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Original Article

Expression of bone modeling markers and formation of bacterial biofilm in middle ear of rats with chronic suppurative otitis media induced by *Pseudomonas Aeruginosa*

Ming Hu[1,2], Ji-Wei Hu[3], Lu He[1], Yun-Sheng Li[1]

[1]*Department of Anatomy, Histology and Embryology, Basic Medical College, Tianjin Medical University, Tianjin, China;* [2]*Department of Otolaryngology Head and Neck, Tianjin First Central Hospital, Tianjin, China;* [3]*Department of Breast Surgery, Tangshan People's Hospital, Tangshan, China*

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Abstract: Objective: To investigate the expression of bone modeling markers and the formation process of bacterial biofilm in middle ear of rats with chronic suppurative otitis media (CSOM). Methods: A total of 60 Sprague-Dawley rats were randomly divided into 2 groups, control group (n=10) and CSOM group (n=50). Rats in CSOM group were infected by *Pseudomonas Aeruginosa* through intratympanic injection to induce CSOM. Bacterial biofilm in middle ear of rats was observed through confocal laser scanning microscope. Expression levels of bone morphogenetic protein (BMP-2), osteoprotegerin (OPG), the receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) and osteopontin (OPN) were determined. Results: Seven days after the induction of CSOM, bacterial biofilm was observed in the middle ear of rats, grew up to the maximum size 14 days after the induction, and declined at 30 days after the induction. mRNA and protein expression of OPG, RANKL, BMP-2 and OPN were significantly higher in the otic vesicle of middle ears of SCOM rats than normal rats. OPG/RANKL significantly decreased at 3 days after the induction, however was significantly higher than control after that. Conclusion: The bacterial biofilm in the otic vesicle of middle ears formed at early CSOM with the bone destruction and bone morphogenetic.

Keywords: Bone modeling marker, bacterial biofilm, middle ear, chronic suppurative otitis media, *Pseudomonas Aeruginosa*

Introduction

Chronic suppurative otitis media (CSOM) is characterized by a perforated tympanic membrane with persistent discharge from the middle ear caused by bacteria, fungi, and viruses, resulting in the inflammation of the mucosal lining. Chronic inflammation of the mastoid cavity and middle ear are characteristic of CSOM; however, the mechanism of chronic infection has not yet been determined [1].

Recently, biofilms have been recognized as being important factors in the pathophysiology of CSOM [2-5]. Biofilms were initially demonstrated in the middle ear of chinchillas with experimentally induced CSOM [5]. In a recent study using Gram-staining peptide nuclear acid (PNA) FISH analyses and microscopy, Homoe et al. [6] found morphological evidence of biofilms in the otorrhea from 5 of 6 (83%) children with CSOM and morphological evidence of biofilms in the mucosa of biopsies from the middle ear in 8 of 10 (80%) adults who had undergone operations for CSOM. Additional studies, based on scanning electron microscopic imaging, showed evidence of biofilm presence in CSOM, with or without cholesteatomas [7-9].

Bone morphogenetic proteins are osteogenic proteins that regulate numerous cellular processes. Specifically, they induce differentiation of cells of the osteoblast lineage and have the ability to increase formation of newbone [10]. Increasing expression level of BMP-2 has been found in patients with active otosclerosis [11]. However, the effect of BMP-2 in CSOM was still unclear.

Osteopontin (OPN) is a secreted bone matrix glycophosphoprotein with diverse actions. OPN is secreted by osteoclasts and osteoblasts and is thought to take part in bone resorption and possibly bone formation by binding to hydroxyapatite [12]. OPN had been reported to participate in the pathological calcification that occurs as a result of chronic otitis media [13].

The TNF family of ligands and receptors are important in bone remodeling and its members, receptor activated NF-κB ligand (RANKL) and osteoprotegerin (OPG), are critical regulators of osteoclastogenesis and have a diverse range of functions and effects on cells other than osteoclasts and osteoblasts [14, 15]. RANKL is expressed by osteoblasts and stimulates osteoclast differentiation and function [14-16]. Whereas OPG, a soluble decoy receptor for RANKL, inhibits osteoclast development and bone resorption [14-16]. The RANKL protein level was found higher in cholesteatoma tissues than in the auditory canal skin and granulation tissues, respectively, while similar OPG protein levels were found in cholesteatoma tissues and the normal auditory canal skin [17].

