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ORIGINAL RESEARCH ARTICLE

Potential Regeneration Capacity of Periodontal Ligament with Autocrine Production of Transforming Growth Factor-Beta 1 and Its Receptors

Jin Gao, Laurence J. Walsh, Anne L. Symons

Abstract

Background: Transforming growth factor-beta1 (TGF-β1) has been extensively studied and reported to be widely expressed in tissues during dentinogenesis and the supporting periodontal tissues. Aims and Objectives: To determine the distribution of TGF-β1 and its receptors in periodontal cells, and to gain insight into the potential actions of TGF-β1 in periodontal tissues. Materials and Methods: Rat periodontal cells were collected from the animal alveolar bone, molar teeth and its surrounding gingivae by cell-explant technique, and primarily characterised and cultured. Cell lysates were detected at the cellular and mRNA levels using in situ hybridization, RT-PCR and Western immunoblots. Results: The mRNA for TGF-β1 was highly expressed in cementoblasts, moderate in osteoblasts and weak in gingival fibroblasts, while TGF-β1 and its receptors (TGF-βR-II and TGF-βR-III) were detected in all periodontal cells. At the protein levels, TGF-β1 and TGF-βR-II were expressed in all periodontal cell types. Conclusion: This study suggests that TGF-β1, TGF-βR-II and TGF-βR-III are produced locally by periodontal cells and play a role in the developing periodontium through regulatory autocrine manners. This would implicate the regeneration capacity in PDL.

Key Words: in situ hybridization;periodontal cells;RT-PCR;TGF β1 and TGF β receptors; western blot analysis

Introduction

TGF-β1 is synthesized by a variety of cells and present in tissues as a latent complex (1). The specific cell surface receptors for TGF-βs (TβR-I, TβR-II and TβR-III) enable a biological response. TGF-β1 has a powerful role in regulating cell proliferation and production of extracellular matrix proteins (2-5). These biological effects may arise from the autocrine activation of TGF-β1 signalling (6,7), and stimulate proliferation of foetal and adult fibroblasts (8). To test the hypothesis that periodontal cells produce TGF-β1 and its receptor in an autocrine manner, this study examined the expression of TGF-β1 and its receptors in primary cultured periodontal cells at both the protein and mRNA levels.

Materials and Methods

Following the approval of the University Animal Research Ethics Committee, rat PDL cells were used for extracting proteins and RNAs, and standard used for culturing on coverslips for undertaking in situ hybridization. All cells were used at passage 3 - 5. Cells (4 x 10^4 cells/ml) were suspended in culture medium (DMEM), and placed onto sterilized coverslips area in triplicate and further cultured for 48 h. After fixation with 4% paraformaldehyde in PBS for 30 min, the cells were determined for the expression of TGF-β1 mRNA using an oligonucleotide TGF-β1 probe or in situ hybridization described by Gao et al 1998 (9).

Cells (2 x 10^6) in 75 cm^2 flasks were scraped from the surface into 500 µL of lysis buffer, whilst DNA chains in the lysates was sheared using a 27-G needle. The protein concentration was determined using a protein assay kit (Bio-Rad, USA). Lysates from periodontal cells were mixed with a loading buffer under reducing conditions, and were loaded (50 µg/lane) and electrophoresed at 100 V using 12% acrylamide SDS-PAGE mini-gels. Lysates were then electroblotted onto a nitrocellulose membrane. Membranes were washed in TBST and blocked with 5% non-fat milk in TBST for 60 min at room temperature, and washed twice in TBST for 15 min.

Polyclonal goat antibodies: latent form of TGF-β1-LAP (1 µg/ml) (R&D Systems, USA), mature form of TGF-β1 (5 µg/ml) (Maine Biotechnology, USA) and a polyclonal rabbit antibody for TβR-II (Upstate Biotechnology, USA), were used to detect TGF-β1 and TβR-II. A monoclonal antibody against vimentin (1 µg/ml) (Sigma, USA) was used to check for equal loading of lysates. Primary antibodies were incubated with the membrane overnight at 4°C, followed by washing with TBST as above, a cocktail of secondary antibody (0.3 µg/ml) of anti-goat/-rabbit/-mouse conjugated with horse-radish

peroxidase were incubated, respectively, for 60 min at room temperature. After washing, the membranes were developed using chemiluminescence solution (Amersham Life Science, England) for 1 min and exposed to X-ray film. Controls were used by non-immune serum and pre-adsorption of the primary antibody by the recombinant proteins (R&D Systems).

