Distribution of Osteopontin in Normal Dog Periodontium (Immunohistochemical Study)

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Abstract: Background: The periodontium is a term describing tooth supporting and investing tissues. It includes gingiva, periodontal ligament, alveolar bone and cementum. This has been always threatened by periodontal diseases which results in its destruction. In order to understand how periodontium responds or how to enforce it against that destruction, its own factors, proteins and cells should be revealed. Osteopontin (OPN) is an extracellular structural protein, and expressed in a variety of tissues including the tooth periodontium. Also it is involved in many vital biological processes such as inflammation, immunity and wound healing expressed. Aim of study: to explore OPN expression in normal dog periodontium. Methods: OPN rabbit polyclonal primary antibody was used to detect its expression in various tissues of the dog pre molar periodontium. Results: OPN was expressed in all tissues of the dog pre molar periodontium differently. It was intense at gingival tissue and periodontal ligaments (PDL). Intense to moderate expression was detected in alveolar bone. But cementum had weak expression. Conclusion: Periodontium undergoes constant physiological remodeling. This remodeling differs among different parts of the periodontium. Accordingly, OPN expression in each part of the periodontium was different.
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1. Introduction

The periodontium, defined as those tissues supporting and investing the tooth, comprises root cementum, periodontal ligament, bone lining the tooth socket (alveolar bone), and that part of the gingival facing the tooth (dentogingival junction) [1]. Periodontal diseases are heterogeneous and include a diversity of infections and inflammatory lesions. Periodontitis is a prevalent disease in human, characterized by loss of connective tissue attachment, alveolar bone resorption, periodontal pocket formation due to apical migration of junctional epithelium, and gingival inflammation which leads enduringly to tooth loss [2.3].

Periodontal regeneration is the reconstruction of the lost tissues as evidenced histologically by new cementum formation, new alveolar bone, and a functionally oriented periodontal ligament. Immunohistochemical studies evaluating the newly formed tissues following periodontal treatment had shown the presence of various matrix molecules such as collagen types I and III, vitronectin, fibronectin, bone morphogenetic proteins 2, 4 and 7, bone sialoprotein and osteopontin (OPN) [4-6]. OPN, also known as a bone sialoprotein1 (BSP1), is an extracellular structural protein that was first identified in osteoblasts [7]. However, several studies have illustrated its expression by a variety of tissues including; bone, dentin, cementum, hypertrophic cartilage, kidney, brain, bone-marrow-, uterus, inner ear, brain cells and specialized epithelia found in mammary salivary and sweat glands. Also it is found in bile, pancreatic ducts, and in distal renal tubules. As well as gut, activated macrophages and lymphocytes [8-12]. Although the secreted protein is incorporated into the matrix in mineralized connective tissues, it also appears in biological fluids, including blood, milk, and seminal fluid [13-12]. OPN is also expressed in the pre-implantation mouse embryo, during differentiation of embryonic stem cells, the rostral hind brain and notochord during neural axis formation [17,18]. This miscellaneous expression would designate OPN for a multiplicity of functions. OPN is involved in diverse of biological events including developmental processes, immunological responses, tumorigenesis, bone resorption, calcification and wound healing (reviewed in Mazzali *et al.*,2002) [19]. Interestingly, OPN level increases in plasma and gingival crevicular fluid from both; sites with periodontal destruction and regenerating periodontium [20-23]. In order to appreciate the role of OPN in periodontal regeneration, expression of OPN in normal periodontal tissues should be examined. To the best of our knowledge, the present study is the first to examine OPN expression in normal periodontal tissues in dogs.

2. Materials and Methods

Animals

Six, 13–16 months old, beagle dogs, weight approximately 10 kg were used. The dogs were obtained from the Animal House, Faculty of Medicine,

Tanta University. Animals were housed under controlled temperature and lighting conditions (12 hours alternating dark and light cycle) with free access to standard food and water. Animals were examined daily for general appearance, activity and weight. The experimental protocol was designed in accordance with the guidelines for the responsible use of animals in research as a part of scientific research ethics recommendations (Reviewed in Russell, 2013) [24].

Euthanasia

Dogs were sacrificed under overdose of anesthesia of Phenobarbital sodium salt. Mandibles of all sacrificed dogs were dissected carefully keeping the gingival attachment intact with bone. Then, each mandible was split into two halves at the midline, all right experimental sides were processed for subsequent immunohistochemical analysis.

