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Vitamin D-binding protein expression in healthy tooth and periodontium: an experimental study both in monkeys *in vivo* and in humans *in vitro*

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Background and Objective: Vitamin D-binding protein (DBP) is a highly expressed plasma protein with many important functions, including transport of vitamin D metabolites, sequestration of actin, control of bone metabolism and modulation of immune and inflammatory responses. Previous results of our study indicated an association between DBP and periodontitis. We hypothesized that periodontium might be another source of DBP in gingival crevicular fluid other than serum.

Material and Methods: DBP expression was examined in dental and periodontal tissues of monkeys by immunohistochemistry, and in primary cells isolated from human dental and periodontal tissues by reverse transcription plus the polymerase chain reaction and immunocytochemistry.

Results: DBP was constitutively expressed and widely distributed in dental and periodontal tissues of primates. Their immunoreaction was evident in gingival epithelium, particularly in junctional epithelium, and in mineralizing areas of the dental pulp, periodontal ligament and bone marrow. Correspondingly, mRNA and protein expression were detected in primary human gingival epithelial cells, dental pulp cells and periodontal ligament cells.

Conclusion: DBP is highly expressed and widely distributed in dental and periodontal tissues, which may take an active part in local host defense and hard tissue metabolism.

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Vitamin D-binding protein (DBP) is a multifunctional and highly expressed plasma protein. It is synthesized predominantly by hepatic parenchymal cells and at lower levels, by tissues such as kidney, testis and placenta (1). As its name suggests, it was initially recognized as the major carrier of vitamin D and its metabolites throughout the body (2). Soon afterwards, it was found that at any one time, only 1–2% of the vitamin D-binding sites were occupied (3). Such an excess came up with the possibility that the primary role of DBP extended far beyond acting solely as a transporter. Thereafter, multiple additional functions have been described,

such as extracellular actin scavenging, complement component 5a (C5a)-mediated leukocyte chemotaxis, macrophage activation and control of bone metabolism (4).

Periodontitis is an inflammatory disorder affecting the supportive tissues of teeth, resulting in attachment loss, formation of periodontal pockets, resorption of the alveolar bone and ultimately tooth loss. Although initiated by bacteria, it is the host immune response that determines the destructive process of the disease, in which cytokines play important roles. The aforementioned functions of DBP might happen to be implicated in periodontitis. As hypothesized, previous studies from our group pointed to an association between DBP and periodontitis. Patients with generalized aggressive periodontitis had significantly higher DBP concentration in plasma while lower in gingival crevicular fluid compared with healthy controls (5,6). Further analysis showed that in healthy conditions, the levels of DBP in gingival crevicular fluid were even higher than those in plasma. This indicated that DBP might play a role in periodontal health and that periodontal tissues might be another source of gingival crevicular fluid DBP other than serum (6). Furthermore, we carried out this study with the use of dental and periodontal tissues of monkeys and tissue cells isolated from human dental and periodontal tissues, to examine systematically the DBP expression and distribution and to infer its possible roles in health conditions.

Material and methods

Tissue sampling

With the approval of the Experimental Animal Welfare Ethical Branch of Peking University Biomedical Ethics Committee (LA2008-006), dental and periodontal specimens were collected from monkeys (*Macaca fascicularis*) from our earlier experiments (7,8). Excised mandibular premolars and molars with their periodontal tissues, from three adult male monkeys (5.5–6.0 years old, weighing 5.1–5.5 kg; Laboratory Animal Center of Academy of Military Medical Sciences, Beijing, China), were first fixed in 10% buffered formalin and then decalcified in 10% EDTA, dehydrated and embedded in paraffin. Microtome serial mesio-distal sections (5 μm) were cut and mounted on to adhesive slides. All sections were examined by hematoxylin and eosin staining.

Vitamin D-binding protein immunohistochemistry

After deparaffinization and rehydration, selected sections were soaked in 3% hydrogen peroxide for 10 min at room temperature to inhibit endogenous peroxidase and digested by 1 mg/mL trypsin for 10 min at 37°C for antigen retrieval. After blocking with 10% normal goat serum at room temperature for 10 min, sections were incubated with rabbit anti-recombinant human DBP polyclonal antibody (1 : 1000; Abcam, Cambridge, MA, USA) or normal rabbit IgG (Santa Cruz, Santa Cruz, CA, USA) at 4°C overnight. The staining of DBP was performed via a polymer/HPR and DAB chromagen system (Zhongshan Golden Bridge Biotechnology, Beijing, China). Finally, the sections were counterstained with hematoxylin. All sections were examined under a digital microscopic system (Olympus BX51/DP72, Tokyo, Japan).

