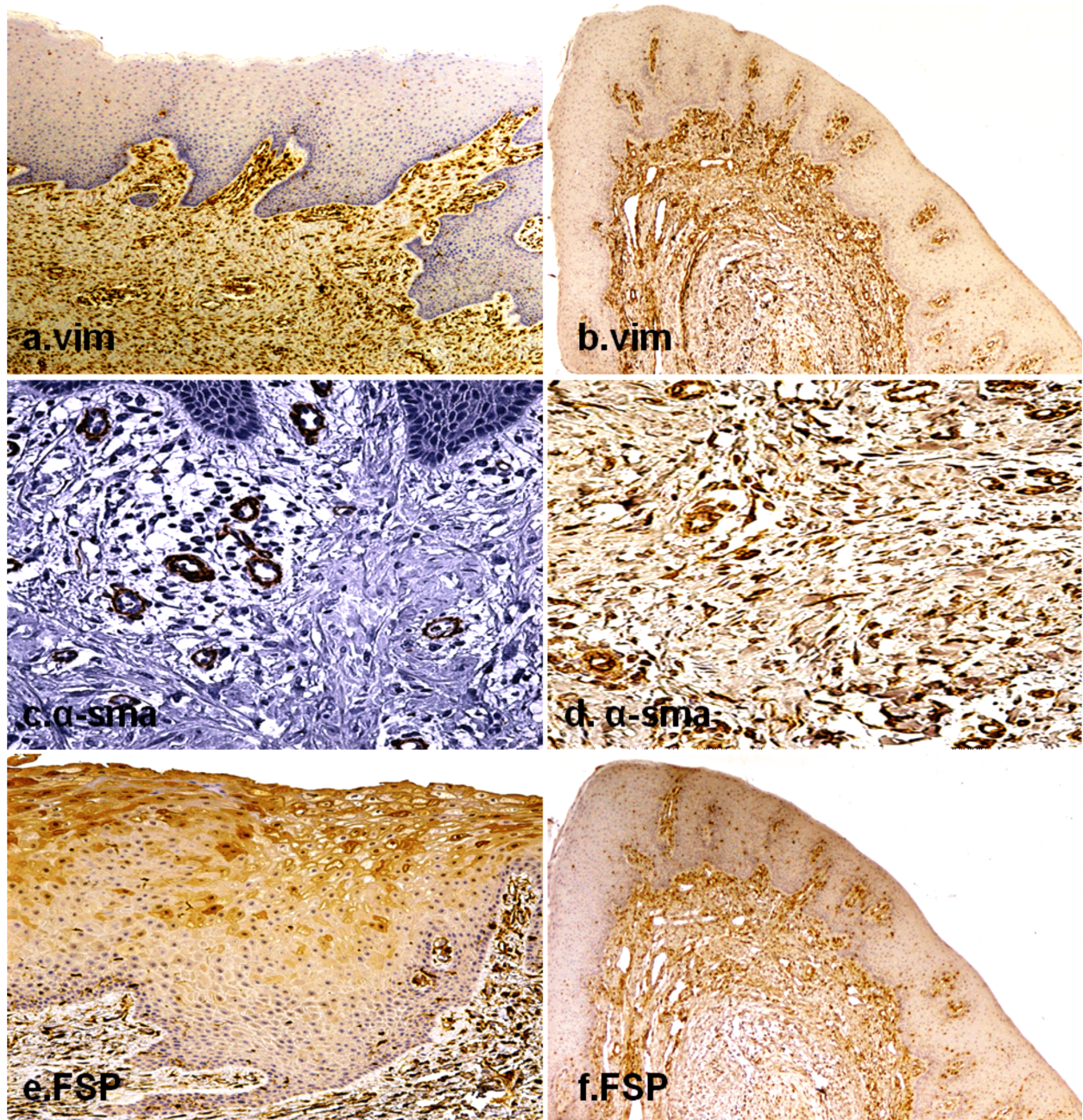


Figure 5 – Immunohistochemical reactions to identify fibroblast phenotypes in the IInd and IIIrd Groups. (a) Vimentin in syndromic fibromatosis, ×100; (b) Vimentin in inherited fibromatosis, ×100; (c) GF – immunoreaction for α-SMA, ×200; (d) Inherited fibromatosis – α-SMA-positive cells in chorion, ×200; (e) Syndromic fibromatosis – many FSP1-positive cells in the epithelium and superficial chorion, ×200; (f) FSP1 in inherited fibromatosis, ×100.