RNA was isolated from cultured cells according to the supplier's directions (Advanced Biotech, UK). The RNA concentration was determined at A_{260} using Gene Quant II, a RNA/DNA calculator (Pharmacia Biotech, England). cDNA was made from 1 µg of total RNA by annealing oligo-dT (at 70°C for 10 min) and reverse transcription (RT) with M-MLV (at 37°C for 1 h). The RNA was then removed by heating (at 99°C for 5 min) and the cDNA product (20 µL) of each cell type was stored at -20°C. The primers for TGF-β1, TβR-II and TβR-III have been reported and characterized previously (10,11). The housekeeping gene β2 -microglobulin (β2MG) was used as an internal control. Primers for rat β2MG (forward: 5'-GGGACCGAGACATGTAATC-3'; reverse: 5'-GAAGATGGTGT GCTCATTG-3') were designed from published sequences (12) using the GCG program Prime (Genetics Computer Group, USA).

Aliquots (2 µL) of each RT reaction were amplified separately for genes above. The PCR protocol was 25 - 30 cycles of denaturing (at 95°C for 1 min), annealing [52°C (β2MG), 60°C (TGF-β1 and TβR-II), and 62°C (TβR-III), respectively, for 30 sec] and primer extension at 72°C for 1 min, which was controlled by a DNA thermal cycler. The polymerase used was Red Hot Taq (Integrated Science, USA) (0.5 U in a 20 µL reaction volume). The sizes of the PCR products were identified on a 2% agarose gel containing 0.4 µg/ml ethidium bromide, observed under UV light and photographed.

To determine the homology of the PCR product, DNA from each PCR reaction product for β2MG, TGF-β1, TβR-II or TβR-III was purified using a Wizard™ PCR Preps DNA Purification System. The DNA products were quantified as above, and used as a template for PCR amplification using forward primers for β2MG, TGF-β1, TβR-II and TβR-III. The standard protocol for Dye Terminator Cycle Sequencing was followed (Perkin Elmer, USA). Analysis of the sequences confirmed that the PCR products were β2MG, TGF-β1, TβR-II and TβR-III, respectively.

Results

Using in situ hybridization, the poly-dT positive control probe gave a strong signal in the cytoplasm of all types of primary periodontal cells (Fig. 1A), while the negative control showed no signal. The mRNA for TGF-β1 was clearly localized in the cytoplasm of cementoblast-like, osteoblast-like, and PDL fibroblast-like cells in vitro (Fig.1B - D).

Western blotting using a polyclonal antibody against LAP (TGF-β1) showed two major bands at 40 and 13 KDa under reducing conditions. Proteins obtained from primary periodontal cells expressed a positive band at 40 KDa (Fig. 2A). The intensity of the 13 KDa band was the strongest for cementoblast-like cells compared with other cell types, and osteoblast-like cells also showed a weak 40 KDa band (Fig. 2A). By comparison, the expression of LAP-TGF-β1 in the established cell lines of neuroblastoma (Fig. 2B) also showed the same pattern and size as the primary culture. Standard LAP (TGF-β1) protein was detected as an approximately 40 KDa band. When the antibody was pre-adsorbed by the TGF-β1-LAP protein for overnight, the intensity of the band was greatly reduced. As expected, no band was detected when the antibody was pre-adsorbed for a further 3 days (Fig. 2A). The intensity of the 73 KDa band corresponding to TβR-II was strongly expressed in each of the four cell types of periodontal cells (Fig. 3A). Staining with Ponceau S, and the detection of vimentin, both showed equal loading of protein per lane (Fig. 3B). Negative controls showed no specific bands.

RNA was extracted from each type of periodontal cells as above, and adjusted to an equal concentration (Fig. 4A). Primers for the rat β2-microglobulin were used to ensure that each lane was loaded with equal amounts of mRNA. All lanes showed equal intensity of the β2-microglobulin PCR product at 130 bp (Fig. 4B). The mRNA for TGF-β1 and its receptors in total RNA extracts from cultured cells was examined by RT-PCR. PCR products for TGF-β1 and its receptor types II and III were detected at 430, 299

and 340 base-pairs (bp), respectively, in all primary periodontal cells described a above (Fig. 4C - E).

