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Insulin-like growth factor-binding protein-2 and -3 in gingival crevicular fluid

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Background and Objective: Insulin-like growth factor-binding proteins (IGFBPs) are crucial regulators of insulin-like growth factor (IGF). They enhance or inhibit IGF functions, but also exhibit IGF-independent effects. In a previous study, we detected, qualitatively, IGFBP-2 and -3 in gingival crevicular fluid using a cytokine antibody array. Here we extended these results using an ELISA to determine the concentrations of IGFBP-2 and -3 in gingival crevicular fluid. In addition, we explored whether the expression of IGFBP-2 and IGFBP-3 correlates with periodontal disease severity.

Material and Methods: Gingival crevicular fluid samples from 92 sites of 12 patients affected with periodontal disease and from 100 sites of 19 healthy volunteers, were collected, divided into two groups and analyzed by ELISA for IGFBP-2 and -3 expression. The potential correlation among probing depth, gingival index and the concentrations of IGFBP-2 and -3 was analyzed.

Results: Positive correlations were observed between the concentration of IGFBP-2 and probing depth and gingival index, but not for IGFBP-3. The IGFBP-2 concentrations at bleeding on probing-positive sites and at sites with a probing depth of ≥ 4 mm were higher than at bleeding on probing-negative sites and at sites with a probing depth of ≤ 3 mm.

Conclusion: These results indicate that IGFBP-2 is a potential novel marker for periodontal disease progression. As IGFBP-2 modulates bone metabolism and cell migration, IGFBP-2 in the gingival crevicular fluid may reflect periodontal disease activity.

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The insulin-like growth factor (IGF) system is well characterized and has profound effects on the growth and differentiation of normal and malignant cells (1). The established components of the IGF family include insulin, IGF-I and IGF-II peptides, IGF-II/mannose 6-phosphate receptors and at least six, high-affinity IGF-binding proteins (IGFBP-1 to IGFBP-6) (2–4). IGF-I and IGF-II are multifunctional peptides that regulate cell proliferation, differentiation, apoptosis and tumorigenesis (5). The family of structurally related IGFBPs bind to IGFs with high affinity and are widely distributed in the serum, tissue and extravascular fluid; thus, they (i) regulate ligand availability and half life in the serum and interstitial compartment; (ii) modulate the IGF/IGF-IR interaction; (iii) augment or inhibit IGF action; and (iv) exhibit IGF-independent activity (6). Hence, as IGFs, IGFBPs are also multifunctional.

The function of the IGF system in oral biology has been reviewed to analyze its role in tooth development, growth, periodontal ligament homeostasis and different pathological conditions (e.g. salivary gland disease) (2). Götz *et al.* (7) reported the localization of both IGF ligands and six binding proteins in the periodontal tissue of

permanent teeth. However, the precise relationship between the IGF system and periodontal disease remains unclear.

We have previously detected IGFBP-2 and -3 in the gingival crevicular fluid, qualitatively, using a cytokine antibody array (8). Elevated serum IGFBP-2 is a predictor of bone resorption (9), and IGFBP-3 is one of the major IGFBPs in serum (10). Therefore, the aim of the present study was to measure the concentrations of IGFBP-2 and IGFBP-3 in the gingival crevicular fluid, and to examine whether the concentrations of IGFBP-2 or -3 correlate with clinical parameters for periodontal disease. This might indicate a direct pathophysiological role in disease progression, which would make IGFB-2 and -3 potential novel molecular therapeutic targets.