In this study, to investigate the expression of bone modeling markers and the formation process of bacterial biofilm in middle ear of rats with chronic suppurative otitis media (CSOM), we established a rat model with CSOM induced by *Pseudomonas Aeruginosa*.

Methods

Animals

Male SPF Sprague-Dawley (SD) rats, weighing approximately 200-250 g, were purchased from Tianjin Tianyao biological technology co., LTD.. Electric otoscopy examination was conducted for each rat in this study. Rats with calcified plaques in tympanic membrane, otopiesis, and infections of external auditory canal, tympanic membrane and middle ear were excluded. A total of 60 rats with intact tympanic membrane and clear light cone were randomly divided into 2 groups, control group (n=10) and CSOM group (n=50).

Experimental procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's Republic of China in 1998, and were approved by the Animal Ethics Committee of Tianjin Medical University.

CSOM model

Pseudomonas Aeruginosa (CCTCC-AB91095) used to induce CSOM in rats, was provided by China Center for Type Culture Collection (CCTCC). The rats were anesthetized with a combination of 10% chloral hydrate (0.4 ml/100 g body weight; Shanghai Mingbo, China) given intraperitoneally. The bilateral middle ears were respectively inoculated with 0.1 ml of a suspension of *Pseudomonas Aeruginosa* with a concentrate of 1.0×10^6 colony forming units (CFU)/ml for each rat in CSOM group. *Pse-udomonas Aeruginosa* was not given to control rats. All procedures were performed under sterile conditions.

The 10 rats of control group were sacrificed at the day of inoculation with an intraperitoneal overdose of chloralhydrate and bilateral otic vesicles were harvested for immunofluorescence (n=5), qRT-PCR (n=5), immunohistochemistry (n=5) and Western blot (n=5). At 3th, 7th, 14th, 21th and 30th day after the inoculation, 10 rats in SCOM group were sacrificed and bilateral otic vesicles were harvested for the same tests.

Immunofluorescence

Attachment adhered to mucosal surface of the otic vesicle was collected under dissecting microscope and fixed in 10% formaldehyde for 24 h, routine paraffin-embedded, and sectioned (5 μm). After dewaxing, the sections were incubated in FITC-ConA (Sigma, USA) in dark at room temperature for 30 min, followed by DAPI (Sigma, USA) incubation in dark at room temperature for 30 min. The images were taken using a confocal laser scanning microscope (Olympus Fluoview FV1000, Japan).

qRT-PCR

Total RNA was extracted from otic vesicles using Trizol reagent (Thermo Fisher, USA) and then reversely transcribed into cDNA using a reverse transcription kit (Thermo Fisher, USA). cDNA was then amplified in a mixture containing 1 μl cDNA, 0.5 μl primer each, 10 μl SYBR Green PCR master (Thermo Fisher, USA), 8 μl RNase-free H_2O under the following conditions:

