Oxytalan-positive peripheral ossifying fibromas express runt-related transcription factor 2, bone morphogenetic protein-2, and cementum attachment protein. An immunohistochemical study

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BACKGROUND: The peripheral ossifying fibroma (POF) represents one of the most common lesions of the periodontal tissues that may originate from the gingival soft tissues, the periosteum, or the periodontal ligament. AIM: To investigate the immunohistochemical expression of runt-related transcription factor 2 (Runx-2), bone morphogenetic protein-2 (BMP-2), and cementum attachment protein (CAP) in oxytalan-positive POF, to establish the use of POF as an in vivo model for the study of the periodontal ligament. MATERIALS AND METHODS: Thirty tumors that presented clinical and histologic features of POF, as well as oxytalan fibers, were included in the study. Immunohistochemical expression of Runx-2, BMP-2, and CAP was evaluated by light microscopy. RESULTS: Runx-2, BMP-2, and CAP were abundantly expressed by POFs; 22 of 30 tumors expressed positive staining for Runx-2, twenty-six tumors for BMP-2, and twenty-five tumors for CAP. The expression of Runx-2 was abundant in POFs where bone was histologically present \((P = 0.04)\) and of BMP-2 in POFs where dystrophic calcifications were present \((P = 0.03)\). CONCLUSION: It is suggested that oxytalan-positive POFs, purportedly originating from the periodontal ligament, express molecules that are specific to bone and cementum (Runx-2, BMP-2), or cementum only (CAP). Thus, the cell populations present in the lesion belong to the mineralized-tissue-forming cell lineages, the cementoblastic or osteoblastic lineage.

Keywords: BMP-2; CAP; cementogenesis; osteogenesis; peripheral ossifying fibroma; Runx-2

Introduction

The peripheral ossifying fibroma (POF) represents one of the most common lesions of the periodontal tissues. Reactive rather than neoplastic in nature, it clinically manifests as a slow-growing gingival mass, usually emerging from the interdental papilla with a sessile or pedunculated base (1) and measuring less than 2 cm in diameter. The color is identical to that of the gingiva or slightly reddish, and the surface may appear ulcerated (2, 3). POF occurs in any age-group, predominating in the second decade of life (4), females are more commonly affected than males (5), and the anterior maxilla is the most common location of involvement (6). The associated teeth are usually not affected. Routine radiographic examination may reveal areas of radiopaque material (7, 8). Histologically, it consists of a highly cellular fibroblastic connective tissue, with focal deposits of bone, cementum, and irregular amounts of dystrophic calcification (2). Areas of dystrophic calcification are more common in early, ulcerated lesions; with time, the ulcer heals and the dystrophic type of calcification seems to mature into bone. The lesion shows a clinically benign behavior, and the prognosis is good. Surgical excision is the treatment of choice, although the recurrence rate can reach 20% (1–4).

The etiology and pathogenesis of POF remain unclear. Trauma or local irritants, such as dental plaque, calculus, microorganisms, masticatory forces, ill-fitting dentures, and poor quality restorations, have been implicated (1, 9). It may originate from the gingival soft tissues or the periosteum, but it is its purported origin from the periodontal ligament, supported by the identification of oxytalan fibers in the areas of calcification (10–13), that makes POF an ideal tissue for the study of hard tissue formation in the periodontal tissues.
Only a few studies have investigated the calcification and ossification process in POF. Shetty et al. (14) identified by polarizing microscopy woven bone, lamellar bone, and cellular and acellular cementum in 22 cases of POF. Yang et al. (15) immunohistochemically detected the presence of bone morphogenetic protein (BMP) in 25 cases of calcifying fibrous epulis. BMP-positive immunostaining was revealed in 60% of the cases and was limited to osteoblasts and fibrous connective tissue surrounding the bone matrix. The authors concluded that the ossifying and cemento-ossifying processes appear to be a result of the excessive proliferation of the periodontal ligament and a metaplastic process occurring in the connective tissue fibers. Ono et al. (16) investigated the expression of BMP-2, BMP-4, osteopontin (OPN), and osteocalcin (OCN) in seven peripheral cemento-ossifying fibromas and five intra-osseous ossifying fibromas. They suggested that POF has only little ability to form hard tissue, as they showed a dominance of BMP-2, BMP-4, OPN, and OCN in the ossifying fibromas, but not in the POFs. Other authors (12, 16, 17) demonstrated that the cellular proliferation in the ossifying fibroma is higher than in the POF, emphasizing the non-neoplastic nature of the POF.