Culture of human primary cells

For confirmation of the results from monkeys, primary human periodontal ligament cells (PDLCs), gingival fibroblasts (GFs) and gingival epithelial cells were cultured with tissue explant methods generally as described previously (9–11). Briefly, PDLCs were obtained by explants dissected from the mid-root of premolars extracted from donors under orthodontic treatment with a sharp scalpel and then minced into small pieces. GFs and gingival epithelial cells were derived from healthy gingival tissues under crown-lengthening surgeries. The gingival epithelium and connective tissues were dissected and minced separately. PDLCs and GFs were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco) at 37°C in a humidified atmosphere of 5% CO_2, while gingival epithelial cells replaced with defined keratinocyte serum free medium (Gibco). After the cells were 80% confluent, primary cells were digested with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA and subcultured. Cells at passages 2–4 were used for the following experiments. The study protocol was reviewed and approved by the ethical board of Peking University School and Hospital of Stomatology (PKUSSIRB-2011007). Human dental pulp cells (DPCs) were kindly donated by Dr. Sainan Wang (Department of Odontology, Peking University School and Hospital of Stomatology).

Detection of vitamin D-binding protein transcription in human primary cells

Total RNA was isolated from the above cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions and was quantified by spectrophotometric absorbance at 260 nm. Approximate 2 μg total RNA was reverse transcribed to cDNA via a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) was then performed with the use of a *Taq* PCR MasterMix (Solarbio Science & Technology, Beijing, China). The primers used were as follows: GAPDH, forward: CGACAGTCAGCCGCATCTT, reverse: CCAATACGACCAAATCCG TTG; DBP, forward: TGGCTACCA CTTTTACATGGTC, reverse: TCAA ACGTGCCACTGGGAAA. The products were analyzed by agarose gel electrophoresis and nucleotide sequencing.

Detection of vitamin D-binding protein expression in human primary cells

Protein of DBP in the above cells was detected by immunocytochemistry. After seeding on glass slides for 8 h,

cells were fixed in 95% ethanol for 30 min at room temperature. Then, 3% hydrogen peroxide was used to inhibit endogenous peroxidase for 10 min at room temperature. After blocking with 10% normal goat serum for 10 min, cells were incubated with the same antibody used above or normal rabbit IgG at 4°C