Material and methods

Subject selection and gingival crevicular fluid sampling

Twelve subjects [nine men and three women; mean age (range): 57.2 (22–91) years] were selected from patients with defined periodontal disease, dental caries, pulpitis, or periapical periodontitis, who had been referred to the Department of Periodontology, Nihon University School of Dentistry Dental Hospital. Nineteen volunteers [13 men and six women; 38.0 (22–72) years] with no dental disease, as diagnosed from clinical and radiographic examinations, were also included in this study. They were free of systemic disease and had received no periodontal treatment or antibiotic therapy for at least 6 mo before examination. To diagnose chronic periodontitis clinically, the probing depth was measured using a periodontal probe (CP-11; Hu-Friedy®, Chicago, IL, USA), and bleeding on probing was recorded after gingival crevicular fluid samples had been obtained. The gingival index was also recorded. The sites from healthy subjects had a probing depth of $\leq$ 3 mm and were bleeding on probing negative. The sites from diseased subjects had a probing depth of $\geq$ 4 mm and were bleeding on probing positive. Gingival crevicular fluid sampling was carried out using a slight modification of the protocol described by Ohshima *et al*. (11). First, the teeth were air-dried and isolated with cotton rolls after removing the supragingival plaque. Gingival crevicular fluid samples were collected by inserting paper strips (Harco Electronics, Winnipeg, MB, Canada) into the sites until the gingival crevicular fluid reached a marked line (approximately 1.0 μL of gingival crevicular fluid was absorbed per strip). In the event of difficulty collecting gingival crevicular fluid (e.g. from only one site in a healthy subject), gingival crevicular fluid was collected from two or more sites selected using the same criteria. Any strips visibly contaminated with blood were discarded, and other sites were selected using the same criteria. The gingival crevicular fluid absorbed by each strip was eluted with 50 μL of phosphate-buffered saline, extracted by vortexing for 30 s and centrifuged at 1000 *g* for 30 s for use in subsequent assays or stored at −80°C until analyzed. One hundred and ninety-two gingival crevicular fluid samples were divided into two groups of 96 samples each. One group was used for determining the IGFBP-2 concentration and the other was used for determining the IGFBP-3 concentration. Intergroup differences between age, probing depth and gingival index were not statistically significant (data not shown). All subjects gave written informed consent that was approved by the Ethics Committee of Nihon University School of Dentistry.

Determination of IGFBP-2 and -3

The IGFBP-2 content in the gingival crevicular fluid was determined using a specific ELISA kit (RayBio® human IGFBP-2 immunoassay; RayBiotech, Norcross, GA, USA), according to the manufacturer's instructions. A gingival crevicular fluid-containing strip was immersed in 50 μL of phosphate-buffered saline, and extracted as described above. Fifty microliters of each specimen was used for the assay. The IGFBP-2 concentration in gingival crevicular fluid was quantified using a standard curve. The IGFBP-3 concentration was determined using a specific ELISA kit (RayBio® human IGFBP-3 immunoassay; RayBiotech) in the same way.

Statistics

The correlation between each of the parameters was analyzed using the Pearson's correlation coefficient. The differences between the groups were analyzed using the Mann–Whitney rank sum test.

Results

The mean concentration of IGFBP-2 in the gingival crevicular fluid was 262.7 (range: 0–1700.6) pg/μL. The mean values of periodontal parameters and of the IGFBP-2 concentration from the healthy and diseased sites are shown in Table 1. All parameters from diseased sites were significantly higher than those from healthy sites.

Table 1. Periodontal parameters and insulin-like growth factor-binding protein-2 (IGFBP-2) concentration of the periodontally healthy and diseased sites

	Healthy (16 subjects, 49 sites)				Diseased (11 subjects, 47 sites)				
Status and level	Mean	SD	Median	Range	Mean	SD	Median	Range	*p*-value
PD (mm)	2.6	0.5	3	2–3	6.8	2.0	6	4–11	< 0.001
GI	0.6	0.5	1	0–1	2.5	0.5	2	2–3	< 0.001
IGFBP-2 (pg/μL)	164.0	109.9	131.5	0–453.5	365.6	340.0	270.0	40.3–1700.6	< 0.01

GI, gingival index; PD, probing depth.

As shown in Fig. 1, the correlation between probing depth and IGFBP-2 concentrations in the gingival crevicular fluid was significant (rho = 0.36; $p < 0.001$). Similarly, the correlation between gingival index and IGFBP-2 concentrations in the gingival crevicular fluid was also significant (rho = 0.38; $p < 0.001$) (Fig. 2). As expected, the probing depth correlated with the gingival index (rho = −0.92; $p < 0.0001$) (data not shown). The mean IGFBP-2 concentrations in bleeding on probing-positive and -negative gingival crevicular fluid samples were 337.3 pg/μL ($n = 48$) and 188.1 pg/μL ($n = 48$), respectively (Fig. 3A); the difference was significant ($p < 0.001$). Similarly, the mean IGFBP-2 concentration in sites with a probing depth of ≥ 4 mm (365.5 pg/μL; $n = 47$) was significantly higher than the mean concentration in sites with a probing depth of ≤ 3 mm (164.0pg/μL; $n = 49$) ($p < 0.001$) (Fig. 3B).