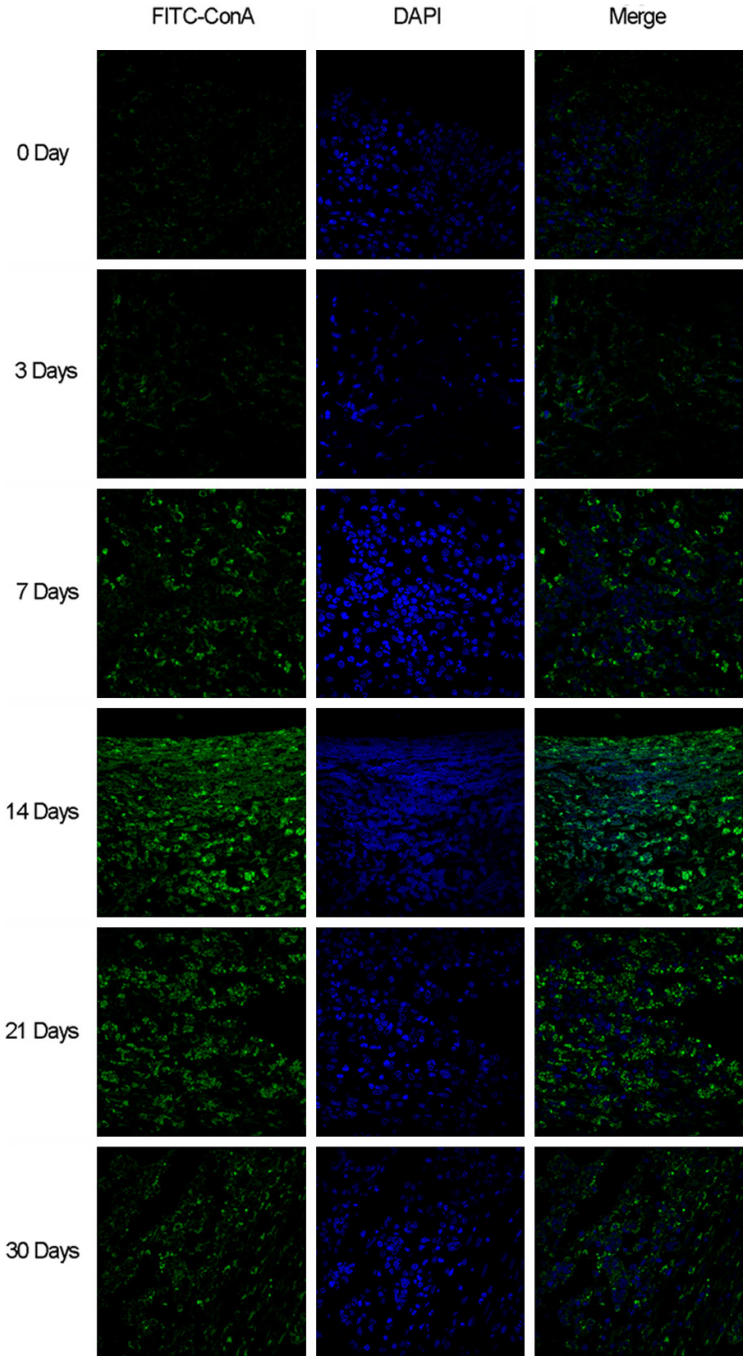

Figure 1. Observation of the bacterial biofilm in the rat middle ear through the confocal microscope.

initial denaturation at 95°C for 5 min; 40 cycles of 95°C for 15 sec, 60°C for 1 min, 72°C for 10 min. Melting curve analysis consisted of 100 cycles of 60-95°C, 0.2°C/sec for fluorescence measurements.

The primers sequences were as follows: BMP-2 forward primer, 5'-TGCTCAGCTTCCATCACGAA-3'; BMP-2 reverse primer, 5'- CCTGCATTTGTTCCC-GAAAA-3'; OPN forward primer, 5'- CCTTCACTGCCAGCACACAA-3'; OPN reverse primer, 5'- CTG-TGGCATCGGGATACTGTT-3'; OPG forward primer, 5'- CCCTGTGCGAAGAGGCATT-3'; OPG reverse primer, 5'- TTTGCTCTTGCGAGCTGTGT-3'; RANKL forward primer, 5'- TCGACTCTGGAGAGCGAAGAC-3'; RANKL reverse primer, 5'- CCA-CGAACCTTCCATCATAGC-3'; GAPDH forward pri-mer, 5'- ATCATGTTTGAGACCTTCAACA-3'; GAPDH reverse primer, 5'- CATCTCTTGCTCGAAGTCCA-3'. Primers were synthesized in the Sangon Biotech (Shanghai) Co., Ltd.

