Expression of insulin like growth factor binding protein-5 in drug induced human gingival overgrowth

Tamilselvan Subramani, Anbazhagi Sakkarai*, Kamatchiammal Senthilkumar*, Soundararajan Periasamy**, Georgie Abraham** & Suresh Rao

Department of Periodontics, Sri Ramachandra Dental College & Hospital, *NEERI, Council of Scientific & Industrial Research, & **Department of Nephrology, Sri Ramachandra Medical College & Research Institute, Deemed University, Chennai, India

Received February 14, 2006

Background & objectives: Insulin like growth factor binding proteins (IGFBPs) control the distribution, function and activity of insulin like growth factors (IGFs) in various cells, tissues and body fluids, thereby modulating their metabolic and mitogenic effects. IGFBP-5, the most conserved IGFBP, can function through IGF or directly play a role in fibrosis. Cyclosporine A (CsA) widely used in organ transplant patients, often causes various side effects including gingival fibrotic overgrowth. This study was carried out to assess the mRNA expression of IGFBP-5 in healthy human gingival, chronic periodontitis and CsA induced gingival overgrowth tissues.

Methods: Total RNA was isolated from gingival tissues collected from eight patients with chronic periodontitis, eight patients with CsA induced gingival outgrowth and an equal number of healthy individuals, and subjected to reverse transcription (RT)-PCR for IGFBP-5 gene expression.

Results: CsA induced gingival overgrowth tissues expressed increased IGFBP-5 mRNA compared to control and chronic periodontitis.

Interpretation & conclusion: Increased mRNA expression of IGFBP-5 in CsA induced gingival outgrowth tissues may be associated with increased collagen synthesis, thereby promoting fibrogenesis.

Key words - Cyclosporine - IGFBP-5 - overgrowth - periodontitis

Insulin like growth factor binding proteins (IGFBPs) are a family of six circulating proteins which bind insulin like growth factors I and II (IGF-I and II) with high affinity and thus control their distribution, function and activity in various cells, tissues and body fluids, thereby modulating their metabolic and mitogenic effects1-3. The liver is the major source of circulatory IGFs and IGFBPs. There are changes in IGFBP expression and concentration in different tissues in many pathological states.
IGFBP-5 is the most conserved of the six IGFBPs. IGFBP-5, a 29 kD glycoprotein, potentiates the action of IGF-I in smooth muscle cells, fibroblasts and osteoblasts. IGFBP-5 also binds with high affinity to extracellular matrix components (ECM) which protects it from proteolysis. Drug-induced gingival overgrowth (DIGO) is known to be caused as side effect of certain drugs such as immunosuppressive agents (cyclosporine A, CsA), antiepileptic drugs (phenytoin) and calcium channel blockers (nifidipine). The pathogenic mechanisms of DIGO involve changes in the metabolism of connective tissue, causing an increased proliferation or decreased apoptosis, increased ECM production, etc., leading to fibrotic overgrowth. In pulmonary fibrosis, IGFBP-5 was found to be increased. Since the gingival overgrowth is similar to pulmonary fibrosis, the present study was carried out to examine the expression of IGFBP-5 mRNA in CsA induced human gingival overgrowth and compared with chronic periodontitis and healthy gingival tissues.

**Material & Methods**

**Materials:** Gingival tissues were collected at the time of resective surgery from marginal papillary and attached gingiva of eight chronic periodontitis patients (aged 35-55 yr; 4 male and 4 female). CsA induced gingival overgrowth tissue samples were obtained from lower anterior and upper posterior of eight patients (3 male and 5 female) aged 40-60 yr. Eight control samples (4 male and 4 female) were also obtained from upper and lower premolar region of healthy individuals, aged 13-17 yr, who have undergone orthodontic treatment. Drug induced gingival overgrowth tissue samples were obtained from cyclosporine-A medicated organ transplant recipients during gingivectomies required to treat drug induced gingival overgrowth. Patients, who were under CsA medication for minimum period of one year, and were taking the average dose of 3.6 mg/kg of body weight per day and developed clinically significant gingival overgrowth within 6 months of intake of the drug were included in the study. All the DIGO samples represented moderate to severe degree of gingival overgrowth. All the chronic periodontitis samples expressed clinical signs of inflammation and were taken from sites with a probing depth greater than 6 mm with evidence of bleeding on probing. The control subjects were periodontally healthy without gingival inflammation and showed no evidence of bleeding on probing with a probing depth less than 4 mm. Gingival tissue samples which would normally be discarded at the time of periodontal surgery were retained if they meet the inclusion criteria. All the patients and healthy subjects were nonsmokers. Subjects, who were medically compromised, or had antibiotics within the preceding 6 months and who had undergone any periodontal therapy within 6 months were excluded from the study. The number of samples was restricted to 8 based on the availability of samples. The study was carried out in Sri Ramachandra Medical College and National Environmental Engineering Research Institute, Chennai, India. The duration of the study was one year from June 2003 to May 2004. All the procedures were performed with appropriate informed consent from all subjects and the study protocol was approved by the Institutional Ethical Committee and Review Board of Sri Ramachandra Medical College and Research Institute, Deemed University.

