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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article http://dx.doi.org/10.1016/j.apjtb.2016.06.012

Regulation of osteoprotegerin expression by Notch signaling in human oral squamous cell carcinoma cell line

Jeeranan Manokawinchoke, Thanaphum Osathanon*, Prasit Pavasant*

Mineralized Tissue Research Unit, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Pathumwan, Bangkok 10330, Thailand

ARTICLE INFO

Article history:
Received 29 Oct 2015
Received in revised form 23 Nov 2015
Accepted 30 Dec 2015
Available online 11 Jun 2016

Keywords:
Notch signaling
Oral squamous cell carcinoma
Osteoprotegerin
DAPT
Jagged1

ABSTRACT

Objective: To investigate the influence of Notch signaling on osteoprotegerin (OPG) expression in a human oral squamous cell carcinoma cell line.

Methods: Activation of Notch signaling was performed by seeding cells on Jagged1 immobilized surfaces. In other experiments, a γ-secretase inhibitor was added to the culture medium to inhibit intracellular Notch signaling. OPG mRNA and protein were determined by real-time PCR and ELISA, respectively. Finally, publicly available microarray database analysis was performed using connection up- or down-regulation expression analysis of microarrays software.

Results: Jagged1-treatment of HSC-4 cells enhanced *HES1* and *HEY1* mRNA expression, confirming the intracellular activation of Notch signaling. OPG mRNA and protein levels were significantly suppressed upon Jagged1 treatment. Correspondingly, HSC-4 cells treated with a γ-secretase inhibitor resulted in a significant reduction of *HES1* and *HEY1* mRNA levels, and a marked increase in OPG protein expression was observed. These results implied that Notch signaling regulated OPG expression in HSC-4 cells. However, Jagged1 did not alter OPG expression in another human oral squamous cell carcinoma cell line (HSC-5) or a human head and neck squamous cell carcinoma cell line (HN22).

Conclusions: Notch signaling regulated OPG expression in an HSC-4 cell line and this mechanism could be cell line specific.

1. Introduction

Osteoprotegerin (OPG) is a decoy receptor regulating receptor and ligand interaction [1]. OPG is known to interact with receptor activator of nuclear factor kappa-B ligand (RANKL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), however, high-affinity binding was demonstrated for RANKL [1]. The role of OPG in osteoclastogenesis is well documented. OPG deficient mice exhibit a severe and early-onset osteoporosis phenotype as well as a high incidence of fractures [2]. Correspondingly, OPG transgenic mice that contained the human OPG promoter had significantly increased bone mass in cortical and trabecular bone [3]. In addition to the effect on bone phenotypes, OPG deficient mice demonstrate high aorta and renal artery calcification, implying the role of OPG in regulating pathological calcification [2]. Furthermore, OPG regulates other biological events, such as immune response, cell survival, and osteoblastic differentiation [1,4,5].

In cancer biology, OPG participates in bone invasion and apoptosis of cancer cells. OPG inhibits TRAIL-induced apoptosis in several types of cancers, including ovarian cancer cells, breast cancer cells, and colon cancer cells [6–8]. A

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*Corresponding author: Assistant Professor Thanaphum Osathanon, DDS, PhD, Mineralized Tissue Research Unit, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand.
Tel: +66 8 2 218 8885
Fax: +66 8 2 218 8870
E-mail: thanaphum.o@chula.ac.th

Professor Prasit Pavasant, DDS, PhD, Mineralized Tissue Research Unit, Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok 10330, Thailand.
Tel: +66 2218 8892
E-mail: prasit215@gmail.com

The experiments using human cell lines were approved by the Human Ethics Committee, Faculty of Dentistry, Chulalongkorn University.

Foundation Project: Supported by a Faculty Research Grant (No. DRF-59010), Faculty of Dentistry, Chulalongkorn University and the 2012 Research Chair Grant, Thailand National Science and Technology Development Agency.

Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

strong negative relationship between endogenous OPG expression and TRAIL-induced cell apoptosis was observed in prostate cancer [9]. Exogenous OPG enhanced the proliferation of an osteosarcoma cell line partly via the nuclear factor kappa-B signaling pathway [10]. Further, a high RANKL/OPG ratio in human non-small cell lung cancer correlated with higher metastatic ability [11]. OPG also promotes endothelial cell survival and vessel formation [12]. Moreover, endothelial cells in malignant lesions express higher OPG levels than those in non-malignant tumors, implying the role of OPG in cancer angiogenesis [12]. Taken together, these data suggest a significant role of OPG in cancer cell behavior.