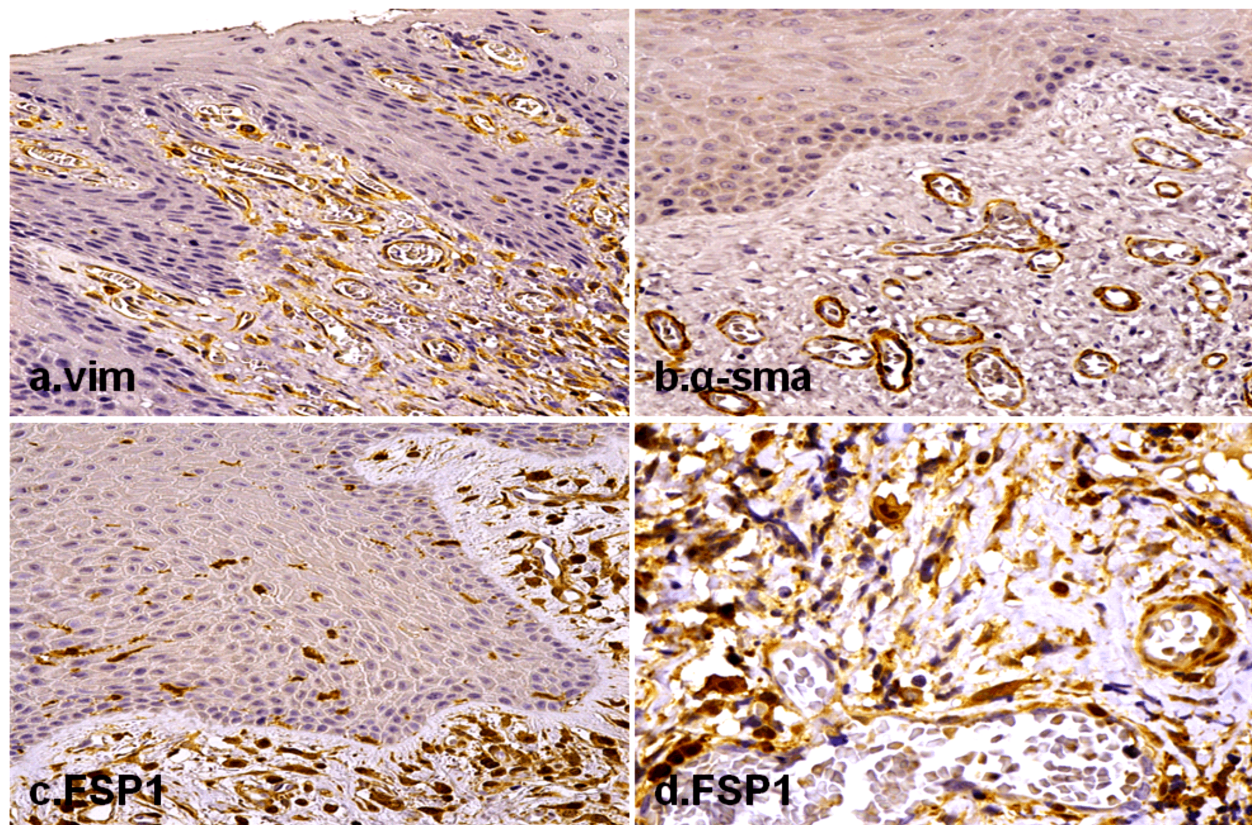


Figure 6 – Immunohistochemical reactions to identify fibroblast phenotypes in idiopathic fibromatosis. (a) Immunoreaction for vimentin, ×200; (b) Reaction for α-SMA is present only in blood vessels, ×200; (c) Few keratinocytes labeled for FSP1 in the basal and prickle cell layers and many in the superficial chorion, ×200; (d) Detailed image of superficial chorion. FSP1 labels fibroblasts, endothelial and pro-inflammatory cells, ×200.

