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Combined effect of TNF-α and cyclic stretching on gene and protein expression associated with mineral metabolism in cementoblasts

Wei Yao[a,b], Xiaona Li[a], Bin Zhao[b], Genlai Du[a], Pengfei Feng[a], Weiyi Chen[a,*]

[a] College of Mechanics, Shanxi Key Laboratory of Material Strength & Structural Impact, Institute of Applied Mechanics and Biomedical Engineering, Taiyuan University of Technology, Taiyuan, Shanxi 030024, China
[b] College and Hospital of Stomatology, Shanxi Medical University, Taiyuan, Shanxi 030012, China

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ABSTRACT

Objective: This work aims to investigate how the combination of TNF-α and cyclic stretching affects the expression of gene and protein associated with mineral metabolism in cementoblasts in vitro.
Design: Cementoblasts were cyclically stretched using the Flexcell tension system 4000 in the presence of 10 ng/ml TNF-α. Subsequently, the gene and protein expression CAP, Col I, BSP, OPG, RANKL and RUNX2 were detected using RT- PCR and ELISA/Western immunoblotting methods, respectively.
Results: Cyclic stretching alone enhanced CAP, Col I, OPG, RANKL and RUNX2 expression in an amplitude manner, while decreased BSP expression. Expression of all these proteins was attenuated in the presence of TNF-α whether the cells were exposed to cyclic stretching or not. The ratio of RANKL/OPG was increased at any stimulation.
Conclusion: This results suggest that TNF-α affected the regulation of gene and protein expression induced by mechanical stimulation in cementoblasts. This may suppress anabolism and promote catabolism of cementum. It suggests that inflammatory cytokine may impair the cementum remodeling under mechanical stimulation.

1. Introduction

Cementum is a thin layer of mineralised tissue that covers the root surface to connect the tooth and its periodontal tissue. The cementum surface is covered by root lining cells, called cementoblasts. Cementoblasts are present on the surface to prevent osteoclasts from contacting the root surfaces and root resorption (D'Errico et al., 1997). Cementoblasts that can be activated by mechanical and biological signals are the key effector cells for secreting new cementum and repairing tooth root. Furthermore, cementoblasts play an important role in periodontal tissue regeneration (Popowics, Foster, Swanson, Fong, & Somerman, 2005).

The tooth movement during orthodontic treatment is based on the alveolar bone absorbing at the pressure side and rebuilding in the stretch side. The adaption and remodeling of bone tissue make the tooth "walk" in the alveolar bone. A more reasonable control of the orthodontic force can effectively promote alveolar bone remodeling without causing root desorption because the cementum has a greater anti-resorption capacity than the alveolar bone. However, more than 90% of patients find different degrees of root desorption in the course of the clinical treatment (Kokich, 2008). The periodontal tissues respond to the orthodontic tooth movement, which is in accordance with all the characteristics of the inflammatory response (Brezniak & Wasserstein, 2002). This response is called the orthodontically induced inflammatory root resorption. The orthodontic force can cause aseptic inflammation in the periodontal tissue and release various cytokines (Uematsu, Mogi, & Deguchi, 1996), including IL-1, IL-6, TNF-α and EGF.

TNF-α is an important pro-inflammatory cytokine in periodontal tissue inflammation; it can inhibit cementoblast mineralisation by inhibiting cell differentiation and promoting apoptosis (Wang et al., 2015). Yoshino, Yamaguchi, and Shimizu (2014) suggest that TNF-α may be an aggravating factor for root resorption during orthodontic treatment. Cementoblast is a kind of force-sensitive cell. Its proliferation, differentiation, maturation, apoptosis and matrix mineralisation are affected by stress (Mullally, 2010). How the combination of inflammatory cytokines TNF-α and mechanical stimulation affects the gene and protein expression associate to mineral metabolism in cementoblast remains unclear.

Cementum remodeling depends on the characteristic gene and protein expression of cementoblasts. Cementum adhesion proteins

* Corresponding author.
E-mail addresses: chenweiyi211@163.com, weiyi.chen0@gmail.com, http://mailto:chenweiyi211@163.com. (W. Chen).

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Table 1
Primer sequences used in the semi-quantitative RT-PCR.

Primer	5′–3′ sequence (forward; reverse)	Size of amplified product (bp)
BSP	For: CAAAAGTGAAGGAAAGCGACGAG Rev: CCGTGGAGTTGGTGCTGGTG	123
CAP	For: GTGAGGAGGACGGCACCAA Rev: AAGCGTACCATAGCAATAGCAAGA	160
Collagen I	For: TGTTGAACTTGTTGCTGAGGGC Rev: GCAGGCGAGATGGCTTATTTG	128
Runx2	For: AACCCACGAATGCACTACCCA Rev: GGAACTGATAGGATGCTGACGAAG	205
OPG	For: AAATTGGCTGAGTGTTTTGGTGG Rev: CACGCTGCTTTCACAGAGGTCA	193
RANKL	For: TCAGGTGTCCAACCCTTCCC Rev: TGCTAATGTTCCACGAAATGAGTC	106
GAPDH	For: GTGAGGCCGGTGCTGAGTATGT Rev: GCAGAAGGGGCGGAGATGA	115

The primers were designed by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). The relative mRNA quantity was calculated using the $2^{-D\Delta Ct}$ method.