**RNA isolation and RT-PCR:** The total RNA was isolated from the gingival tissues by single step, acid guanidium thiocyanate-phenol-chloroform extraction method. Briefly, gingival tissue was homogenized with 1 ml of solution containing 4 M guanidium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5 per cent sarcosyl; 0.1 M 2-mercaptoethanol. 100 µl of 2M sodium acetate, pH 4; 1 ml of H₂O-saturated phenol; 200 µl of a 49:1 mixture of CHCl₃: isoamyl alcohol (Merck, India) were added to the homogenized tissues and mixed thoroughly by inversion after addition of each reagent. The final mixture was shaken vigorously on vortex for 10 sec and transferred to ice for 15 min then centrifuged at 10,000 xg for 20 min at 4°C. The RNA-containing aqueous phase was transferred to a fresh tube, and the DNA and proteins which should be present in the phenol
and at the interface was discarded. One volume of isopropanol was added to the aqueous phase and precipitated at -20°C for at least 1 h and centrifuged at 10,000 x g for 20 min at 4°C. The pellet was transferred to a 1.5 ml microcentrifuge tube and precipitated the RNA again in 1 volume of isopropanol (or two volumes of ethanol) at -20°C for at least 1 h. After an hour, it was centrifuged at high speed (13,000 x g in a microcentrifuge) for 10 min at 4°C. The RNA pellet was washed in 75 per cent ethanol, centrifuged again and vacuum desiccated. The total RNA was transcribed to cDNA using First Strand cDNA synthesis Kit (Qbiogene, USA) for RT-PCR according to manufacturer instructions. The IGFBP-5 was designed from the known sequence, as IGFBP-5: 5’-GGCTCCGAATCTAAGTGCTG-3’ (sense) and 5’-GCAGCCCTGTCTCACTAACC-3’ (antisense). The primers (Biocorporals, India) were predicted to amplify 457 bp base pairs. As a positive control β-actin primer designed as: 5’-AAGGATTTCTATGTGGGCA-3’ (sense) and 5’-CATCTCTTGCTCGAAGTCTC-3’ (antisense), which were predicted to amplify a 300 base pair DNA fragment. The amplification profile was as follows: initial denaturing at 94°C for 5 min, followed by denaturing at 94°C for 90 sec; annealing at for 60 sec; extension at 72°C for 60 sec. The cDNA was amplified for 25 cycles for IGFBP-5 and 36 cycles for β-actin; followed by a step of 7 min at 72°C to extend the partially amplified products. The PCR products were electrophoresed on 1.5 per cent agarose gel and visualized by ethidium bromide staining. The gels were photographed and their image data were analyzed for band quantification using 1D image analysis software (Applied Biosystems, USA). The relative amount of each IGFBP-5 gene expression was calculated as the ratio of the individual IGFBP-5 to the intensity of β-actin gene products as the control. The relative expression of each IGFBP-5 gene from control, diseased and drug induced gingival overgrowth tissues were compared.

The linear regression curve for IGFBP-5 in DIGO was performed against the control tissue and the differences in the levels of expression of IGFBP-5 in different groups of patients and healthy controls were analyzed using ANOVA for the significance.

Results & Discussion

IGFBP-5 was expressed in all examined tissue samples collected from DIGO, chronic periodontitis and healthy subjects. The level of the IGFBP-5 mRNA expression from chronic periodontitis and DIGO tissue samples was found to be more than that in the uninflamed tissues (Fig. 1). On the other hand, DIGO tissue samples showed significantly increased expression of IGFBP-5 mRNA compared to chronic periodontitis and control (Fig. 2). The linear regression curve of IGFBP-5 in DIGO against healthy control tissues (Fig. 3) showed the increased expression of IGFBP-5 in DIGO tissue samples. Binding proteins are produced locally by different tissues and could therefore act in local regulation. IGFBPs modulate the half-life and activity of IGFs. IGFBP-5 is unique, in that it is the only binding protein that has been shown to consistently stimulate cell proliferation in vitro. IGFBP-5 is produced locally in all tissues and plays a major role in decreasing apoptosis and increasing proliferation. IGFBP-5 enhanced the mitogenic actions of IGF-I on cultured fibroblasts and smooth muscle cells by associating with extracellular matrix (ECM) near the target cell. However, IGFBP-5 also has an important role in controlling cell survival, differentiation and apoptosis.