Table 2 – Synthetic presentation of the semiquantitative evaluation of immunohistochemical reactions

Antibody	I st Group				II nd Group		III rd Group		IV th Group	
	Focal reactive fibrosis		Orthodontic device							
	E	LP	E	LP	E	LP	E	LP	E	LP
Vim	-	+++	-	+	-	+++	-	+++	-	+
α-SMA	-	+	-	++	-	+	-	+++	-	+
FSP1	++	++	++	++	++	+++	+++	+++	++	++

E: Epithelium; LP: Lamina propria; Vim: Vimentin; α-SMA: α-Smooth muscle actin; FSP1: Fibroblast-specific protein-1.

In all cases, we observed thickening of the gingival chorion with significant accumulation of fibrillar collagen in the form of thick bands with different directions. In most cases, collagen fibers ascend from the deep chorion to the papillary one so that the loose connective tissue of the papillae is replaced with dense tissue. We also observed areas of inflammatory tissue confounding with fibrous tissue. Interesting was its distribution in relation to other structural elements of the chorion. Granulation tissue, as we did not note acute inflammatory infiltrate, was located either immediately below the papillary chorion at the top of epithelial ridges and among them, either in the form of small nodules of pro-inflammatory tissue located in the angles between collagen bundles.

A microscopic study carried out by Uzel *et al.* on tissues collected from subjects with inherited fibromatosis shows collagen broad bands with few fibroblasts located in the depth of connective tissue. Semiquantitative determination in these cases show reduced inflammation, less represented than in the control group, while the level of fibrosis was significantly higher compared to the control (3.33 on a scale up to 4) [36]. The authors concluded

that ECM synthesis is independent of local factors such as inflammation in inherited GF. Following the logic of the argument, one can assume that at least in these cases the presence of inflammation with its entire cascade of cytokines released locally do not influence neither the mitotic capacity of fibroblasts nor the collagen synthesis. A hypothesis that can be launched in this condition is that genetic factors determine the recruitment of highly secreting fibroblast phenotypes.

As mentioned above, our results indicate a variable degree of inflammation in all sections examined. It is difficult to quantify inflammation by usual histological methods, as pro-inflammatory tissue is not located in defined areas that can be evaluated by histomorphometry, the granulation tissue being spread in different areas as we have shown above. On the other hand, it is extremely difficult to obtain cases totally free of inflammatory elements even in the IInd and IIIrd Groups, considered outside the inflammatory pathogenesis, as gingival pathology itself involves the accumulation of plaque by increasing gingival volume, the gingival sulcus becoming the site of bacterial accumulation. In fact, there are cases

described in the literature associating GF with severe periodontitis [38, 52].

These observations lead us to conclude that at least in fibrotic overgrowth included in the Ist and IVth Groups, where the inflammatory reaction was more important, fibrotic lesions cannot be considered isolate of the inflammatory microenvironment from the gingival tissue. An argument is the coincidence of clinical onset of GF with dental eruption onset, when fibroblasts are activated by inflammatory cells involved in tissue resorption or because of the mechanical trauma [9]. Regardless of abundance, inflammatory infiltrate was composed mainly of lymphocytes and plasma cells forming the granulation tissue located mainly perivascular. Detailed examination revealed the existence of activated macrophages and mast cells sometimes undergoing degranulation. Mast cells present in normal gingival mucosa are located close to blood vessels, nerves and epithelial basement membrane, which ensure their role in homeostasis and participation in primary immune response [53, 54]. An important number of plasma cells was reported in drug-induced GO [55]. In chronic gum inflammation, chemical mediators released by mast cells participate in both tissue damage and maintain a barrier of defense for those structures [56]. It is recognized that heparin, histamine and TNF- α released by mast cells in gingival inflammation affect the proliferation of fibroblasts, the synthesis and degradation of ECM and the synthesized chymase stimulates the synthesis of MMP-9 and lysis of epithelial basement membrane [57] which facilitates the penetration of epithelial layers and EMT. Mast cells are directly involved in this pathogenic pathway by increasing the amount of ECM in fibrotic GO. Among the many chemical mediators contained in granulations of mast cells is endothelin-1 (ET-1) [58] that acts in autocrine manner to initiate the degranulation of mast cells. At the same time, ET-1 induces the expression of a great number of MMP and matrix proteins in cell cultures, such as collagen, laminin, fibronectin [59, 60]. Further studies are needed to confirm these mechanisms.

