ABSTRACT

Objectives: The aim of this study was to detect the presence of myofibroblasts and transforming growth factor-beta1 in fibrous and ossifying-fibrous epulis and their possible contribution to the collagenous connective tissue formation. The correlation between the myofibroblasts and the degree of inflammatory infiltration was also examined.

Material and Methods: The presence of myofibroblasts as well as transforming growth factor-beta1 was examined in twenty cases of fibrous epulis and 22 ossifying fibrous epulis, using immunohistochemistry.

Results: Myofibroblasts positive for alpha smooth muscle actin and vimentin but negative to desmin were found in 20% and 45% in fibrous epulis and ossifying fibrous epulis, respectively. Myofibroblasts were distributed in areas with and without inflammatory infiltration and their presence in inflammatory areas was not related with the degree of inflammatory infiltration. A percentage of 21 - 60% of fibroblasts and chronic inflammatory cells expressed transforming growth factor-beta1 in all cases.

Conclusions: These data suggest that transforming growth factor-beta1 and myofibroblasts contribute to the formation of collagenous connective tissue in fibrous epulis and ossifying fibrous epulis. Myofibroblasts are mainly presented in ossifying fibrous epulis than in fibrous epulis. It seems to be no relationship between the presence of myofibroblasts and the degree of inflammatory infiltration of the lesions.

Keywords: myofibroblasts; transforming growth factor beta1; epulis; fibroma.

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INTRODUCTION

Myofibroblasts (MFs) are specialized fibroblasts characterized by the presence of contractile apparatus [1] and are responsible for synthesizing enzymes involved in extracellular matrix degeneration, tissue remodelling and wound healing [2]. Furthermore, these cells are involved in several fibrotic diseases, such as pulmonary fibrosis, interstitial lung fibrosis, liver cirrhosis, renal fibrosis and scleroderma, thus being responsible for overproduction of extracellular matrix molecules, such as collagen type I [3,4]. Although fibroblasts are considered to be the main progenitor cells of MFs, pericytes and vascular smooth muscle cells may be transformed into MFs [5].

Transforming growth factor-beta (TGF-β) is a large family of structurally related growth and differentiation factors including activins and bone morphogenetic proteins. There are three TGF-β isoforms, TGF-β1, TGF-β2 and TGF-β3 with distinct and overlapping activities, such as control of mesenchymal cell proliferation and differentiation, wound healing and extracellular matrix production [6,7]. TGF-β1 is present in epithelia, connective tissue and mononuclear inflammatory cells [7]. It is well documented that TGF-β1 plays the principal role in the trans-differentiation of fibroblasts into MFs [4], as well as fibroblasts' proliferation and collagen secretion in pathologic conditions [8,9].

Fibrous epulis (FE) or peripheral fibroma and ossifying fibrous epulis (OFE) are composed of common reactive fibrous overgrowths of the gingiva caused by chronic irritation. FE consists of interlacing collagen bundles but areas with chronic inflammatory infiltration may also be present [10,11]. On the other hand, OFE is composed of high fibrocellular tissue containing little or plenty of bone, cementum-like material, and dystrophic calcification and peripherally, there is a less fibrocellular tissue that is infiltrated by chronic inflammatory cells, mostly lymphocytes and plasma cells. The stratified epithelium of the lesion may be ulcerated [11,12].

The expression of TGF-β1 in both FE and OFE has not been investigated in detail, so far. Few studies have focused on the presence of MFs. Their controversial results were based on a small number of cases [13-15]. Furthermore, the relation of MFs and the inflammatory infiltration was described only in one study on FE [16]. The aim of the present study was to detect immunohistochemically the presence of myofibroblasts and transforming growth factor-beta1 in fibrous and ossifying fibrous epulys in an attempt to note their possible contribution in the formation of collagenous connective tissue of the lesions. Also, a possible relationship between the presence of myofibroblasts and degree of inflammatory infiltration was examined.