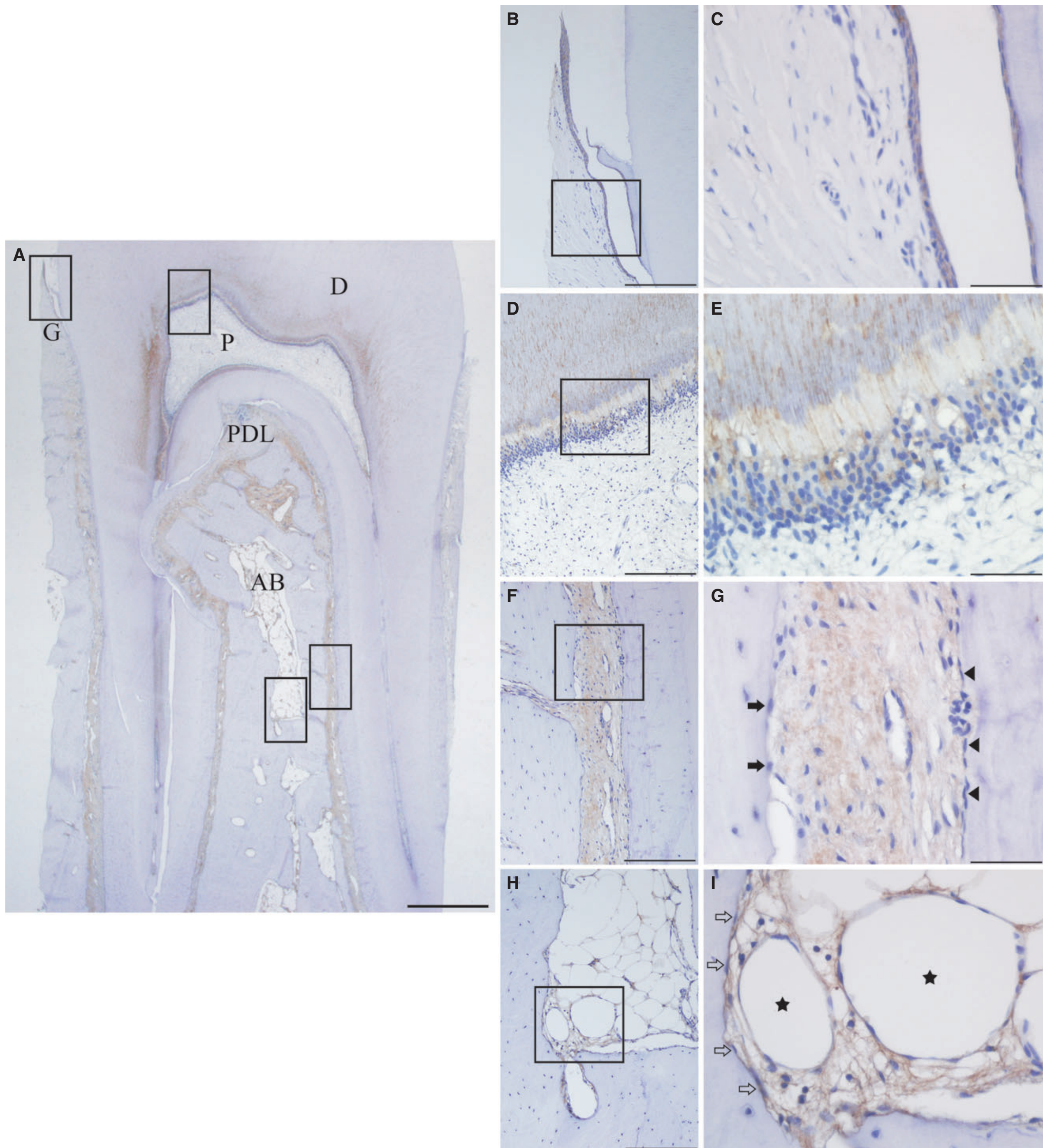

Fig. 1. (A) Immunohistochemistry of vitamin D-binding protein in dental and periodontal tissue; (B, D, F, H) magnification of the boxed areas in gingiva, pulp, periodontal ligament and alveolar bone of (A), respectively; (C, E, G, I) high magnification of the boxed areas in (B, D, F, H), respectively. AB, alveolar bone; D, dentin; G, gingiva; P, pulp; PDL, periodontal ligament. Solid arrows, osteoblasts in periodontal ligament lying next to the alveolar bone; solid triangles cementoblasts in periodontal ligament lying next to the cementum; asterisks, fat cells in alveolar bone marrow; hollow arrows, osteoblasts in alveolar bone marrow lining the marrow cavities. Scale bars: (A) 1 mm; (B, D, F, H) 200 μm; (C, E, G, I) 50 μm.

overnight. The staining of DBP was performed in the same way and hematoxylin was used for counter-staining.

Results

Vitamin D-binding protein expression in dental and periodontal tissues of monkeys *in situ*

DBP was detected in gingival epithelium, dental pulp, periodontal ligament and bone marrow (Fig. 1). Specifically, in gingival epithelium, DBP was strongly positive in all layers of the junctional epithelium (Fig. 1B and 1C), evenly positive from stratum basale to stratum granulosum of the oral and sulcular epithelium (from other samples, Fig. 2), while much stronger in the superficial hydropic degenerated cells of the sulcular epithelium (Fig. 2C solid triangles) and much weaker in stratum corneum of the oral epithelium (Fig. 2B). Moreover, the staining seemed to become weaker from junctional epithelium and sulcular epithelium to oral epithelium. In gingival connective tissue, most cells were DBP-negative (Fig. 2A), with only some GFs near the inflammatory zone weakly stained (Fig. 2C hollow triangles). In addition, some polymorphonuclear leukocytes (Fig. 2C hollow arrow) and mononuclear cells (Fig. 2C solid arrow) beneath the sulcular epithelium also expressed DBP. In the pulp, cells in the pulp core were negative for DBP (Fig. 1D). However, odontoblasts lying next to the dentin were significantly positive, some of whose cytoplasmic processes extending into the dentinal tubules even showed DBP immunoreactivity (Fig. 1E). Periodontal ligament was diffusely positive for DBP (Fig. 1F), with osteoblasts (Fig. 1G solid arrows) lying next to the alveolar bone and cementoblasts (Fig. 1G solid triangles) lying next to the cementum were evenly positive. In alveolar bone marrow (Fig. 1H), fat cells (Fig. 1I asterisks) and osteoblasts (Fig. 1I hollow arrows) lining the bone marrow cavities were DBP positive as well. Results of the negative controls confirmed the specificity of the immunoreaction (data not shown).