Tissue preparation

Specimens comprising of bone segments carrying the premolar teeth, were fixed in 10% buffered formalin for 24 h and decalcified in 10 % neutral-buffered EDTA at room temperature for three months. Then, they were dehydrated through ascending graded series of ethanol, and processed into paraffin. 5 µm mesio-distal serial sections were cut and mounted on commercially available positively charged glass slides to be ready for immunohistochemical staining.

Immunohistochemical Staining

Immunohistochemical labelling was performed using the avidin–biotin–complex (ABC) method. Representative sections, taken from the central part of the wax block, were deparaffinized in xylene and re-hydrated through a descending series of ethanol concentrations. The sections were washed with TBS (20 mM Tris- HCl,150 mM NaCl, pH 7.4). Then they were incubated in 0.3% H2O2 in dH_2O at room temperature (30 min) to inhibit endogenous peroxidase. Antigen retrieval was performed according to the manufacture instructions. Slides were placed in 100 µl blocking solution (Abcam), for 30 minutes at room temperature. Rabbit polyclonal primary antibody to OPN (Cat. No. ab8448, Abcam, Cambridge, UK) was applied at recommended dilutions at 4ºC overnight. Sections were washed in 1X Phosphate buffered saline (PBS) and then incubated with HRP-conjugated Goat anti -Rabbit IgG at 1/10000 dilution (in blocking buffer) for 1 hour at room temperature in a humidified chamber. To perform peroxidase visualization; sections were incubated in ABC solution for 1 hour at room temperature. Color reaction was then developed by adding DAB solution (0.5 mg/ml DAB and 0.1% H_2O) onto the sections. When color reaction was satisfactory, it was stopped by rinsing with H2O for 5-10 minutes, and then sections were counterstained with hematoxylin for 2 minutes. Sections were gradually dehydrated and mounted with coverslips. Immunohistochemical staining was assessed using Leica light microscope.

3. Results

OPN expression in different parts of the periodontium will be described as soft and hard tissue expression. Soft tissue includes gingiva and PDL while hard tissues have bone and cementum. Each will be depicted separately.

In gingiva, OPN was expressed in both gingival epithelium and connective tissue. In epithelium, there was diffuse moderate staining for OPN in the extracellular matrix. Also, some epithelial cells showed nuclear staining. Nevertheless, OPN staining in gingival connective tissue was sparse. It was more limited to blood vessel walls (Figure 1, A). Whereas in periodontal ligaments, OPN expression was intense (Figure 1, B). This intensity was dispersed thought the PDL tissue. It was expressed in fibroblasts, lining of blood vessel walls and extracellular matrix. But, staining in extracellular matrix in front of cementum (Figure 1, D) was less diffused compared to that in front of alveolar bone (Figure 1, C).

In bone, there was moderate to intense OPN staining. Moderate staining was detected in Sharpey's fibers (White arrow heads in figure 2, A) and the longitudinal lamellae of alveolar bone (black arrowheads in figure 2, A). Also reversal lines showed similar staining as previous structures (White arrow in figure 1, C). Expression of OPN in osteocyte was diverse. Some cells showed peri nuclear staining (Black arrowheads in figure 1, B) while others showed nuclear one (White arrowheads in figure 1, B) with their staining ranged from moderate to intense. Whereas bone marrow tissues showed intense staining (Figure 1, C). On the other hand, in cementum, OPN expression was weak in both; cellular and acellular cementum. Incremental lines of cementum showed weak to mild staining (White arrows in figure 2, B), Also, cementocytes showing mild perinuclear staining. But cells lining the cementum surface showed moderate staining. However, cementodentinal junction showed negative expression (Figure 2, B).