Of particular interest among factors that regulate bone formation are BMP-2 and runt-related transcription factor 2 or core binding factor 1 (Runx-2, Cbfa-1). BMP-2 is a growth factor that plays an important role in osteoblastic commitment and differentiation, enhancing bone formation (18), while Runx-2 is a transcription factor required for osteoblastic commitment and expression of the osteoblastic phenotype (19, 20). Cementogenesis shares many similarities with bone formation, as more than twenty non-collagenous proteins that control osteoblast function are also present in cementoblasts (21–24). However, cementum-derived attachment protein (CAP), a 56- or 65-kDa collagenous protein that promotes the attachment of mesenchymal cells on extracellular matrix (25, 26), has been identified in mature cementum and cementoblasts, but not in bone or osteoblasts (26–28). The role of epithelium in cementoblast differentiation is largely speculative, but many researchers agree that root sheath epithelial cells or their products are involved in the directed migration of pre-cementoblasts (29). However, the persistence of epithelial cells in the functioning periodontal ligament is known; the root sheath eventually migrates away from the root surface and forms a network of epithelial cells in the periodontal ligament area, known as the epithelial cell rests of Mallassez (29), identified by the expression of cytokeratins (30).

The aim of this study was to investigate the immunohistochemical expression of Runx-2, BMP-2, and CAP proteins in oxytalan-positive POF. Such an investigation could support the use of POF as an in vivo model for the study of the periodontal ligament and can contribute to our level of understanding of the regulatory mechanisms regarding bone and cementum formation and resorption in periodontal tissues. This can be of particular interest in periodontology, with research investigating novel therapeutic strategies aiming to regenerate periodontal tissues lost as a result of periodontal disease.

Materials and methods

Tissue

This is a retrospective study on archival tissue material. Seventy-eight tumors diagnosed during a 5-year period (2006–2011) as ‘peripheral ossifying fibroma,’ ‘peripheral fibroma,’ or ‘peripheral odontogenic fibroma’ were collected. All tumors had been fixed in 10% buffered formalin (24–48 h) and embedded in paraffin, without prior decalcification. Representative hematoxylin and eosin stained tissue sections were re-evaluated based on standard microscopic criteria (31), and thirty cases in total were selected based on the adequacy of clinical information, the quality of tissue specimen, and the presence of oxytalan fibers. In each case, the presence of bone trabeculae, cementum-like material, and dystrophic calcifications was recorded, according to standard microscopic criteria (2). The presence of rests of odontogenic epithelium was identified by immunohistochemistry (see below). Data on age, gender, and location of the cases were retrieved from the respective biopsy request forms.

Gordon and Sweet’s staining for oxytalan fibers

Four-μm-thick sections were incubated in potassium permanganate solution for 5 min, washed in 5% oxalic acid until clear, incubated in iron alum solution for 10 min, and washed and incubated in silver solution. They were subsequently treated with 10% formaldehyde solution until gray black, 0.5% gold chloride, 5% hypo and nuclear-fast red solution (32).

Immunohistochemistry

Four-μm-thick sections were cut and heated overnight at 37°C. Immunohistochemical staining for Runx-2 and CAP was performed in the Ventana BenchMark XT® fully automated slide preparation system (Ventana Medical Systems, Tucson, AZ, USA), using the iView DAB detection kit (Ventana Medical Systems) with a standard streptavidin–biotin–peroxidase technique. Antigen retrieval was performed by treating the sections with Cell Conditioning CC1 (Ventana Medical Systems) at 95°C for 30 min for Runx-2 mouse monoclonal antibody and for 60 min for CAP mouse monoclonal antibody. Primary antibodies were Runx-2 antibody (1:40; 27-K; Santa Cruz Biotechnology) and CAP antibody (1:25; G-3, Santa Cruz Biotechnology, Dallas, TX, USA). For cytokeratin and BMP-2, the VECTASTAIN Universal Quick Kit (Vector Laboratories, Inc., Burlingame, CA, USA) was used. The antigen retrieval of BMP-2 was performed by treating the sections with CINtec solution (3 × 5 min) (MTM Laboratories, Inc., Westborough, MA, USA) in a microwave at boiling point. Following rinsing with distilled water, the sections were incubated in pepsin solution for 8 min, washed with distilled water, and treated with PBS. The primary antibodies were multicytokeratin mouse monoclonal antibody (1:100; NCL-L-AE1/AE3; Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK) and BMP-2 goat polyclonal antibody (N-14, Santa Cruz Biotechnology). Positive controls were human placenta tissue sections for Runx-2 and CAP, human intestine for BMP-2, and skin for cytokeratins. To create negative controls, primary antibodies
were substituted by non-immune serum of the same specificity.