Vitamin D-binding protein expression in human cells isolated from dental and periodontal tissues *in vitro*

Reverse transcription plus the PCR revealed the transcription of DBP mRNA in human PDLCs, GFs, DPCs and gingival epithelial cells (Fig. 3). Relatively, DBP was primarily expressed in gingival epithelial cells, less in DPCs and the least in PDLCs. The transcripts of DBP mRNA in GFs could not be detected in this condition. Immunocytochemistry confirmed the results of reverse transcription-PCR. DBP staining was most prominent in gingival epithelial cells, followed by DPCs and PDLCs, and negative in GFs (Fig. 4). Generally, the results were consistent with those observed in dental and periodontal tissues of the monkeys *in vivo*.

Discussion

DBP is characterized by widespread distribution into the tissues and can be determined most abundantly in plasma and less in cerebrospinal fluid, seminal fluid, saliva and breast milk (12). As far as we know, this is the first study to identify systematically the expression and distribution of DBP in dental and periodontal tissues both in monkeys *in vivo* and in humans *in vitro*. Significantly, gingival epithelial cells, DPCs and PDLCs, these unique cells from dental and periodontal tissues, all expressed DBP to some extent. Combined with our previous results that in healthy conditions the levels of DBP in gingival crevicular fluid were higher than those in plasma (6), it is reasonable to

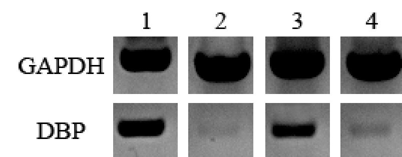

Fig. 3. Messenger RNA expression of DBP in primarily cultured human gingival epithelial cells (lane 1), gingival fibroblasts (lane 2), dental pulp cells (lane 3) and periodontal ligament cells (lane 4). GAPDH was used as an internal control. DBP, vitamin D-binding protein.

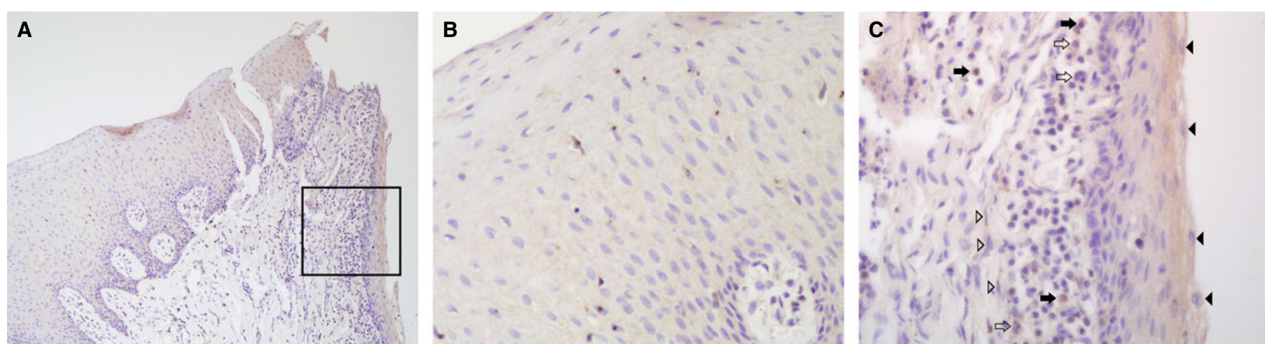

Fig. 2. (A) Immunohistochemistry of vitamin D-binding protein in gingiva; (B, C) high magnification of the boxed areas in oral epithelium (left) and sulcular epithelium (right), respectively. Hollow arrows, polymorphonuclear leukocytes; solid arrows, mononuclear cells; hollow triangles, weakly positive gingival fibroblasts; solid triangles, superficial hydropic degenerated cells of the sulcular epithelium. Scale bar: (A) 200 μm; (B, C) 50 μm.