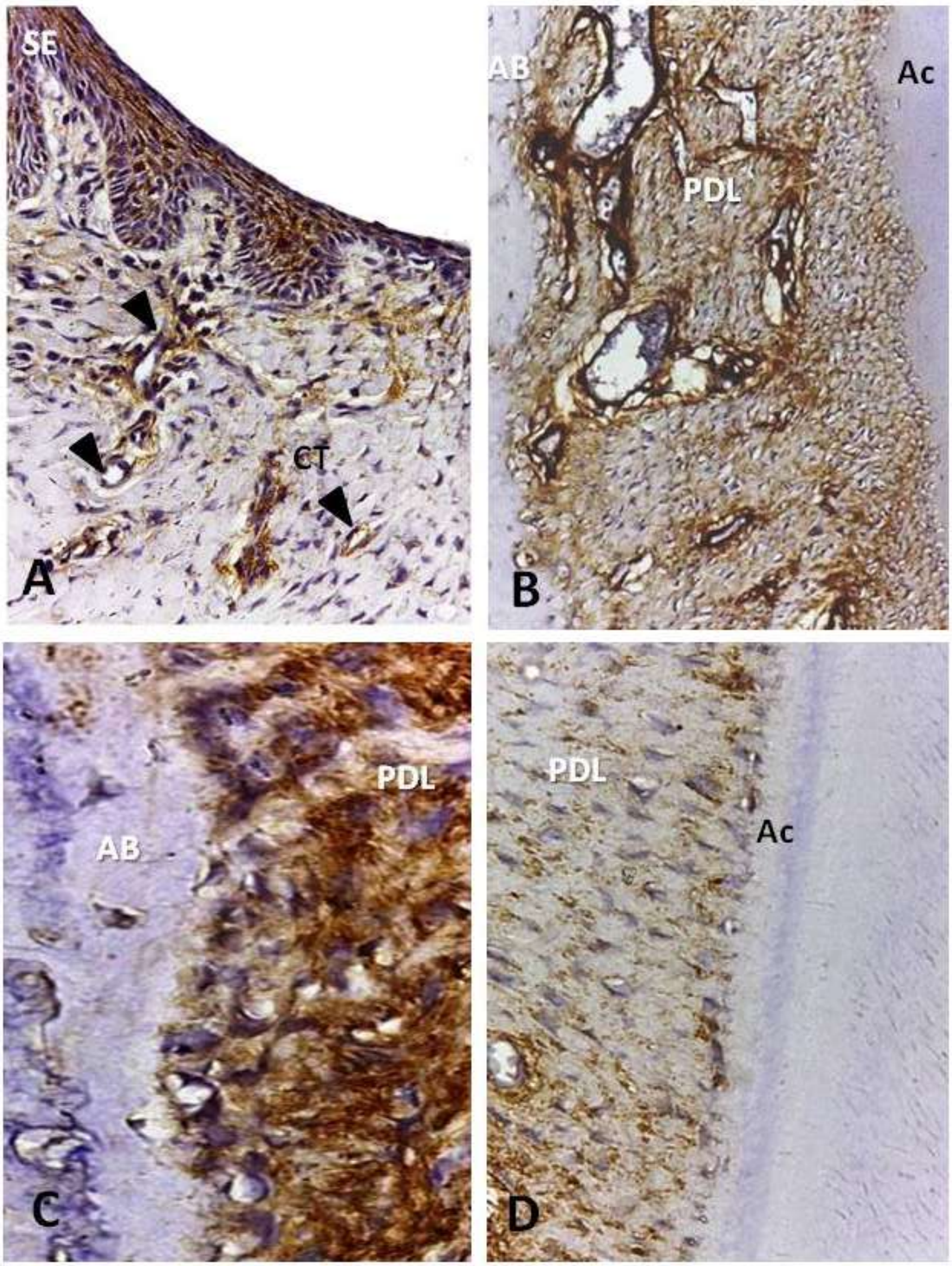

Figure 1: Immunolocalization of OPN in dog gingiva and PDL (A) Shows diffuse moderate to intense reaction in extracellular matrix of sulcular epithelium in addition to some nuclear staining. In gingival connective tissue, the intense reaction is sparse, more limited to the walls of blood vessels (Black arrowheads). (B) Shows intense diffuse staining in the extracellular matrix, fibroblasts and lining of blood vessels walls. (C) Shows intense staining along the border of alveolar bone. (D) Shows intense but less diffuse staining along the border of cementum. (SE) Sulcular Epithelium, (CT) Connective Tissue, (PDL) Periodontal Ligaments, (Ac) Acellular cementum, (AB) Alveolar Bone. (DAB. Original Mag. A, C and D X 40 and BX10).