Immunohistochemistry

Otic vesicles of rats were harvested and fixed in 4% paraformaldehyde, decalcified in 10% EDTA for 7 to 10 days, routine paraffin-embedded, and sectioned (5 μm). After dewaxing, antigen retrieval and blocking, the sections were incubated in primary antibodies (goat anti-BMP-2, goat anti-OPG, goat anti-RANKL, or goat anti-OPN, 1:150 dilution, Santa Cruz, USA) for 1 h at room temperature, and then were incubated in second antibodies (1:200 dilution, Santa Cruz, USA). DAB staining was used to examine the expression of BMP-2, OPG, RANKL or OPN in otic vesicles under light microscope.

Western blot

Otic vesicles were cut into pieces, and total protein was extracted with RIPA lysate (3 ml/1 g sample) containing PMSF (10 mg/ml). The protein concentration was determined by the Bradford method. 50 μg sample protein per lane was separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene-difluoride (PVDF) membrane. The membranes were incubated in 5% skimmed milk,

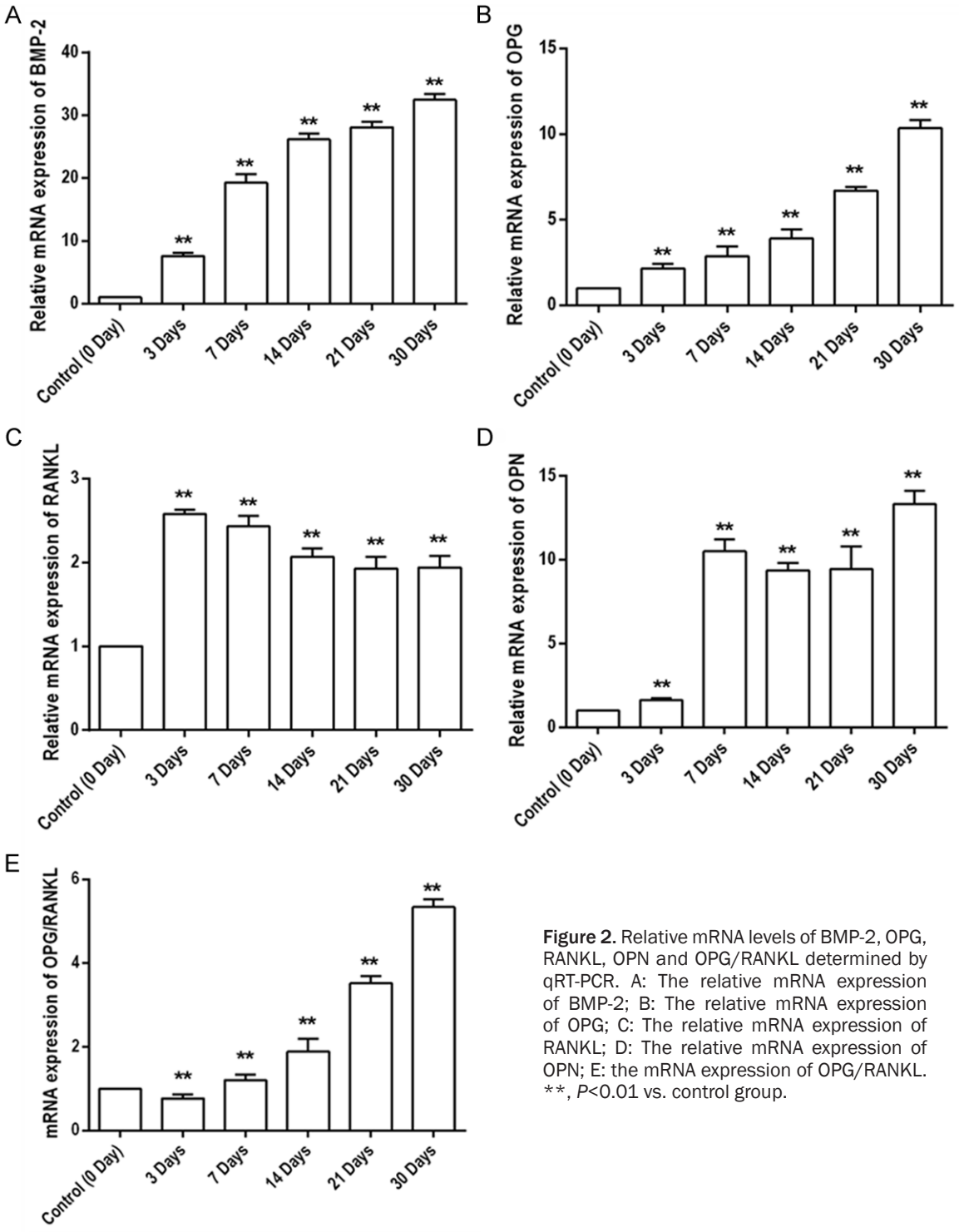

Figure 2. Relative mRNA levels of BMP-2, OPG, RANKL, OPN and OPG/RANKL determined by qRT-PCR. A: The relative mRNA expression of BMP-2; B: The relative mRNA expression of OPG; C: The relative mRNA expression of RANKL; D: The relative mRNA expression of OPN; E: the mRNA expression of OPG/RANKL. **, $P<0.01$ vs. control group.

overnight at 4°C and for 1 h at 37°C with primary antibodies (goat anti-BMP-2, goat anti-OPG, goat anti-RANKL, or goat anti-OPN, 1:150 dilution, Santa Cruz, USA). β-actin was used as a loading control. The membranes were exposed to the negative films to develop target bands after incubated with enhanced chemiluminescence (Santa Cruz, USA). The intensities of bands were quantitated by LabWorks 4.5 software (UVP, USA).

Statistics analysis

SPSS 18.0 was used for statistical analysis. All data were expressed as mean ± SD ($x \pm s$), and the statistical differences among different time