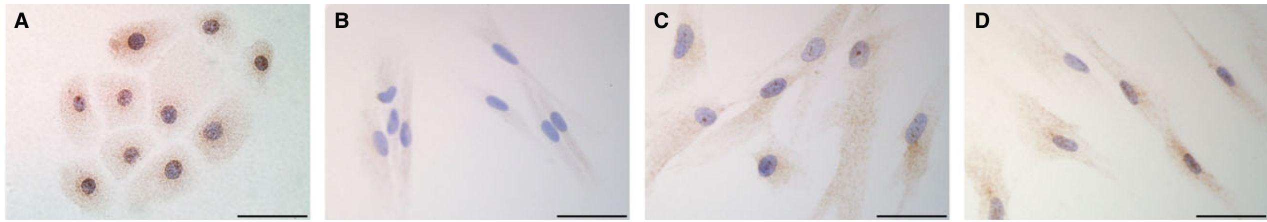

Fig. 4. Protein expression of vitamin D-binding protein in primarily cultured human human gingival epithelial cells (A), gingival fibroblasts (B), dental pulp cells (C) and periodontal ligament cells (D). Scale bar: 50 μm.

presume periodontal tissues to be another source of gingival crevicular fluid DBP other than serum.

DBP is known for its role as a transporter of vitamin D. Vitamin D has a multilevel role in maintaining periodontal health, including strengthening epithelial barrier, stimulating antimicrobial peptides, enhancing macrophage activities and reducing bone loss (13). However, binding to DBP is a requisite for vitamin D to be transported within the organism, facilitating access to tissues and cells, as well as regulating the amount of vitamin D available (14). Previous results from our group demonstrated that *in vitro* human GFs and PDLCs could uptake 25-hydroxyvitamin D_3 to synthesize 1,25-dihydroxyvitamin D_3 so as to make it possible for vitamin D to act in an autocrine/paracrine manner in these cells (15). Our finding of DBP expression in periodontal tissues helps to make these further convincing in the periodontal tissues *in vivo*.

In addition to vitamin D, DBP also acts as binding protein of actin (14). Upon cell lysis, actin is released into the extracellular environment, where it goes on to nucleation and polymerization and has the potential to block and damage the microvasculature (16). Periodontitis has been shown to be associated with endothelial and microvascular dysfunctions (17). Thus, the presence of DBP in periodontal tissues, which binds actin with high affinity (18), must have contributed to the local protection against the detrimental effects of actin and partially explain the significant decrease of gingival crevicular fluid DBP levels in periodontitis (6).

DBP is also implicated in the immune system and host defense. DBP was suggested to be an important scavenger of endotoxin (19). And the concentrations of DBP were ever used to predict organ failure in peritonitis (20). DBP was also reported to be associated with the surface of a large number of cells, such as neutrophils, fibroblasts, B and T cells, etc. (21), which was also shown in our results (Fig. 2C). When binding to C5a, DBP can enhance the C5a-mediated neutrophil and monocyte chemotaxis (22,23). This can act as the first defense in the periodontium and determine the switch for initiation or resolution of periodontitis particularly at the initial stage. During inflammation, activated T and B cells are able rapidly to transform the DBP into a potent macrophage-activating factor (MAF) (24). Thereafter, this DBP-MAF can activate macrophages for the benefit of the host defense (25). However, if the inflammation overreacts, DBP-MAF can induce apoptosis in activated macrophages via caspase induction as well (26). Therefore, the expression of DBP in the periodontium, particularly in the junctional epithelium, may represent an important mechanism for local protection in periodontal health and inflammation.

Furthermore, DBP-MAF is also involved in bone metabolism. In cases of inflammation, the production of DBP-MAF could contribute to bone resorption through its effects on modulating osteoclast calcium sensing (27). In an *in vitro* assay, DBP-MAF was much more potent for stimulating bone resorption in comparison to interferon-γ (28). In contrast, in an *in vivo* assay, the same protein was able to promote new bone formation upon injection into rat models (29). These paradoxical results suggest that DBP might have a more generalized regulating role in bone formation and remodeling. As alveolar bone is the most dynamic in the skeletal system, the abundance of DBP in periodontal ligament and bone marrow might be critical for mineralization homeostasis in healthy tissues.

In conclusion, our study for the first time systematically determined the expression and distribution of DBP in dental and periodontal tissues. Tissue cells, including gingival epithelial cells, DPCs and PDLCs, were all able to synthesize DBP, which may be responsible for local host defense and hard tissue metabolism. Further research on DBP expression during periodontal inflammation and its function in periodontal tissues should be carried out for understanding its roles in periodontitis.

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Competing interests

The authors declare no conflicts of interest related to this study.

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