Discussion

PDL cells are reported to primarily synthesize TGF-β1 and secrete the protein locally in variety of tissues (8,13-15). Since TGF-β1 has a short half-life in the circulation, its autocrine production by periodontal cells may take place on a demand basis, when this protein is required to maintain periodontal homeostasis (16). The periodontal cementoblasts, fibroblasts and osteoblasts play a key role in maintaining homeostasis of the periodontium under physiological conditions, and in propagating new cells and producing extracellular matrix needed for tissue repair and regeneration during pathological conditions (17,18). Previous reports indicated that TGF-β1 induced fibroblasts to synthesise proteoglycans, such as biglycan and decorin (19). When interacting with cells, TGF-β1 binds to a specific cell membrane receptor with high affinity (20). There is essentially no cross-reactivity between TGF-β1 and receptors for other growth factors. TGF-β receptors located on the cell surface or within cells may modulate the interaction of TGF-β1 with the target cells via the availability of binding sites (20,21).

This study has provided evidence to demonstrate that cultured periodontal cells express mRNA for TGF-β receptor types II and III, as well as TβR-II proteins. This suggests that these cells are able to synthesize those proteins and secrete into the local tissues. The presence of TGF-β1 and its receptors in periodontal tissues and cells both in vivo and in vitro strongly supports the view that TGF-β1 acts in an autocrine manner. The intercellular region of the TβR-II receptor has a serine/threonine (S/T) kinase domain and S/T kinase activity which is a common characteristic of TGF-β receptors in this superfamily of proteins for their signal transduction (22). Additionally, the Smad proteins have also been demonstrated to be important for signal transduction for TGF-β1 from receptor into nucleus (21,23). Betaglycan (TβR-III) could be involved in capturing and retaining TGF-β1 within the cellular environment for presentation to the signalling receptors (21,24). Thus, both receptors may be involved in regulating the actions of TGF-β1 in the periodontium.

In this study, the 13 KDa band detected using western blotting may be a proteolytic breakdown product of the latency-associated protein, although proteinase inhibitors were properly used. To eliminate the possibility that the 13 kDa may be a non-specific band, we have undertaken additional experiments. When cellular proteins were diluted 2.5, 5, 10 and 20 times, the intensity of the varied band showed from strong to weak. Omission of the primary antibody resulted in no band being present, whilst cellular proteins blocked the reaction of the primary antibody. The 40 KDa band, a monomer form of LAP (1), demonstrates the presence of LAP (or TGF-β1) in periodontal cells. This suggests that periodontal cells contain components of the LAP-TGF-β1 complex. This interesting result demonstrates that the expression of LAP (or TGF-β1) within periodontal cells is similar, but the degradation of this protein occurs more readily in cementoblasts, moderately in periodontal ligament and gingival fibroblasts, and less so in osteoblasts. RT-PCR analysis of total cellular RNA demonstrated the detectable presence of mRNA for TGF-β1 and its receptor types II and III in rat periodontal cells (11). The ability to screen for a broad range of cytokines and the stability of periodontal cells in culture makes an excellent model for studying normal and diseased periodontal tissues.

Overall, this study demonstrates the presence of TGF-β1and its receptor type II and III in primarily cultured periodontal cells. The expression of these molecules at the protein and the mRNA levels suggests the likelihood that an autocrine production of TGF-β1 be associated with expression of its receptors in vitro. It is implicated that the regeneration capacity is naturally present in PDL.

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Authors Affiliations: 1. Dr. Jin Gao, School of Medicine and Dentistry, James Cook University, PO Box 6811, Cairns, QLD 4870, 2. Dr. Laurence.J.Walsh, 3. Dr. Anne L. Symons, School of Dentistry, The University of Queensland, 200 Turbot Street, Brisbane, QLD 4000, Australia.

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Address for Correspondence
Dr. Jin Gao,
School of Medicine and Dentistry,
James Cook University,
PO Box 6811, Cairns,
QLD 4870, Australia.
Email: jin.gao@jcu.edu.au

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