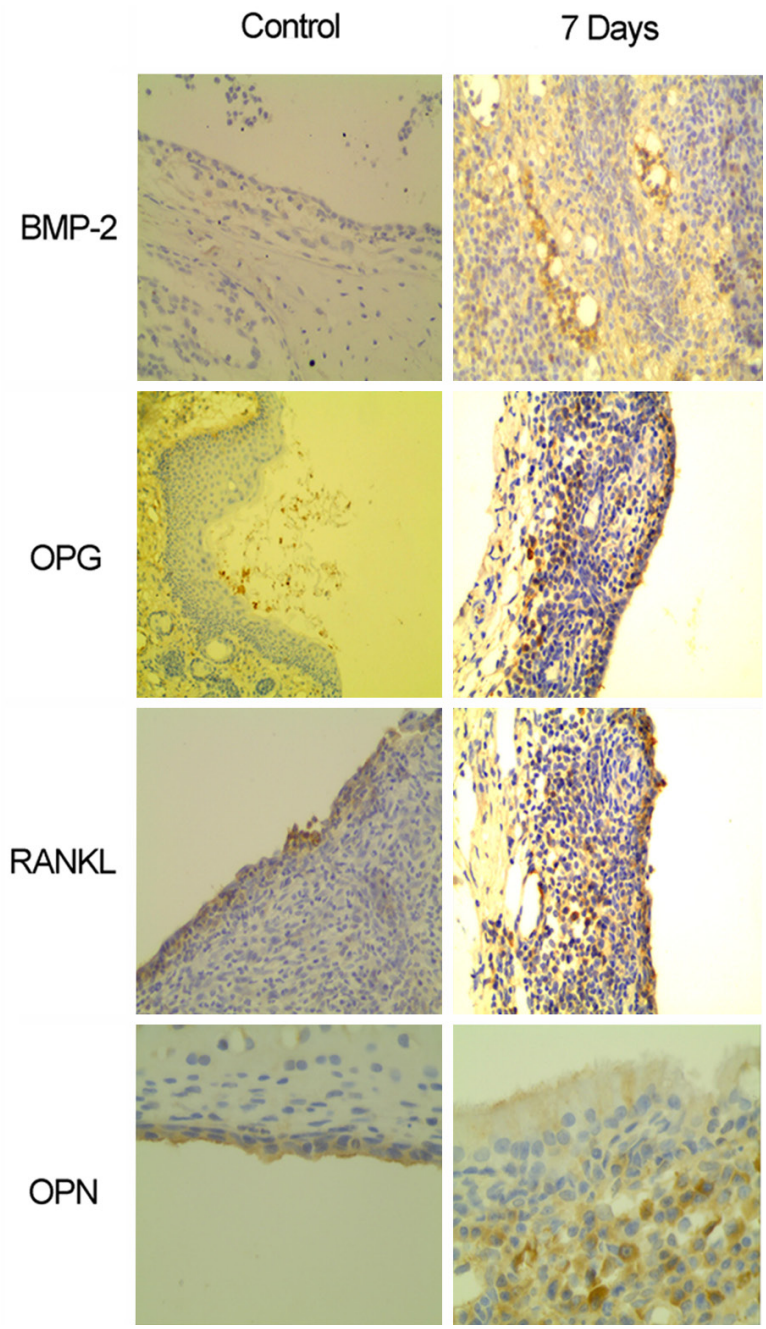

Figure 3. Immunohistochemistry examination of BMP-2 (×200), OPG (×200), RANKL (×200) and OPN (×400) in otic vesicles of rats. Protein expressions of BMP-2, OPG, RANKL and OPN were all weakly positive in control rats. Positive results of the four proteins were observed at each time point after the induction of CSOM. The positive staining of BMP-2 mainly located in the cytoplasm. OPN expressed in the cytoplasm of submucosal fibroblast-like cells and osteoblasts. OPG and RANKL both expressed in the cytoplasm of tympanic mucosa.

points were assessed by one-way ANOVA. The two groups were compared using student *t*-test. $P<0.05$ indicated a significant difference.

Results

The formation of bacterial biofilm in rats with CSOM

We observed the formation of the bacterial biofilm in the rat middle ears through the confocal microscope. In CSOM group, a few sporadic green dots were observed on the surface of middle ear mucosa 7 days after the induction (**Figure 1**). Mature biofilms were observed in rat middle ear both 14 and 21 days after the induction. Green dots gathered into a cluster and mist was observed. At 30th day after the induction, bacterial biofilm presented signs of recession. There were hardly any exopolysaccharides observed in middle ears of mice in control group.

