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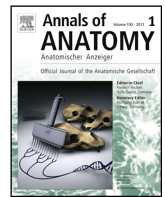

Research article

Autoregulation of insulin-like growth factor 2 and insulin-like growth factor-binding protein 6 in periodontal ligament cells in vitro

A. Konermann[a,*], S. Lossdörfer[a,1], A. Jäger[a,2], Y. Chen[b], W. Götz[a,1]

[a] *Department of Orthodontics, University of Bonn, Germany*
[b] *Department of Orthodontics, Guangdong Provincial Stomatological Hospital, Guangzhou, PR China*

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SUMMARY

The insulin-like growth factor (IGF) system plays an important role in tissue development and presumably also governs pathophysiology of the periodontal ligament (PDL).

It has been the aim of this study to elucidate the specific expression pattern of IGF2 and IGFBP6 in PDL cells and to determine whether PDL cells feature autoregulatory mechanisms upon exposure to these IGF components.

Human PDL cells ($n = 6$) were exposed to IGF2 (100 ng/ml), IGFBP6 (450 ng/ml, 675 ng/ml, 1125 ng/ml) or a combination of 100 ng/ml IGF2 and 675 ng/ml IGFBP6 for 1, 3 or 5d. qRT-PCR was run for *IGF2*, *IGFBP6*, *Ki67*, *ALP*, *osteocalcin*. Immunocytochemical quantification was performed for IGF2 and IGFBP6.

Results showed a time-dependent increase in IGF2 and IGFBP6 gene expression, as opposed to a general decrease at the protein level. At the transcriptional and protein level, challenge with IGF2 and IGFBP6 dampened the expression of both molecules at all time points investigated. Only in the case of IGF2 did combined treatment with IGF2 and IGFBP6 contrarily increased protein expression in both nuclear and cytoplasmatic structures compared to the vehicle treated controls. Analyses of PDL cell proliferation and differentiation revealed *Ki67* downregulation by IGF2 and IGFBP6 alone or in combination. Beyond this, the osteogenic differentiation potential of PDL cells was suppressed as *ALP* and *osteocalcin* expression was reduced.

Our results indicate that IGF2 and IGFBP6 appear to govern various regulatory feedback mechanisms in PDL cells. Thus, the functional properties of these molecules in oral structures are presumably self-controlled under impact of different biological processes such as expression levels of these IGF components, cell proliferation and differentiation.

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1. Introduction

Insulin-like growth factors (IGFs) comprise a family of endocrine, paracrine and autocrine polypeptides consisting of the ligands IGF1 and IGF2, two receptors (IGF1R, IGF2R), at least 6 IGF-binding proteins (IGFBPs) and IGFBP proteases. IGF1 and IGF2 are involved in various cellular processes including differentiation, proliferation, morphogenesis, growth, apoptosis, control of metabolic functions, tissue repair and carcinogenesis (Denley et al., 2005; Pavelic et al., 2007; Chao and D'Amore, 2008; Gallagher and LeRoith, 2010; Maki, 2010; Annunziata et al., 2011). IGF1 is a part of the growth hormone (GH)-IGF1 axis, in which GH stimulates the synthesis of IGF1 in various organs by negative feedback mechanisms, while IGF2 is believed to play an important role during prenatal development (Kaplan and Cohen, 2007; Chao and D'Amore, 2008). Most of the cellular effects of both IGFs are mediated by binding to the IGF1R, which induces its autophosphorylation via tyrosine kinase activity. This, in turn, causes interaction with certain cellular substrates mediating the transmission of mitogenic, metabolic and anti-apoptotic signals (Mauro and Surmacz, 2004; Laviola et al., 2007). The IGFBPs exhibit different binding affinities to their ligands and are essential to coordination and regulation of the biological activities of IGFs. Located both in the circulation and in most target tissues, they act as carrier proteins, transport the IGFs out of the circulation to target tissues and prolong their half-lives by protecting them from proteolytic degradation (Le Roith et al., 2001; Firth and Baxter, 2002). Depending on the extracellular or cell membrane-associated localization,