Staining was evaluated by light microscope using the three-scale semiquantitative system modified by Vered et al. (33, 34): absent/limited (<5% of cells stained), intermediate (5–50% of cells stained), and abundant (>50% of cells stained).

Statistical analysis of staining reaction (absent/present) of the Runx-2, BMP-2, and CAP antibodies in the tumors where bone trabeculae, cementum-like material, dystrophic calcifications, and odontogenic epithelium were present was performed using chi-square test ($P < 0.05$).

**Results**

Nineteen patients were female, and eleven were male. Most lesions were located on the lower gingiva ($n = 18$), followed by the upper gingiva ($n = 12$). Based on hematoxylin and eosin stained tissue sections, 22 of 30 tumors (73.3%) showed bone trabeculae, ten (33.33%) showed cementum-like material, and 15 (50%) showed dystrophic calcifications (Fig. 1). In total, in 23 of 30 tumors (76.6%), some form of hard tissue was present. Histochemistry for oxytalan fibers (Fig. 2) was utilized as a marker of periodontal ligament origin (11, 18) and immunohistochemistry for keratins to identify the presence of rests of odontogenic epithelium within the lesional tissue. Six of thirty tumors (20%) showed keratin-positive rests of odontogenic epithelium.

Runx-2, BMP-2, and CAP were expressed in the stromal cells of POF, both in fibrous and hard-tissue-forming areas. Runx-2 expression was nuclear or cytoplasmic; 22 of 30 tumors (73.3%) were positive for Runx-2, of which 21 (70%) expressed the protein in >50% of stromal cells (Fig. 3a). BMP-2 expression was cytoplasmic; 26 of 30 POFs (86.7%) were positively stained for BMP-2, of which 25 (83.3%) expressed BMP-2 in >50% of stromal cells (Fig. 3b). CAP expression was cytoplasmic; 25 of 30 POFs (83.3%) were positively stained for CAP, of which 15 (50%) expressed the protein in >50% of stromal cells (Fig. 3c). Results for each antibody are shown in Fig. 4. Runx-2, BMP-2, or CAP were not expressed by bone trabeculae, cementum-like material, but occasionally dystrophic calcifications reacted for BMP-2.

Chi-square statistical analysis was performed to evaluate the correlation between the expression of Runx-2, BMP-2, and CAP antibodies and the presence of bone, cementum, dystrophic calcifications, or odontogenic epithelium in the tumors. Runx-2 expression was associated with the presence

**Figure 1** (a) Bone trabeculae, (b) cementum-like material, (c) dystrophic calcifications (arrows), and (d) rests of odontogenic epithelium (arrows) in POF (hematoxylin and eosin stain, original magnification ×400).

**Figure 2** Thin oxytalan fibers enter the surface of bone trabeculae in a POF (Gordon and Sweet stain, original magnification ×400).
of bone ($\chi^2 = 3967; \text{d.f.} = 1, P = 0.0464$); BMP-2 was associated with the presence of dystrophic calcifications ($\chi^2 = 4615; \text{d.f.} = 1, P = 0.03169$), and CAP was associated with the absence of rests of odontogenic epithelium ($\chi^2 = 3.811; \text{d.f.} = 1, P = 0.05$). No statistical significance was found for any other comparison. No correction for multiple comparisons was performed, due to the small number of values compared.

Discussion

In the present immunohistochemical study, expression of Runx-2, BMP-2, and CAP was shown in the stroma of most oxytalan-positive POFs. Runx-2 expression and BMP-2 expression were associated with the presence of bone trabeculae and dystrophic calcifications, respectively, but CAP expression was not associated with the presence of cementum-like material, while the latter did not express the protein.

Runx-2 is essential for osteoblast differentiation, acting as a positive regulator in the commitment of multipotent mesenchymal cells to the osteoblastic lineage (35, 36). It induces the expression of major bone matrix protein genes in osteoblast progenitors, allowing the cells to acquire the osteoblastic phenotype, hence to form bone (37–39). Along with BMP-2 that has a pivotal role in bone formation and remodeling processes, Runx-2 has also been implicated in cementoblasts’ differentiation and proliferation, as cementum is largely composed of proteins similar to those of bone matrix. Runx-2 is expressed during tooth root development, and Runx-2 and another protein, osterix, regulate bone and tooth development and may be required for the differentiation of multipotent mesenchymal cells into cementoblasts (40–42).