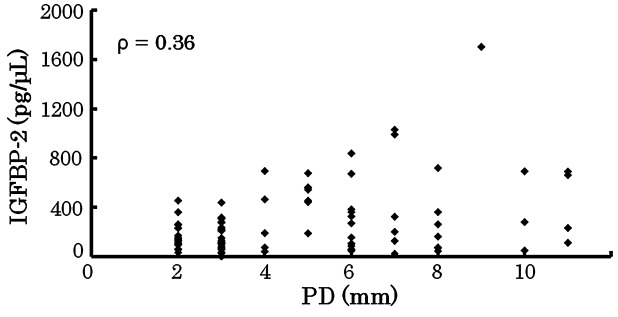

Fig. 1. Correlation between probing depth (PD) and the concentration of gingival crevicular fluid insulin-like growth factor-binding protein-2 (IGFBP-2) at 96 sites. Correlation analysis ($\rho = 0.36$; $p < 0.001$).

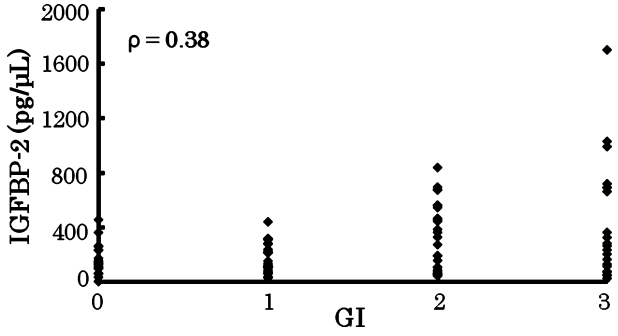

Fig. 2. Correlation between gingival index (GI) and the concentration of gingival crevicular fluid insulin-like growth factor-binding protein-2 (IGFBP-2) at 96 sites. Correlation analysis ($\rho = 0.38$; $p < 0.001$).

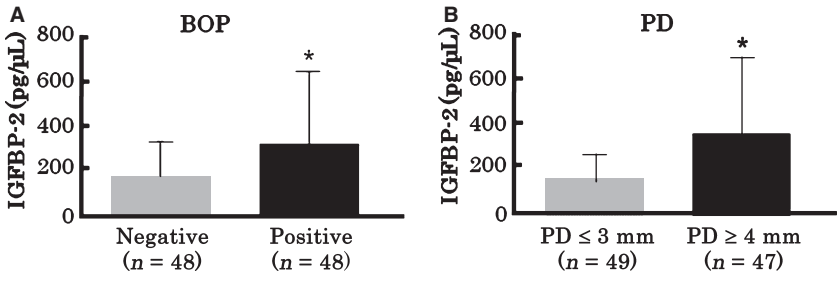

Fig. 3. Insulin-like growth factor-binding protein-2 (IGFBP-2) concentration in gingival crevicular fluid with or without bleeding on probing (BOP) (A) ($p < 0.001$), or probing depth (PD) ≥ 4 mm (subjects with periodontal disease) or ≤ 3 mm (healthy subjects) (B) ($p < 0.001$).

The mean IGFBP-3 concentration in gingival crevicular fluid was 36.0 pg/μL (range: 0–177.3 pg/μL). The mean values of periodontal parameters, and the IGFBP-3 concentration, from the healthy and diseased sites are shown in Table 2. The clinical parameters had significantly higher values in diseased sites than in healthy sites, but there was no significant difference between the concentration of IGFBP-3 in diseased and healthy sites. As shown in Fig. 4, the correlation between probing depth and IGFBP-3 concentrations in gingival crevicular fluid was not significant (rho = −0.15; $p = 0.142$). Similarly, the correlation between gingival index and IGFBP-3 concentrations in gingival crevicular fluid was also not significant (rho = −0.05; $p = 0.678$) (Fig. 5). Probing depth correlated with gingival index (rho = 0.86; $p < 0.0001$; data not shown).

The mean IGFBP-3 concentrations in bleeding on probing-positive and -negative gingival crevicular fluid samples were 31.3 pg/μL ($n = 47$) and 40.3 pg/μL ($n = 49$), respectively (Fig. 6A); the difference was not significant ($p = 0.214$). Similarly, the IGFBP-3 concentrations in sites with a probing depth of ≥ 4 mm (33.8 pg/μL; $n = 45$) were not significantly higher than those in sites with a probing depth of ≤ 3 mm (37.9 pg/μL; $n = 51$; $p = 0.642$) (Fig. 6B).