* Corresponding author at: Department of Orthodontics, Dental Clinic, University of Bonn, Welschnonnenstr. 17, 53111 Bonn, Germany. Tel.: +49 228 287 22426; fax: +49 228 287 22588.
E-mail addresses: konermann@uni-bonn.de, anna.konermann@gmx.de (A. Konermann), s.lossdoerfer@gmx.de (S. Lossdörfer), andreas.jaeger@ukb.uni-bonn.de (A. Jäger), yuxuechen@gmail.com (Y. Chen), wgoetz@uni-bonn.de (W. Götz).
[1] Welschnonnenstr. 17, 53111 Bonn, Germany. Tel.: +49 228 287 22433; fax +49 228 287 22588.
[2] Welschnonnenstr. 17, 53111 Bonn, Germany. Tel.: +49 228 287 22449; fax +49 228 287 22588.

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IGFBPs can promote or inhibit IGF actions. IGFBPs may also have IGF-independent effects on cell functions like gene expression regulation or apoptosis (Baxter, 2000; Mohan and Baylink, 2002).

Among the binding proteins, IGFBP6 is unique because of its N-terminal disulfide linkages and its marked binding preference for IGF2 (Bach, 2005). It is a potent inhibitor of the interaction between IGF2 and its receptor IGF1R, thus preventing major functions of IGF2, e.g. induction of proliferation, differentiation, cell adhesion, or colony formation (Firth and Baxter, 2002; Bach, 2005). These inhibitory effects on growth and differentiation also seem to be IGF-independent in various cell lines, e.g. human neuroblastoma cells (Grellier et al., 2002). Furthermore, IGFBP6 was found to suppress alkaline phosphatase (ALP) activity and the differentiation of both murine and human osteoblasts (Yan et al., 2001; Strohbach et al., 2008). Another IGF-independent action of IGFBP6 is the induction of cancer cell migration (Fu et al., 2007), but recent findings also indicate a function as an oncosuppressor (Kuo et al., 2010).

The IGF system presumably has an important function in the development and pathophysiology of oral tissues including teeth, jaw muscles, tongue, mucosa and salivary glands (Werner and Katz, 2004; Hoeflich et al., 2007). In this role, the periodontium and notably the periodontal ligament (PDL) are regarded as important reservoirs of IGF system components. Among these, IGF2 and its specific binding protein IGFBP6 can be detected in the PDL (Götz et al., 2001, 2003, 2006a,b), but their precise biological functions and local modifications by external factors remain to be elucidated. In light of their influence on various cellular processes, a balance between IGF2 and IGFBP6 may play a role in the homeostasis of the PDL by autocrine and paracrine interactions and exert effects on processes like proliferation and osteogenesis.

It has been the aim of this study to investigate whether PDL cells display autoregulatory mechanisms in the expression pattern of both IGF2 and IGFBP6 upon exposure to these IGF system components with regard to an effective control of tissue homeostasis and adaptation to functional requirements of the periodontium.

2. Materials and methods

2.1. Cell culture

Human PDL cells from 3 donors were explanted from the middle third of the roots of premolars from donors aged between 12 and 14 years, showing no clinical signs of periodontitis. Tooth extraction has been conducted for orthodontic reasons, with informed parental consent and following an approved protocol of the ethics committee of the University of Bonn.

PDL cells were isolated according to standardized protocols as previously described (Lossdörfer et al., 2005). Prior to experimental use, PDL cells were screened for the expression of mesenchymal marker genes, namely alkaline phosphatase, osteocalcin, parathyroid hormone receptor, bone morphogenetic protein-2 and -4, bone morphogenetic protein receptor-1a, -1b and -2, integrin A6, integrin B4, transforming growth factor-b1 and cyclin D1. Purity analyses were performed by means of quantitative real-time polymerase chain reaction (qRT-PCR), and, in addition, cells were examined and characterized morphologically by transmitted-light microscopy.

Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Invitrogen), 1 μg/ml penicillin/streptomycin (Invitrogen), 0.5 μl/ml plasmocin (InvivoGen, San Diego, CA, USA), and 0.005% L-ascorbic acid (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C in a humidified 5% CO_2 atmosphere. Fourth passage cells were plated in 24-well plates at a seeding density of 10,000 cells/well and cultured to confluence. Medium was changed every other day.

2.2. IGF2 and IGFBP6 administration

Confluent PDL cells were continuously exposed to IGF2 (100 ng/ml; Merck, Darmstadt, Germany), IGFBP6 (450 ng/ml, 675 ng/ml, 1125 ng/ml; Abcam, Cambridge, UK), or a combination of 100 ng/ml IGF2 and 675 ng/ml IGFBP6 for 1, 3 or 5d. For each experimental group, vehicle-treated cultures served as controls.

2.3. qRT-PCR

Total RNA ($n = 6$) was isolated (RNeasy mini kit; Qiagen, Hilden, Germany), mRNA concentration was measured (Nanodrop spectrophotometer; Thermo-Fischer Scientific, Wilmington, DE, USA) and reverse transcribed to cDNA (Amersham-Pharmacia-Biotech RT kit; Amersham Biosciences, Piscataway, USA). qRT-PCR was operated with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and primers for *IGF2*, *IGFBP6*, *Ki67*, *ALP*, *osteocalcin*, and *GAPDH* as housekeeping gene (QuantiTect Primer Assay; Qiagen) on a light-cycler (Roche, Mannheim, Germany) using the light cycler software 3.5.3. Prior to experimental analyses, stable expression of GAPDH under the influence of IGF2 and IGFB6 exposure was tested and verified (data not shown). Water was used as negative control and amplifications were performed in duplicate for each sample.

For comparison, the ΔΔCt method was used. Through this method, a single sample, represented in our experiments by cells of the control group, was designed as a calibrator and used for comparison of the gene expression level of any unknown samples (Livak and Schmittgen, 2001).

2.4. Immunocytochemistry

In order to examine the effect of the above treatment regimen on IGF2 and IGFBP6 protein expression, 4th passage PDL cells ($n = 6$) were cultured on glass cover slips (20,000 cells/cover slip) for 24 h prior to exposure to IGF2, IGFBP6 or a combination of both factors for 1, 3, 5d as described above. Thereafter, cells were fixed and permeabilized using a commercially available kit (Fix&Perm; Bioresearch GmbH, Vienna, Austria). Incubation with anti-human IGF2 (Biomol, Hamburg, Germany) was performed at a dilution of 1:200 in tris-buffered saline (TBS)/1% bovine serum albumin (BSA) at 37 °C for 1 h and incubation with anti-human IGFBP6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:50 in TBS/BSA at 37 °C over night. Chromogen staining of bound antibodies was done using the Dako EnVision+ System consisting of a horseradish peroxidase (HRP)-labeled anti-mouse/anti-rabbit polymer immunglobulins (Dako, Glostrup, Denmark) and 3,3′-diaminobenzidine (DAB; Dako). Samples were counterstained with Mayer's hematoxylin and cover slipped for light microscopical analysis.

In order to prove the specificity of the immunoreactions, negative controls were carried out (i) omitting the primary antibody, (ii) omitting both the primary and secondary antibody and using TBS/BSA instead and (iii) substituting an isotype matched mouse IgG-1 (Medac, Hamburg, Germany) for the primary antibody. Positive controls were carried out using human liver and kidney tissue sections carrying known IGFs or IGFBPs (Gotz et al., 2005).

Images were captured by transmitted-light microscopy (Axioskop 2, Carl Zeiss Jena GmbH, Jena, Germany).

2.5. Immunocytochemical quantification

Immunocytochemical quantification of the staining intensities in the samples utilized for immunocytochemistry was performed in order to evaluate and compare the expression of IGF2 and IGFBP6 in PDL cells. Image processing was done using Adobe Photoshop 6.0 and ImageJ software (NIH; http://rsb.info.nih.gov/ij/). The 8-bit RGB

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