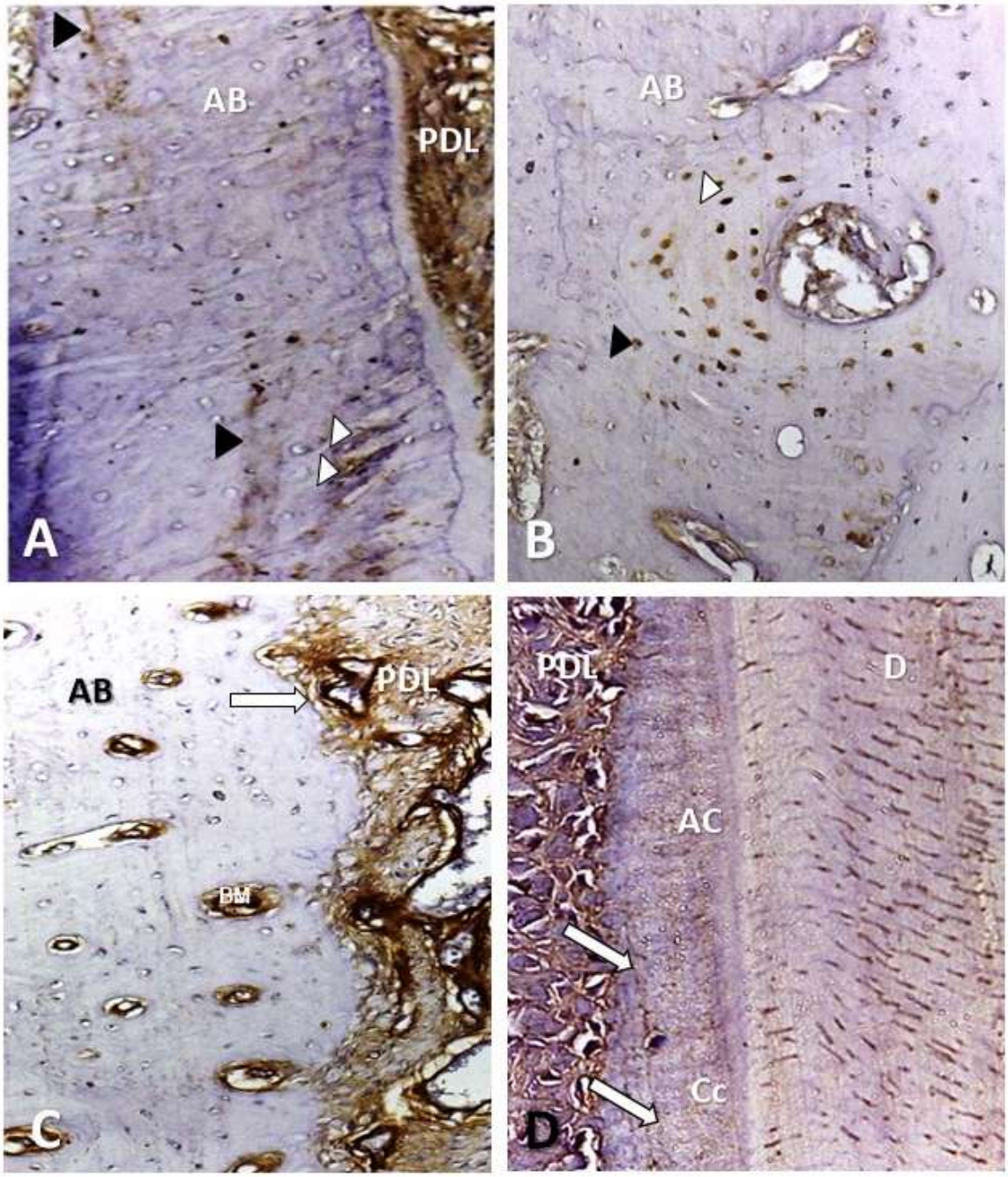

Figure 2: Immunolocalization of OPN staining in dog alveolar bone and cementum. (A) Shows moderate staining in Sharpey's fibers (White arrowheads) and longitudinal lamellae of alveolar bone proper parallel to the tooth surface (black arrowheads). While some scattered osteocytes show intense nuclear staining. (B) Shows nuclear and peri nuclear staining of osteocytes in Haversian system of supporting alveolar bone (White and black arrow heads respectively). (C) Shows staining of BM tissues and reversal lines (White arrow). (D) Shows OPN staining in cementum. In cementum, OPN has weak staining in both, acellular and cellular cementum. Cells lining the cementum show moderate staining. Also, Incremental lines of cementum show weak to mild staining (White arrows). In addition, cementocytes show mild perinuclear staining. Whereas cementodentinal junction shows no staining. (AB) Alveolar Bone, (PDL) Periodontal Ligaments, (BM) Bone Marrow. (Ac) Acellular cementum. (Cc) Cellular cementum. (D) Dentin. (DAB. Original Mag. A and B X10, C and D X 40).