Discussion

To our knowledge, this is the first study to clearly demonstrate that the concentration of IGFBP-2 in the gingival crevicular fluid significantly correlates with probing depth and gingival index.

High serum levels of IGFBP-2 have been reported as the strongest predictor of low bone mineral density, particularly among men and postmenopausal women, independent of age and bioavailable sex steroids (9,12,13). IGFBP-2 has been shown to have an inhibitory effect on the anabolic action of IGF-I and IGF-II on bone (14–17) and thus elevated levels of IGFBP-2 could lead to a decrease in bone forma-

Table 2. Periodontal parameters and insulin-like growth factor-binding protein-3 (IGFBP-3) concentration of the periodontally healthy and diseased sites

Status and level	Healthy (15 subjects, 51 sites)				Diseased (9 subjects, 45 sites)				*p*-value
	Mean	SD	Median	Range	Mean	SD	Median	Range	
PD (mm)	2.4	0.5	2	2–3	6.0	2.2	5	4–11	< 0.001
GI	0.3	0.5	0	0–1	2.4	0.5	2	2–3	< 0.001
IGFBP-3 (pg/μL)	37.9	50.6	14.2	0-177.3	33.8	45.9	15.8	0-153.9	

GI, gingival index; PD, probing depth.

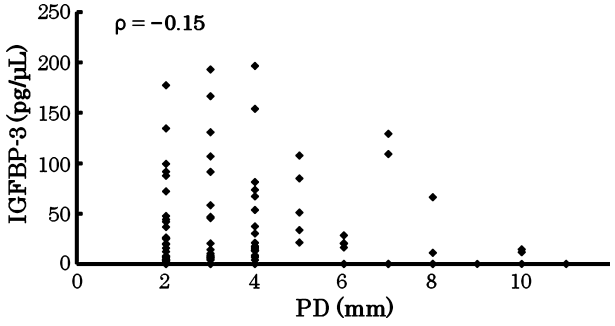

Fig. 4. Correlation between probing depth and the gingival crevicular fluid insulin-like growth factor-binding protein-3 (IGFBP-3) concentration at 96 sites. Correlation analysis ($\rho = -0.15$; $p = 0.142$).

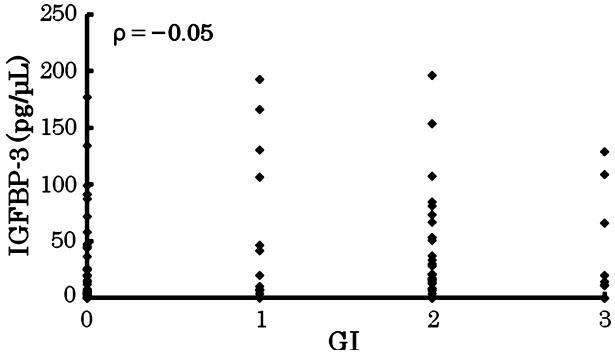

Fig. 5. Correlation between gingival index (GI) and the gingival crevicular fluid insulin-like growth factor-binding protein-3 (IGFBP-3) concentration at 96 sites. Correlation analysis ($\rho = -0.05$; $p = 0.678$).

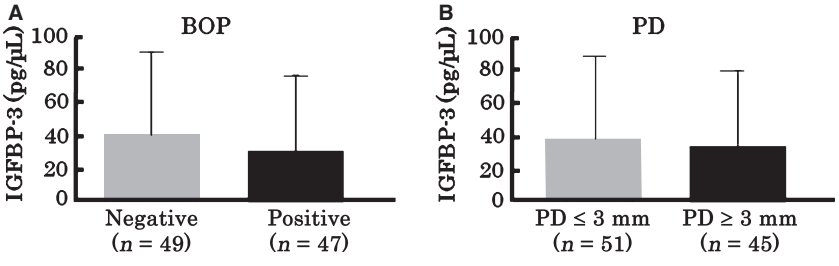

Fig. 6. Insulin-like growth factor-binding protein-3 (IGFBP-3) concentration in gingival crevicular fluid with or without bleeding on probing (BOP) (A), and a probing depth (PD) of ≥ 4 mm (subjects with periodontal disease) or ≤ 3 mm (healthy subjects) (B). (A) $p = 0.214$. (B) $p = 0.642$.

tion by interfering with the action of IGF. These findings suggest that elevated serum IGFBP-2 may have a negative effect on bone metabolism in aging men and women. Therefore, it is likely that high IGFBP-2 levels in the gingival crevicular fluid may reflect negative bone metabolism in periodontitis-affected sites.