MATERIAL AND METHODS

Twenty cases of FE and 22 cases of OFE were retrieved from the archives of the Department of Oral Medicine and Oral Pathology, Dental School of Aristotle University of Thessaloniki, Greece. Cases with and without inflammatory infiltrations in both FE and OFE were included. Serial, 4 μm sections from paraffin-embedded tissues stained with haematoxylin and eosin (for the confirmation of diagnosis) and for immunohistochemistry, as well. Patients had given informed consent and the whole study was performed according to the Declaration of Helsinki II.

The inflammatory infiltration was graded as absent, mild, moderate and severe. In cases with different degrees of inflammatory infiltration, the most frequent degree was recorded in association with the presence of MFs.

Monoclonal antibodies against alpha smooth muscle actin (alpha-SMA), vimentin, and desmin and polyclonal antibody against TGF-β1 were used. Endogenous peroxidase activity was blocked with 3% H2O2 for 10 min at room temperature. Then the sections were pre-treated for antigen retrieval (Table 1).

### Table 1. Source, clone and pretreatment for antigen retrieval

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Clone</th>
<th>Pretreatment</th>
<th>Dilution</th>
<th>Incubation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha smooth muscle actin</td>
<td>Dako, Glostrup, Denmark</td>
<td>1A4</td>
<td>No treatment</td>
<td>1:100</td>
<td>30</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Dako, Glostrup, Denmark</td>
<td>V9</td>
<td>Microwave Citrate buffer 0.01M pH 7.2, 95°C, 15 min</td>
<td>1:100</td>
<td>30</td>
</tr>
<tr>
<td>Desmin</td>
<td>Biocare, Carmino Diablo, CA, USA</td>
<td>D33</td>
<td>Microwave Citrate buffer 0.01M pH 7.2, 95°C, 15 min</td>
<td>1:100</td>
<td>30</td>
</tr>
<tr>
<td>Transforming growth factor-beta1</td>
<td>Spinge Biosense, Pleasaton, CA, USA</td>
<td>Polyclonal</td>
<td>Microwave Citrate buffer 0.01M pH 6, 95°C, 10 min</td>
<td>1:25</td>
<td>30</td>
</tr>
</tbody>
</table>
Sections that were intended for the detection of alpha-SMA, vimentin and desmin were incubated with normal rabbit serum, and those for the detection of TGF-β1 with normal mouse serum at a dilution of 1:20 for 30 min at room temperature. Sections were incubated with monoclonal and polyclonal antibodies (Table 1) and sections incubated with normal mouse serum were used as negative control. Envision/horse radish peroxidase (HRP) ChemMate/TechMate detection system (Dako, Glostrup, Denmark) was performed for the detection of all antigens using the autostainer Ventana (Ventana Med Systems Inc Tuscon, AZ, USA), and the reaction was developed using diaminobenzidine. Haematoxylin was used as counterstain. Sections from a leiomyoma and placenta were used as positive control for desmin and TGF-β1, respectively.

Alpha-SMA and desmin were used in conjunction with morphology to identify MFs, which are usually appeared as spindle shaped and sometimes stellate. MFs’ presence was counted as a percentage of all spindle shaped and stellate cells, as reported previously [14]: (0) no staining, (+) weak staining of 1% - 20% of all spindle shaped and stellate cells, (++) intense staining of 21% - 60% spindle shaped and stellate cells and (+++) intense staining of more than 60% of spindle shaped and stellate cells. Fibroblasts and inflammatory cells expressing TGF-β1 were evaluated together using the method mentioned above. Five hundred cells from 5 fields of each section were enumerated as the percentage of positive MFs and cells expressing TGF-β1. Sections were examined by two of the authors (AE, DA) independently of each other. Sections were re-examined when there were differences, and discussion was occasionally necessary to establish uniformity.

**Statistical analysis**

Statistical analysis was performed using the chi-squared test ($\chi^2$) and statistical significance level was defined at $P = 0.05$.

**RESULTS**

MFs positive for alpha-SMA and vimentin but negative for desmin were found in 4 of 20 cases (20%) of FE. Half of the cases did not contain inflammatory cells and 2 of these cases showed a percentage of intense presence of MFs (Figure 1A). In the rest 10 cases of FE, where areas with and without inflammatory cells were concurrently presented in the same case, two of the cases with weak and intense presence of MFs were observed, respectively (Table 2). The inflammatory infiltration in these cases consisted of plasma cells lymphocytes and occasionally macrophages (Figure 1B).