BMP-2, OPG, RANKL and OPN mRNA levels in rats with CSOM

BMP-2, OPG, RANKL, OPN and GAPDH mRNA levels were determined using a fluorescent quantitative PCR. And then the concentration of the target mRNA was calculated (target mRNA relative concentration = target mRNA concentration/GAPDH mRNA concentration). The relative concentration levels of BMP-2, OPG, RANKL and OPN mRNA (CSOM group/control group) were shown in **Figure 2**. All the four kinds of mRNAs increased very significantly ($P<0.01$) after the induction of CSOM. The mRNA expressions of BMP-2 and OPG increased over time. RANKL mRNA expression had a downtrend after the induction. The mRNA expression of OPN at 7 days after the induction was higher than 3, 14 and 21 days, however lower than 30 days after the induction. The

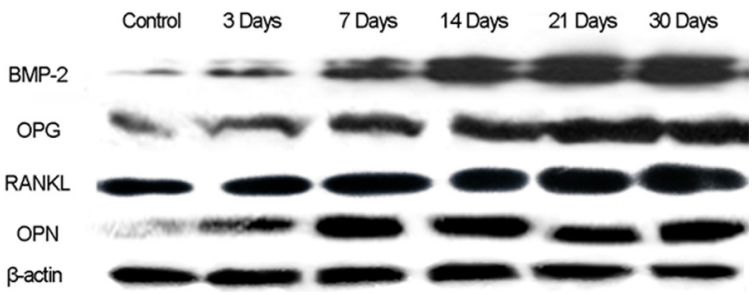

Figure 4. Western blot of OPN, BMP-2, OPG, RANKL and β-actin.

mRNA expression of OPG/RANKL significantly decreased at 3 days after the induction, however was significantly higher than control after that with a rising trend.