Cells from the lamina propria are represented in particular of fibroblasts with their variants, fibrocytes, blood vessels and cells migrated from the blood – pro-inflammatory elements. Our results indicate a relatively high incidence of gingival fibroblasts. As shown in Table 2, the three antibodies used for identification of fibroblast populations have shown differences sometimes noticeable between groups and sometimes in the same group.

These results are consistent with observations that show not only that the cells classically known as fibroblasts actually represent a population extremely heterogeneous in origin, differentiation and function in each organ, but that in the same host tissue, these cells may exhibit significant variations with respect to the physiological or pathological circumstances. Recent studies show that under normal circumstances but especially in fibrotic tissues the origin of fibroblasts is only slightly, if not absent, in the local fibroblasts, most of them originating in the bone marrow (fibrocytes), epithelial or endothelial cells [49–51, 61, 62]. One example is kidney fibrosis in which 36% of the fibroblasts derive from trans-differentiation of epithelial cells after EMT, 14–15%

from fibrocytes, stem cells of bone marrow [62, 63] and the others after local proliferation of fibroblasts [64]. Following this heterogeneity of origin, there are different markers that are currently used to highlighting fibroblasts but not all are equally reliable. Vimentin, a marker that has imposed itself over time to highlight cells of mesenchymal origin, by labeling intermediate filaments in their cytoplasm, appears to be suitable for *in vitro* identification of fibroblasts from all tissues [65, 66]. Only some subpopulations of fibroblasts express collagen type I [64, 67, 68]; similarly, only certain subpopulations express α -SMA, a marker for myofibroblasts, also called activated fibroblasts [69, 70] being even advanced the hypothesis that α -SMA could label actually cells detached from blood vessel walls in injuries due to local pathology [66, 71].

Our results have indicated a high number of mesenchymal elements positive for vimentin in the Ist Group, samples from patients with FRGO, while patients from the same group who developed fibrosis as a result of wearing an orthodontic device had a small number of vimentin-positive cells, similar to those from the IVth Group. α -SMA-positive fibroblasts showed the highest incidence in cases of fibromatosis produced by the presence of an orthodontic device and in those with syndromic fibromatosis. This reaction does not seem to be related to the abundance of inflammatory tissue, as the IIIrd Group presented the smallest number of pro-inflammatory cells. This similarity between the two conditions, one being induced and the other genetic is difficult to explain, but must have in mind that both situations are characterized by a large amount of fibrous ECM and the presence of numerous active fibroblasts.

The presence of myofibroblasts in chorion is controversial, as opposed to the fibroblasts present in the periodontal ligament that normally express smooth muscle myosin [72]. Some authors argue that in the granulation tissue progressively fibroblasts become the majority population and takes myofibroblasts phenotype, including expression of α -actin [73, 74]. Therefore, differentiated myofibroblasts can long remain silent or disappear by apoptosis after wound healing. It suggests that the depletion of myofibroblasts in the tissue may be responsible for storage a disorganized and fibrotic ECM [75]. This is probably the situation that occurred in other cases of GF when the number of α -SMA-positive cells was very low, if not reduced only to cells from blood vessel walls. There are reports in the literature referring to the presence of variable number of myofibroblasts in inherited GF and drug-induced gingival hyperplasia [25, 76, 77]. Schmitt-Gräff *et al.* showed that the subcutaneous administration of TGF β 1 in experimental animals results in the appearance of granulation tissue in which the α -SMA-positive myofibroblasts are particularly abundant, whereas administration of other profibrogenetic factors (PDGF, TNF α) do not induce formation of α -SMA in myofibroblasts [78]. The authors concluded that fibroblasts, relatively undifferentiated cells can be differentiated in relation to many physiological stimuli or microenvironments [78]. Recent data confirm this hypothesis and show that three conditions are simultaneously necessary for the differentiation of fibroblasts into myofibroblasts: (1) accumulation of profibrogenetic factors, mainly TGF β 1, (2) the presence of specialized matrix proteins, such as fibronectin variants,

and (3) presence of enhanced extracellular stress that determine stimulation of matrix remodeling [79–81].