Inflammatory infiltration and MFs were not found in 5 of 22 cases of OFE. In the other 17 cases of OFE, in the periphery of the central, highly fibrocellular and mizeralized part of the lesion, chronic inflammatory infiltration from plasma cells, lymphocytes and occasional macrophages could be observed. The presence or absence of MFs in the central part and areas with different degrees of inflammatory infiltration around the central part, as well as the percentages of

**Table 2. Presence of myofibroblasts (MFs) in different areas of 10 cases of fibrous epulis**

<table>
<thead>
<tr>
<th>Areas with inflammatory infiltration</th>
<th>Number of cases with MFs</th>
<th>Number of cases</th>
<th>Degree of inflammatory infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>Moderate</td>
</tr>
<tr>
<td>Areas without inflammatory infiltration</td>
<td>2</td>
<td>10</td>
<td>Absent</td>
</tr>
</tbody>
</table>

[Figure 1. Fibrous epulis. A = myofibroblasts positive for alpha smooth muscle actin in area without inflammatory infiltration. B = in area with severe inflammatory infiltrate (hematoxylin and eosin stain, original magnification x100).]

[Table 2. Presence of myofibroblasts (MFs) in different areas of 10 cases of fibrous epulis]
MFs in 17 cases of OFE are presented in Table 3 and appeared in Figure 2A, B. Although, the frequency of MFs between FE and OFE was statistically significant (P < 0.001), however, the presence of MFs in areas with inflammatory cells was not related to the degree of inflammatory infiltration in both FE and OFE (Tables 2 and 3).

TGF-β1 expression was intensely seen in fibroblasts, plasma cells, lymphocytes and macrophages in both FE and OFE (Figure 3A, B). Also, a percentage of 21 - 60% of cells expressing TGF-β1 was found in all examined cases.

**DISCUSSION**

The results of the current study showed that MFs and TGF-β1 are likely involved in the collagen formation

Table 3. Myofibroblasts (MFs) in different areas of 17 cases of ossifying fibrous epulis, degrees of inflammatory infiltration and percentage of myofibroblasts

<table>
<thead>
<tr>
<th>N</th>
<th>MFs in the central part</th>
<th>MFs in areas without inflammatory infiltration around the central part</th>
<th>MFs in areas with inflammatory infiltration around the central part</th>
<th>Degree of inflammatory infiltration</th>
<th>MFs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>mild</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>severe</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>mild</td>
<td>1 - 20</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>mild</td>
<td>1 - 20</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>moderate</td>
<td>1 - 20</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>severe</td>
<td>21 - 60</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>moderate</td>
<td>21 - 60</td>
</tr>
</tbody>
</table>

N = number of cases.

**Figure 2.** Ossifying fibrous epulis. A = myofibroblasts positive for alpha smooth muscle actin in central area (hematoxylin and eosin stain, original magnification x200). B = Beyond the vessels, no positive for alpha smooth muscle actin myofibroblasts are presented in area with severe inflammatory infiltrate around the central area of the lesion (hematoxylin and eosin stain, original magnification x100).