(CAPs) are specific cementoblast markers that can significantly promote periodontal cell adhesion and migration in the root surface (Metzger, Weinstock, Dotan, Narayanan, & Pitaru, 1998). Type I collagen (COL I) is a mineralised bone matrix protein, which is an important marker of osteogenic differentiation (Ignatius et al., 2005). Bone sialoprotein (BSP) is a multifunctional extracellular matrix protein; it is an important marker protein used to study cementogenesis. BSP can mediate adhesion, differentiation and mineralisation of osteoblast-like cells (AO et al., 2014). Osteoprotegerin (OPG) is a soluble,decoy receptor belonging to the TNF receptor superfamily. The receptor activator of the nuclear factor-kappa B ligand (RANKL) is a member of the TNF ligand superfamily which has a membrane-bound form as well as a soluble form. The RANKL and OPG ratio is the key to determine the maturity and function of osteoclasts (Khosla, 2001), which also expresses in the cementoblasts (Dalla-Bona et al., 2008). RUNX2 is a pivotal force-sensitive transcription factor, whose expression can be promoted by stress, and can regulate the mineralised gene expressions, thereby affecting osteoblast differentiation and maturation and comprehensively promoting osteogenesis (Marie, 2008).

In this study, cementoblasts were cyclically stretched using the Flexcell tension system 4000 in the presence of 10 ng/ml TNF-α. The gene and protein expression associated with mineral metabolism in cementoblasts were investigated.

2. Materials and methods

Cementum and bone are both mineralised tissues. Cementoblasts, like osteoblasts, are mature mineralising cells (Matthews et al., 2016). The OCCM30 cells (kind gift from Professor Martha J. Somerman, Washington University) were an immortalised murine cementoblast cell line widely used to represent primary cementoblasts(D'Errico, Berry, Strayhorn, Windle, & Somerman, 2000). The OCCM30 cells underwent two cyclic stretching (1 Hz; i.e., 6% and 12%) (Tang, Lin, & Li, 2006) and two different TNF-α concentrations (i.e., 0 and 10 ng/ml; R&D Systems, Minneapolis, MN, USA) (Sanchavanakit, Saengtong, Manokawinchoke, & Pavasant, 2015) within 12 h using the Flexercell Strain Unit (Flexercell 4000; USA) in a tissue incubator (5% CO_2, 37 C). The stretching was conducted using Bioflex Loading Stations with a diameter of 25 mm per well resulting in an equally uniform stretching in all directions. The static groups were handled in the same manner, but without cyclical deformation. Our experiments were grouped into six as follows: (1) static control group;(2) 10 ng/ml TNF-α stimulation; (3) cyclic stretching of 6% strain; (4) TNF-α combined with cyclic stretching of 6% strain; (5) cyclic stretching of 12% strain; (6) TNF-α combined with cyclic stretching of 12% strain. The cells and supernatants from the cell cultures were collected for gene and protein detection, respectively, at the end of each experiment.

2.1. Real-time polymerase chain reaction (RT-PCR)

The total RNA from the cells was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was generated from the total RNA by reversing transcription using the PrimeScript™ RT reagent kit (TaKaRa Biotechnology Co., Dalian, China). The cDNA products were amplified with SYBR® Premix Ex Taq™ II (TaKaRa Biotechnology Co., Dalian, China) and the StepOnePlus™ RT-PCR System (Applied Biosystems, USA) using the gene specific primers (Table 1):

2.2. Enzyme-linked immunosorbent assay (ELISA)

The ELISA method was used to measure the BSP, OPG and RANKL levels in the cell supernatant. Highly specific quantitative sandwich ELISA kits for mouse BSP, OPG and RANKL were purchased from R&D Systems (Minneapolis, MN, USA). The instruction manuals were followed for the commercial kits. The optical density was measured in a microplate reader set to 450 nm with wavelength by a spectrophotometer (Multiskan Go, Thermo Scientific, USA).

2.3. Western immunoblotting

Cells were lysed and protein was extracted using RIPA (Beyotime Biotechnology Co., Shanghai, China). Equal amounts of proteins were loaded on a 10% denaturing sodium dodecyl sulfate polyacryla-mide gel electrophoresis (SDS-PAGE) and transferred by electrophoresis to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% fat-free milk in tris-buffered saline tween buffer (TBST) and then incubated overnight with primary antibodies (Table 2). The membranes were

Table 2
List of antibodies used for western immunoblotting studies.

Antibody	Ab. Dilution	Source
Mouse anti-Collagen I	1:500	Hebei Bio-HighTechnology, China
Mouse anti-BSP	1:300	Hebei Bio-HighTechnology, China
Mouse anti-OPG	1:500	Hebei Bio-HighTechnology, China
Mouse anti-RANKL	1:200	Hebei Bio-HighTechnology, China
Rabbit anti-RUNX2	1:500	Hebei Bio-HighTechnology, China
Mouse anti-actin	1:1000	Hebei Bio-HighTechnology, China
Goat anti-mouse IgG	1:1000	ZSGB-bio Technology, China
Goat anti-rabbit IgG	1:1000	ZSGB-bio Technology, China