Protein expression of BMP-2, OPG, RANKL and OPN in rats with CSOM

As the results of immunohistochemistry, protein expressions of BMP-2, OPG, RANKL and OPN were all weakly positive in control rats (**Figure 3**). Positive results of the four proteins were observed at each time point after the induction of CSOM. The positive staining of BMP-2 mainly located in the cytoplasm. OPN expressed in the cytoplasm of submucosal fibroblast-like cells and osteoblasts. OPG and RANKL both expressed in the cytoplasm of tympanic mucosa.

As shown on the results of Western blot, protein expressions of BMP-2, OPG, RANKL and OPN in CSOM rats were significantly higher than control at each time point after the induction (**Figures 4**, **5**). The protein expression of OPG/RANKL significantly decreased at 3 days after the induction, however was significantly higher than control after that. The expression of BMP-2, OPG and OPG/RANKL had a rising trend, while the expression of RANKL had a downtrend after the induction. The expression of OPN had a highest expression at 30 days after the induction and a second highest expression at 7 days after the induction.

Discussion

CSOM is a common but intractable infectious ear disease. Roland [18] suggested that the biofilm might be the reason for CSOM. Michael *et al.* [19] re-ported that biofilms were statistically more common in patients with CSOM compared with control patients undergoing otologic surgery for other otic diseases. In our study, we found biofilms in middle ears of rats with CSOM induced by *Pseudomonas Aeruginosa* through the confocal microscope 7 days after the induction. And the biofilms were mature 14 days after the infection.

Biofilms are structured communities of bacteria cells whose formation is a critical component of prokaryotic survival in hostile environments [20]. These communities are formed from bacteria implanted in extracellular polymeric substances (EPS) and composed of polysaccharides, proteins, and nucleic acids. Biofilms are well designed and consist of bacterial towers separated by channels that both provide nutrients to the bacteria and eliminate the waste in the system [21]. With signaling and communication, bacteria are able to develop these complex structures critical to their preservation in threatening situations [22].

The life cycle of biofilms includes three phases: adhesion, growth and release. Primarily, trace organics form a neutralizing layer on the tissue surface, making it easier for bacteria to adhere to it [23]. Then the biofilm becomes mature with the cellular growth and the increased production of EPS. Once the biofilms are mature, planktonic bacteria will be released into systemic circulation [24].

In our study, there was a great increase in the expression of RANKL 3 days after the induction of CSOM, and OPG/RANKL was significantly lower than control. It indicated that destruction and absorption of the bone were promoted in the middle ear at the early stage of CSOM. It might be because EPS could stimulate the high expression of RANKL. After that, OPG/RANKL increased gradually, suggesting the calcium deposition and bone formation in the middle ear.

The expression of BMP-2 increased significantly after the induction, suggesting that bone formation existed over the course of CSOM. In addition, BMP-2 might also have correlation to the inflammatory responses in the middle ear of CSOM rats.