Our results are consistent with some literature data who also reported a reduced number of α -SMA-positive fibroblasts in FRGO [25, 82]. Data from the literature state that myofibroblasts express α -SMA, vimentin, fibronectin and non-muscle myosin [71]. Our results are in disagreement with these observations, because not always all the number of vimentin-positive mesenchymal cells was found also as α -SMA-positive cells. In this respect, we are in agreement with the observations made by Kisseleva and Brenner, which showed that there are differences between differentiation and expression of markers between fibrocytes and fibroblasts [62]. Fibrocytes are cells involved in skin, kidney, liver and lung fibrosis. They have dual phenotypic features between fibroblasts and lymphoid cells, and are defined as CD45+ cells able to synthesize collagen, with bone marrow origin where they represent $\leq 1\%$ of the cell population. Due to tissue injuries, their number increases, and after replication they reach the damaged tissue. The percentage of these cells varies in relation to the damaged tissue (5–25%) [83, 84]. Fibrocytes in culture can differentiate into α -SMA-positive myofibroblasts because of stimulation by TGF β 1. The authors suggest that the role of these cells in tissue is not limited to fibrillogenesis but they fulfill the role of intermediary in signaling between immune and fibrogenetic cells. This observation is based on the fact that the fibrocytes expressed lymphoid markers (CD45, MHC II, MHC I), myeloid markers and adhesion molecules (CD54, ICAM-1) but also fibroblast markers (Thy-1, α -1 collagen). Fibrocytes also secrete growth factors and cytokines, which stimulate the local deposition of ECM (e.g., TGF β 1) [85]. A second type of fibroblast-like cells derived still from bone marrow is represented by fibroblasts, which are different from fibrocytes by the fact that do not express myelo-monocytic markers and do not overexpress α -SMA *in vitro* [86]. They are the main cells responsible for pulmonary fibrosis. In liver fibrosis, however, was described a population of cells recruited after stimulation with carbon tetrachloride, cells which express collagen, vimentin, desmin and α -SMA [87]. These observations are arguments in favor of fibroblast heterogeneity that differ not only between cavitory and solid organs, but also between cells resident in the skin to gingival fibroblasts [88].

The third marker used to highlight fibroblast phenotype in GO was S100A4, referred also as FSP1 (fibroblast-specific protein 1). This protein discovered in 1995, highly specific to fibroblasts, belongs to the S100 protein family. Has been implicated in the progression of fibrosis in various organs (kidney, liver, joints, heart, nervous system, lung) [89, 90]. FSP1 is a specific marker for both fibroblasts and cells, which undergo EMT [91, 92]. The possibility that fibroblasts derive from EMT was recently emphasized for drug-induced GO by nifedipine, phenytoin and cyclosporine [50, 51, 93]. According to some authors, the main source of fibroblasts in connective tissues, especially in the case of fibrotic pathologies, is EMT. The main argument is that epithelial cells in culture undergo EMT and are specific labeled by FSP1 [49, 62, 64]. Our results indicate many FSP1-positive cells in the lamina propria, but also in the epithelium, the greatest number reported for syndromic fibromatosis, as shown

in Table 2. As can be seen, the three antibodies had a heterogeneous expression in different groups and even in all the cases from a group. Cases in which mesenchymal cells are labeled by all the three antibodies are represented by those from the IInd Group and only a part with fibrosis caused by wearing orthodontic devices. This draws attention to a marked phenotypic heterogeneity of fibroblasts in gingival fibrosis, myofibroblasts being least represented and the cells positive for FSP1 most commonly present. Further studies are needed to argue the role of EMT in recruitment of cells highly active in ECM synthesis in gingival fibrosis.

Conclusions

The storage of collagen fibers, increase of fibroblast number and frequent presence of inflammatory infiltrate are histological issues that characterize fibrotic GO. The incidence of granulation tissue varies in relation to etiology, but the frequency of its presence point the attention to its involvement in collagen metabolism imbalance. The phenotype of fibroblasts is different in gingival fibrosis in relation to the risk factor, at present the most common being vimentin-positive and FSP1-positive fibroblasts. Myofibroblasts are rare in gingival fibrosis, the most numerous being in local lesions caused by wearing orthodontic devices and in syndromic fibromatosis. Further studies are required to elucidate the manner in which the active fibroblasts are recruited in relation to the etiologic factor of GO.

Conflict of interests

The authors declare that they have no conflict of interests.

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Author contribution

All authors have equal contributions to the study and the publication.

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