However, the action of IGFBP-2 is complex and has thus far not been understood in detail. IGFBP-2 binds IGF with high affinity, thereby preventing the interaction of IGF with IGF receptors (17). Alternatively, IGFBP-2 stimulates the action of IGF by harboring IGF-II in the extracellular matrix (ECM), and IGF-II in complex with IGFBP-2 and glycosaminoglycans in the ECM is bioavailable for IGF receptor activation (17), because IGFBP-2 is able to bind different components of the ECM such as vitronectin, laminin, collagens and fibronectin (18). Proteolysis of IGFBPs also regulates IGFBP functions that alter IGF affinity and/or association with cell membranes or ECM proteins and ultimately modulates the targeting of IGF with cell membrane-bound receptors (19,20).

The IGF-independent activities of IGFBPs include cellular migration and the stimulation or inhibition of proliferation, and pro-apoptotic activity (21–23) has also been reported. Carboxy-terminal fragments of IGFBP-2 exert a mitogenic effect on growth plate chondrocytes comparable with that of equimolar concentrations of IGF-I (24). An IGFBP-2-binding protein, IIp45, antagonizes IGFBP-2 (25). Therefore, the local microenvironment should be considered to describe the role of IGFBP-2 in bone metabolism.

One of the IGF-independent functions of IGFBP-2 is the stimulation of

cell migration (26). IGFBP-2 contains an RGD sequence, binds to integrin α5 and induces glioma cell motility (26). Recently, we reported that Malassez-derived epithelial cells, gingival epithelial cells and periodontal ligament fibroblasts secrete IGFBP-2 (27). IGFBP-3 is the major IGFBP in adult serum (concentration range: 2200–4600 ng/mL), and IGFBP-2 appears to be less prevalent (concentration range: 50–270 ng/mL) (28). In the present study we detected higher amounts of IGFBP-2 than of IGFBP-3 in the gingival crevicular fluid. Thus, the local production of IGFBP-2 by Malassez-derived epithelial cells, gingival epithelial cells and periodontal ligament fibroblasts might be the major source of IGFBP-2 in gingival crevicular fluid. As we were unable to detect IGF-I in the gingival crevicular fluid from either healthy or diseased sites (8), IGFBP-2 in periodontal pockets may enhance epithelial cell apical migration together with hepatocyte growth factor (11), laminin and fibronectin (29), independently of IGF ligands.

IGFBP-3 in the gingival crevicular fluid did not correlate with clinical parameters. However, it cannot be ruled out that this factor plays a crucial role in periodontal disease. IGFBP-3 has multiple roles both in systemic and in local regulation (30) and has been widely accepted as a stimulator of IGF action in bone, in contrast to IGFBP-2 (31). High concentrations of IGFBP-3 in serum have also been associated with an increased risk of premenopausal breast cancer (5,30). Because of its high concentration in serum, the balance between local and systemic IGFBP-3 in periodontal tissue remains to be elucidated. It has been reported that IGFBP-3 localizes to the nucleus of several cultured cell lines, including epidermal keratinocytes (32,33), in the regulation of epidermal homeostasis, possibly via regulating the initial stages of keratinocyte terminal differentiation (34,35). These reports suggested that IGFBP-3 may be associated with homeostasis in periodontal tissue, rather than being involved in the progression of periodontitis.

A novel function of IGFBP-2 has been reported in IGFBP-2 null mice, where the roles of IGFBP-2 in gender-specific changes in bone turnover and skeletal architecture were described (36). It was shown that IGFBP-2 is critical for optimal trabecular bone acquisition in male mice. By contrast, the absence of IGFBP-2 in female mice resulted in increased cortical bone acquisition with minimal effects on the trabecular compartment. IGFBP-2 has been reported to support mouse hematopoietic stem cell expansion (37). Taking into account the results of the above studies and the periodontal tissue microenvironment, further studies are required to establish whether IGFBP-2 could be a novel marker, and thus a therapeutic target, in periodontal disease progression.

Conclusions

We have established an elevated concentration of IGFBP-2 in gingival crevicular fluid of patients with periodontitis, and suggest that IGFBP-2 plays a crucial role in bone metabolism and cell migration. However, the precise role of IGFBP-2 in periodontal disease progression needs to be characterized in greater detail. Further studies are required to describe precisely the individual roles of IGFBPs in periodontitis.

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