4. Discussion

The extensive incidence of periodontal diseases along with the awareness that damaged or lost tissues could be repaired or regenerated has developed great interest in the factors events, proteins and cells regulating their formation and maintenance [27]. It has been reported that, the usage of animal models in periodontal research is a necessary step prior to entering into clinical trials and understanding the origin and evolution of pathology in humans. In this study, beagle dogs were used, as they have been one of the most widely used animal models in periodontal research [28]; especially for the study of the histopathological aspects of the disease and for developing new therapeutic procedures [29,30]. However, little is known about normal expression of growth factors and matrix proteins in their periodontium.

OPN is a secreted non-collagenous bone matrix protein. It was named for its function as a bridge between cells and minerals. OPN is synthesized at the highest levels in bone and epithelial tissues. It is also expressed in a variety of other cell types including macrophages, activated T cells smooth muscle cells and endothelial cells [31-35]. In addition, OPN is synthesized by preosteoblasts, osteoblasts and osteocytes. As well as bone cells, OPN is synthesized by extraosseous cells, as well as by odontoblasts, certain bone marrow cells and several types of cultured fibroblasts and epithelial-derived cell lines can secrete open [36].

In dog's gingiva, OPN was expressed differently between both epithelium and connective tissue. Though both had the same intensity, it was diffuse in epithelium in contrast to connective tissue, where it was limited to blood vessels. In a study comparing the expression of bone associated macromolecules by human gingival and periodontal ligaments fibroblasts, OPN had weak expression in the extracellular matrix and cells of gingival connective tissues. In contrast, Cultures of porcine gingival fibroblasts derived from healthy explants of gingiva showed strong OPN mRNA band when cultured with alpha MEM supplemented with 15% fetal calf serum [37]. OPN was reported to be expressed in human tissues with surface epithelium lining suggesting a protective role in interaction between these epithelial surfaces and the external environment [38]. Gingival tissue is continuously subjected to mechanical and bacterial aggression and epithelial cells contribute to the innate host response in all human epithelia, including oral epithelia [39,40]. These together suggest a similar protective role of OPN in gingival epithelium.

In PDL, OPN expression was intense throughout the whole tissue. This contrasts with McKee and Nanci, 1996 who stated the absence of OPN in the PDL proper using a rat model [41]. On the other hand, Rincon et al., 2005, demonstrated strong OPN mRNA expression in cultures of PDL fibroblasts and epithelial cell rests of Malassez derived from healthy explants of periodontal ligament [37]. Also, Ivanovski et al., 2001 reported strong OPN expression in extracellular matrix of human PDL. They proposed that, strong expression within the PDL gives further support to the theory that cells from the periodontal ligament have the ability to facilitate hard tissue formation and hence play a role in periodontal regeneration. Also, the same group explained the comparatively weak localization of OPN within the connective tissue of the gingiva compared with PDL as those two closely apposed but anatomically unique tissues are synthesized and maintained by distinct fibroblast populations [42].

OPN is abundant in bone matrix. It is synthesized by preosteoblasts, osteoblasts and osteocytes [43]. It has been linked with bone formation and bone resorption.

While OPN is not essential for bone resorption, it is required for osteoclast stimulation [44]. It may act as a means of attachment for these cells to the bone surface. In rats periodontium, alveolar bone was intensely stained for OPN along the trabecular surfaces, and in general the predominant staining pattern was oriented parallel to the longitudinal axis of the tooth [20]. Also, cultured porcine bone cells showed strong OPN expression [37]. In the present study, alveolar bone had moderate OPN staining in Sharpey's fibers, the longitudinal lamellae of alveolar bone, reversal lines and osteocytes. In contrast, bone marrow tissues showed intense staining. OPN in cementum is believed to guide and stabilize the interfibrillar mineralization pattern in this tissue [45]. In a study examining OPN and bone sialoprotein expression in regenerating rat PDL and alveolar bone, OPN was present along cellular and acellular cementum and was particularly intense along the cementodentinal junction [20]. This in part matches with our results which showed OPN expression in both; cellular and acellular cementum but negative cementodentinal junction. Also, OPN mRNA positive signal was detected at cellular cementum, and the surface of the acellular cementum within the PDL space of the 7-week-old rat tooth during physiological tooth movement [46]. Similarly, weak staining for OPN in rat's acellular and cellular cementum was demonstrated [41].

In conclusion

OPN is a multifunctional cytokine involved in diverse functions like cell adhesion, migration, survival and proliferation of cells. Thus, OPN expression in normal dog's periodontium coincides

with its continual remodeling. Also, it contributes, in part, to its defense action.

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