**Figure 3.** Fibroblasts and inflammatory cells express transforming growth factor-beta 1 in areas with chronic inflammatory infiltrate. A = fibrous epulis. B = ossifying fibrous epulis (hematoxylin and eosin stain, original magnification x100).
in FE and OFE, but in contrast no relationship was found between the presence of MFs and the degree of inflammatory infiltration in both FE and OFE. Immunohistochemically, MFs may have a variable phenotype including those that express only vimentin (V type); vimentin and alpha-SMA (VA type); vimentin, alpha-SMA and desmin (VAD type) and vimentin, alpha-SMA, smooth muscle myosin heavy chains and/or desmin (VAM or VAMD type). The expression of alpha-SMA is considered to be the main biochemical marker of myofibroblastic differentiation [17]. In the current study the detection of MFs was based on the combined immunohistochemical profile (vimentin and alpha-SMA) as well as their morphology indicating that MFs of FE and OFE belong to the VA type. Interestingly, the presence of MFs in FE has not been reported in previous studies [13,15]. This finding could be explained by the small number of cases in previous studies and differences in the immunohistochemical procedure. The findings of the present study regarding OFE are in accordance to a previous study by Garcia de Marcos et al. [14] that reported the presence of MFs positive for vimentin and alpha-SMA, whereas, Damasceno et al. [15] did not reveal the presence of MFs. The results of our study suggest that MFs may contribute in the formation of collagenous connective tissue more frequently in OFE than in FE. FE is considered to originate from mesenchymal cells of gingival and periosteum [11], and OFE is considered to originate from cells of periodontal ligament and periosteum [12]. Although immunohistochemical studies did not reveal the presence of MFs [13,18], cell cultures of fibroblasts from healthy gingiva and periodontal ligament demonstrated that a few cells express alpha-SMA [19,23]. It remains to be determined whether the alpha-SMA positive cells in gingiva and periodontal ligament represent a permanent or modulated fibroblastic population. Differentiation from fibroblast into MF phenotype has been proposed to be dependent on local environmental cues, including accumulation of biologically active TGF-β1 [20], extracellular matrix-integrin interactions [21] and mechanical stress [1]. The reverse MF-to-fibroblast differentiation is also possible under insulin-like growth factor-I [22], and basic fibroblast growth factor influence [23]. Possibly the absence of MFs in many of our cases may be due to alterations or lack of local environmental cues, or the presence of insulin like growth factor-I and/or basic fibroblast growth factor.

TGF-β1 is believed to participate in fibroblastic differentiation and alpha α-SMA expression in fibroblasts in vitro and in vivo [1]. Connective tissue cells, macrophages, neutrophils, lymphocytes and plasma cells express TGF-β1 [7,24] that has paracrine and autocrine effect [5]. Our results showed that MFs were not always present in areas containing different degree of inflammatory infiltration. Similar findings were reported in another study of FE, as well [16]. These results suggest that TGF-β1 of chronic inflammatory cells possibly is not always involved in the recruitment of mesenchymal cells and their trans-differentiation into MFs. Noteworthy, MFs were constantly presented in cases with different degree of chronic inflammatory infiltrate in obstructive pancreatitis [25], whereas in oral squamous cell carcinoma, MFs tended to be inversely related to the infiltration with mononuclear inflammatory cells [26]. Extracellular matrix molecules and cytokines may affect the bioactivity of TGF-β1. Fibronectin domain ED-A is crucial for myofibroblastic phenotype induction [27], whereas decorin, tumour necrosis factor-α, interferon-γ, and interleukin-1 inhibit the trans-differentiation of MFs [28-31]. In the current study 21 - 60% of fibroblasts and chronic inflammatory cells found to express TGF-β1 in all cases of FE and OFE. This result may indicate that there was no relationship between the presence of MFs and percentage of cells expressing TGF-β1. Although the expression of TGF-β1 was similar in all examined cases of FE and OFE, it can be hypothesized that the absence of MFs in many cases may be due to the lack of other than TGF-β1 local environmental cues, or presence of extracellular matrix molecules/cytokines affecting TGF-β1 bioactivity. Further investigation is needed to examine the local environmental cues and growth factors/cytokines implication in the differentiation of MFs in FE and OFE. TGF-β1 has a marked effect on extracellular matrix composition by attracting fibroblasts and leading to the synthesis and secretion of extracellular matrix molecules, such as collagen type I, fibronectin and tenascin [32,33]. It is possible that autocrine and/or paracrine effect of TGF-β1 of fibroblasts cause collagen overproduction, as well.

CONCLUSIONS

The results of the present study suggest that transforming growth factor-beta 1 likely contributes in the formation of collagenous connective tissue in fibrous epulis and ossifying fibrous epulis, whereas myofibroblasts more often in ossifying fibrous epulis than in fibrous epulis. Also, there is no obvious relationship between the presence of myofibroblasts and the degree of inflammatory infiltration in both fibrous epulis and ossifying fibrous epulis.

ACKNOWLEDGMENTS AND DISCLOSURE STATEMENTS

The authors declare that they have no conflict of interests.
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