Gao et al. (43) showed immunohistochemical expression of BMP (BMP-3, or other members of the BMP family except BMP-2, -4, -6, -12) in a number of odontogenic tumors, namely cementifying fibroma, benign cementoblastoma, dentinoma, compound odontoma, and odontogenic fibroma, but not by the calcified cementum-like structure of those tumors. In this study, most of the BMP-positive odontogenic tumors were those forming dentine, cementum, or enamel, suggesting that BMP might play an important role in the formation of calcified dental tissues. Yang et al. (15) reported that 15 of 25 ‘calcifying fibrous epulides’ showed positive BMP staining in bone-forming areas, dense fibrous tissue with features of periodontal ligament, peripheral connective tissue fibers, and osteoblasts surrounding bone matrix. Ossification appeared to be more commonly associated with proliferating periodontal fibers, and it did
not usually occur in areas associated with dense chronic inflammatory infiltration. In a study by Ono et al. (16), BMP-2 expression was considered consistent with the higher ability of central ossifying fibroma to form hard tissue compared to peripheral cemento-ossifying fibroma, while in accordance with our study, they did not find BMP-2 in hard tissues. Hirata et al. (44) suggested that Runx-2 and osterix co-localize in mature cementoblasts, odontoblasts, and osteoblasts, triggering the formation and secretion of cementum, dentine, and bone, respectively. BMP-2 has also been shown to enhance cementogenesis in vitro and induce osteogenic markers in periodontal ligament cells in vivo (45), as well as the differentiation of early undifferentiated progenitors of the periodontal ligament population toward the osteoblastic and the cementoblastic lineages (46).

In our study, the stromal cells of most oxytalan-positive POFs (22/30) expressed Runx-2 and/or BMP-2 (26/30) throughout the tumor, indicating the presence of cells that express an osteoblastic and/or cementoblastic phenotype. The expression of BMP-2 is in accordance with previous studies (15, 16), indicating the ability of those tumors to form hard tissues. A possible explanation for the absence of BMP-2/Runx-2 expression in some lesions is that they are too immature, less differentiated, and unable to synthesize detectable amounts of the proteins. Statistically significant association was present between Runx-2 expression and the formation of bone trabeculae, as well as BMP-2 expression and the presence of dystrophic calcifications, but no statistically significant association was found between any of the proteins and cementum-like material. This could indicate that in oxytalan-positive POFs, Runx-2 is associated with bone formation and BMP-2 with the calcification process, but as those associations are of marginal statistical significance, more cases need to be evaluated.

CAP is specifically associated with cementoblast differentiation and cementum matrix formation (47–49). Liu et al. (50) and BarKana et al. (51) suggested that the level of CAP binding might be an indicator of the commitment of a progenitor clone to the mineralized-tissue-forming cell lineage. As CAP can be isolated from cementum matrix only (28, 29), and high levels of CAP are produced by cementoma-derived cell lines (29, 30), CAP is strongly associated with the cementoblastic phenotype and the tendency of a precursor cell to undertake the cementoblastic lineage. In our study, the stromal cells of most POFs (25/30) expressed CAP, indicating differentiation of cementoblastic precursors toward the cementoblastic lineage. A possible explanation for the finding that CAP was expressed in POFs where no cementum-like material was detected is that cells present in these lesions may be non-committed, undifferentiated precursor cells with a tendency to commit to the cementoblastic lineage. In addition, CAP expression was not statistically associated with the presence of cementum-like material, while the cementum-like material did not react for CAP, in accordance with previous studies suggesting that there is no cementum production in POFs (15, 43). This may, also, explain the lack of association of CAP expression with the presence of odontogenic epithelium, as in normal odontogenesis, the epithelium has a significant role in the migration of pre-cementoblasts and the initiation of cementum formation (29, 30).

In conclusion, the present immunohistochemical study shows that oxytalan-positive POFs, purportedly originating from the periodontal ligament, express Runx-2 and BMP-2 proteins that are present both in bone and cementum, as well as the cementum-specific protein CAP. Those proteins may also participate in the differentiation and commitment of undifferentiated mesenchymal cells into the osteoblast or cementoblast phenotype. Thus, oxytalan-positive POFs may harbor the mineralized-tissue-forming cell populations of the periodontal ligament. The establishment of oxytalan-positive POF as an in vivo model for the study of the interactions of cells that belong to the osteoblastic and cementoblastic lineage may be utilized in elucidating the pathways involved in osteogenesis and cementogenesis that will lead to complete and successful periodontal regeneration. Further studies need to be conducted that will include the use of freshly isolated, immunophenotyped, and functionally characterized cells from the lesions, to strengthen these conclusions and their potential impact in the field.