ECM associated IGFBP-5 may act by accumulating IGF-I near its receptor by modulating the interaction of IGF-I. The expression of IGFBP-5 gene is cell type specific. High levels of IGFBP-5 mRNA have been found in fibroblasts, glioblastoma cells, skeletal muscle cells, osteoblasts, and chondrocytes. In our study we found IGFBP-5 mRNA in healthy human gingival tissues. McCarthy et al have shown that IGFBP-5 mRNA was increased 2.4 fold in inflamed rat colon compared with control. Similar to earlier studies, our results showed that the expression of IGFBP-5 mRNA was 4-fold increased in chronic periodontitis tissues.
Fig. 1. IGFBP-5 mRNA expression in human gingival tissue samples obtained from patients with cyclosporine-A induced gingival overgrowth (DIGO), chronic periodontitis (CP), and normal healthy subjects (Control). Total RNA was isolated and RT-PCR was performed using gene specific primers. β-actin was used as the internal control for PCR.

Fig. 2. mRNA expression of IGFBP-5 in human gingival samples obtained from patients with cyclosporin-A induced gingival overgrowth (DIGO), chronic periodontitis (CP), normal healthy subjects (Control). Patients with CsA-induced gingival overgrowth had higher IGFBP-5 mRNA in gingival tissues than chronic periodontitis and healthy subjects. **P<0.001 vs control (n=8 in each case).

compared to control. Zimmermann et al demonstrated the expression of IGFBP-5 mRNA in rat intestinal smooth muscle and increased IGFBP-5 mRNA in chronically inflamed intestine. The role of gingival fibroblasts proliferation in the setting of inflammation is poorly understood but is likely caused by several inflammatory mediators including IGF-I. Kelly et al suggested that IGFBP-5 may play an important role in the enhancement of the fibrogenic actions of IGF-I.

IGFBP-5 induced production and deposition of collagen and fibronectin in primary adult lung fibroblasts. Unlike most of the other binding proteins, which act as competitive inhibitors of IGF-I receptor, IGFBP-5 acts to enhance IGF-I actions. IGF-I increases the synthesis of both IGFBP-5 and collagen. Moreover, IGF-I’s biological activity on fibroblasts includes stimulation of collagen production and downregulation of collagenase production, suggesting that IGF-I may be an important mediator in the development of gingival fibrosis through IGFBP-5. Feghali et al have demonstrated a 20-fold increase in IGFBP-5 expression in fibroblast from the fibrotic skin of patients than control skin. The interaction of IGFBP-5 to collagen type I and fibronectin in ECM may form the basis for the initiation of signal and its perpetuation since stimulation of collagen and fibronectin production provides additional IGFBP binding partners in the ECM, thus generating an uncontrolled loop of increased ECM components and increased binding.
sites for IGFBPs. IGFBPs contribute to the propagation of the fibrotic phenotype, but our observation of increased expression of IGFBP-5 implicates IGFBPs in the initiation phase of fibrosis, where the inflammation factor was uncontrolled. This is further supported by other findings showing increased IGFBP-5 mRNA levels in fibrotic skin of patients with systemic sclerosis\textsuperscript{23,24}.

Further support comes from micro array analysis that revealed increased IGFBP-5 mRNA levels in human idiopathic pulmonary fibrosis lung tissues and increased IGFBP-5 in lung tissues of bleomycin-treated mice\textsuperscript{25,26}. The greater expression of IGFBP-5 in periodontal ligament fibroblast (PDLF) together with IGF-I induced reduction of apoptosis in PDLF, suggests a potential role of IGFBP-5 in the upregulation of IGF-I pathway\textsuperscript{10}. Taken together, these observations suggest that IGFBPs may be involved early in the cascade of fibrosis.

In this pilot study, we kept the methodology to the minimum and the limitation of the study was not carrying out the protein estimation. In conclusion, drug induced gingival tissues showed increased mRNA expression of IGFBP-5 compared to chronic periodontitis and healthy gingival tissues which may be associated with increased collagen synthesis, thereby promoting fibrogenesis. Further studies need to be done to understand the mechanism.

**References**


22. Feghali CA, Pilewski JM, Knauer AV. Increased IGFBP levels in IPF fibroblasts contribute to the fibrotic process. *Am J Respir Crit Care Med* 2002; 165 : A361.


*Reprint requests:* Dr R. Suresh, Professor & Head, Department of Periodontics, Sri Ramachandra Dental College & Hospital, Sri Ramachandra Medical College & Research Institute, Deemed University, Porur, Chennai 600116, India e-mail: chennai_dentist@yahoo.co.in