In oral cancer, OPG administration led to decreased mandibular bone invasion by transplanted human oral squamous cell carcinoma (OSCC) cells in nude mice [13]. In addition, OPG injected mice exhibit higher terminal deoxynucleotidyl transferase dUTP nick end labeling positive cancer cells compared with the control group, indicating that OPG promotes OSCC cell apoptosis [13]. Furthermore, OPG significantly inhibits RANKL-induced OSCC cell migration *in vitro* [13]. Another report indicated that OSCC promotes bone invasion via the suppression of OPG expression in the host stromal cells [14]. These results imply an important function of OPG in OSCC cell behavior.

Notch signaling is a highly conserved signaling pathway that regulates various cell functions in development, disease, and regenerative processes. It has been shown that Notch signaling regulates OSCC cell behavior [15,16]. In addition to the roles of Notch signaling in OSCC, it has been demonstrated that Notch signaling regulates OPG expression in osteocytes [17]. However, the relationship between Notch signaling and OPG expression in human OSCC cells remains unknown. Thus, the present study investigated the regulation of OPG expression by Notch signaling in a human OSCC cell line.

2. Materials and methods

2.1. Cell culture and treatment

Two human OSCC cell lines (HSC-4 and HSC-5) and one HNSCC cell line (HN22) were used in the present study. The HSC-4 and HSC-5 cell lines were gifts from Professor Teuro Amagasa (Tokyo Medical and Dental University, Japan). The HN22 cell line was kindly provided by Professor J. Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, USA). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 unit/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B at 37 °C in a humidified 5% carbon dioxide atmosphere. All cell culture reagents were purchased from Gibco BRL (Carlsbad, USA).

For Notch signaling activation, Jagged1 immobilization on tissue culture surfaces was performed according to our previous publication [18]. Briefly, tissue culture plates were coated with recombinant protein G (50 μg/mL) for 16 h, followed by bovine serum albumin (10 mg/mL) for 2 h and recombinant human Jagged-1/Fc (R & D systems, USA) for 2 h. The surfaces were washed with sterile phosphate buffer solution between each step. Subsequently, 75 000 cells/well were seeded on the Jagged-1 modified surfaces in 24-well plates and maintained in culture medium for 48 h. For the inhibition experiment, the cells were seeded on 24-well plates as described above and treated with a γ-secretase inhibitor (DAPT 20 μmol/L, Sigma–Aldrich, USA) for 48 h.

2.2. ELISA

Culture medium was collected 48 h after treatment and used to determine the secreted OPG levels. OPG protein expression was measured using a human OPG/TNFRSF11B DuoSet kit R & D Systems, USA) according to the manufacturer's instructions. The absorbance was examined at 450 nm. The results were normalized to the control and presented as fold change.

2.3. Real-time PCR

Total cellular RNA was isolated using Isol-RNA Lysis reagent (5 Prime, USA). RNA (1 μg) was converted to cDNA using a reverse transcriptase kit (Promega, USA). FastStart® Essential DNA Green Master (Roche Applied Science, Indianapolis, IN, USA) was employed for PCR in a Lightcycler Nano realtime PCR machine (Roche Applied Science, USA). The expression values were normalized to glyceraldehyde-3-phosphate dehydrogenase expression and then normalized to the control results. The primer sequences are shown in Table 1.

Table 1

Primer sequences.

Gene		Primer sequences	Accession no.
GAPDH	F	5′-TCATGGGTGTGAACCATGAGAA-3′	NM_002046.3
	R	5′-GGCATGGACTGTGGTCATGAG-3′	
OPG	F	5′-AGCTGCAGTACGTCAAGCAGGA-3′	NM_002546.3
	R	5′-TTTGCAAACTGTATTTCGCTCTGG-3′	
HES1	F	5′-AGGCGGACATTCTGGAAATG-3′	NM_005524.2
	R	5′-CGGTACTTCCCCAGCACACTT-3′	
HEY1	F	5′-CTGCAGATGACCGTGGATCA-3′	NM_012258.3
	R	5′-CCAAACTCCGATAGTCCATAGCAA-3′	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; F: Forward; R: Reverse.

2.4. Microarray database analysis

Publicly available microarray expression database analysis was performed using connection up- or down-regulation expression analysis of microarrays software [19,20]. Briefly, the microarray dataset Gene Expression Omnibus (GEO) series were identified using the keywords “JAG1 or Jagged1”. The expression profile studies in human cancer cells containing three or more samples in each experimental group were included in our analysis. The GEO series and their platforms (GSE14995 and GSE36051) were downloaded from the GEO repository [21]. The differential expression of OPG (TNFRSF11B) mRNA was evaluated. Statistical analysis was performed using the two independent samples student's *t*-test, which is an extension of the connection up- or down-