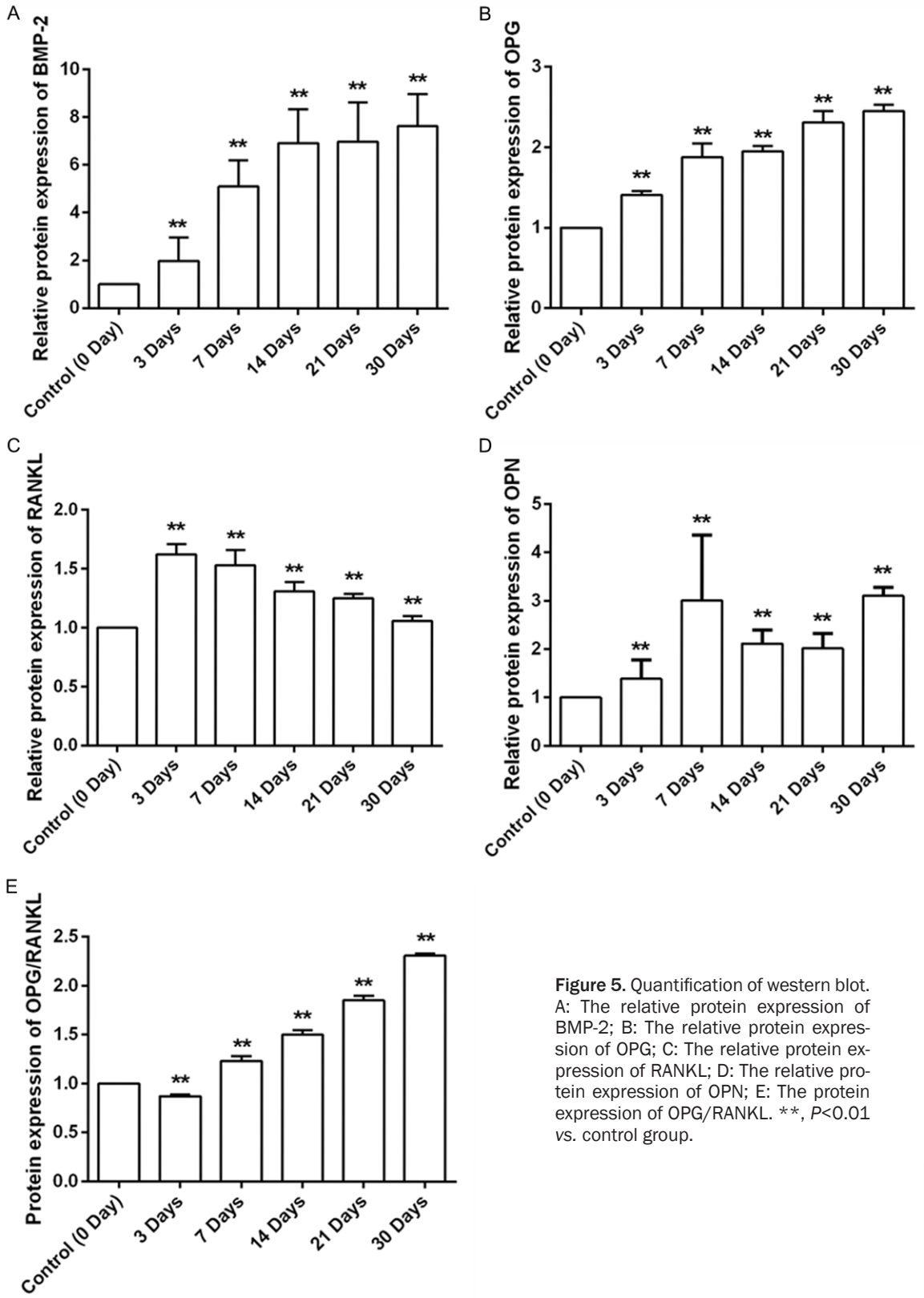

Figure 5. Quantification of western blot. A: The relative protein expression of BMP-2; B: The relative protein expression of OPG; C: The relative protein expression of RANKL; D: The relative protein expression of OPN; E: The protein expression of OPG/RANKL. **, $P<0.01$ *vs.* control group.

OPN, related to pathological calcification in the middle ear, could regular the bone formation. In this study, OPN were significantly higher in CSOM rats than control rats. OPN had a dual

role in both anti-inflammatory and pro-inflammatory. The 2 time points with the highest expression of OPN in our study was 7th and 30th day after the induction. OPN might promote the inflammatory responses at the early stage of CSOM and played an anti-inflammatory role at the later stage of CSOM.

In conclusion, the bacterial biofilm in the otic vesicle of middle ears formed at early CSOM with the bone destruction and bone morphogenetic. Further studies of the relations between bacterial biofilms and bone modelling markers would be needed to reveal the mechanism of CSOM.

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Disclosure of conflict of interest

None.

Address correspondence to: Yun-Sheng Li, Department of Anatomy, Histology and Embryology, Basic Medical College, Tianjin Medical University, 22 Qixiangtai Road, Tianjin 300070, China. E-mail: liyunsheng25@163.com

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