Reduction of osteocalcin expression in aged human dental pulp

T. Muramatsu[1,2], H. Hamano[2], K. Ogami[3], K. Ohta[1,4], T. Inoue[1,5] & M. Shimono[1,2]

[1]Oral Health Science Center, and Departments of [2]Pathology, [3]Complete Denture Prosthodontics, [4]Biochemistry and [5]Clinical Pathophysiology, Tokyo Dental College, Chiba, Japan

Abstract

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Aim To investigate the expression of osteocalcin mRNA in young and in aged human dental pulp tissue to determine the characteristics of osteocalcin expression.

Methodology Human dental pulp tissues of the third molars were obtained from healthy young (17–23 years) and aged (>50 years) subjects, and total RNA was extracted. Osteocalcin mRNA expression was determined by RT-PCR and by quantitative real-time RT-PCR (QRT-PCR). The threshold cycle (Ct) value, which reflects the amount of PCR, was calculated and the difference between the value in young and aged pulp was statistically analysed.

Results Osteocalcin mRNA was detected in all samples of human dental pulp tissue homogenates by RT-PCR analysis. Osteocalcin mRNA was expressed in young adult dental pulp but was decreased in aged human dental pulp. QRT-PCR analysis also showed a reduced expression of osteocalcin mRNA in aged human pulp. Expression of osteocalcin in young human pulp was significantly higher (about sixfold) than in aged pulp ($P < 0.01$, Mann–Whitney U-test).

Conclusion Reduction of osteocalcin expression may be associated with the loss of viability in human dental pulp tissue, and may be a characteristic of aged human dental pulps.

Keywords: aging, dental pulp, human, osteocalcin, quantitative RT-PCR.

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Introduction

Aged pulp tissues are associated with various characteristics including reduction of the dimensions of the pulp chamber, fibrosis, atrophy, loss of cellularity, dyscalcification and degeneration of odontoblasts (Bernick & Nedelman 1975, Stanley 1978, Ketterl 1983). Reduction of chamber size is a typical phenomenon in teeth from older individuals (Oi *et al.* 2004) and it frequently leads to difficulty in clinical treatment of the pulp. The phenomenon suggests that aged dental pulp still has the ability to create dentine but at a diminished rate (Woods *et al.* 1990). However, the aged pulp has not been well characterized, because markers to study it have not been established.

Osteocalcin is a vitamin K-dependent and non-collagenous dentine and bone matrix protein. Osteocalcin is synthesized by osteoblasts and odontoblasts, and is a well-known marker of viability, differentiation and osteogenic ability in those cells (Camarda *et al.* 1987). Excessive mineralization with growth plate closure was detected in rats treated chronically with vitamin K-antagonist warfarin that was well used for patients with myocardial or brain infarction clinically (Price *et al.* 1982), suggesting osteocalcin had an important role to inhibit excessive mineralization. Furthermore, the function of osteocalcin has been studied using knockout mice, and osteocalcin has been shown to inhibit excess-creating bone matrix (Ducy

Correspondence: Takashi Muramatsu, DDS, PhD, Department of Pathology, Tokyo Dental College, 1-2-2, Masago, Mihama-ku, Chiba 261-8502, Japan (Tel.: +81 432703782; fax: +81 432703784; e-mail: tmuramat@tdc.ac.jp).

et al. 1996). However, the function of osteocalcin in dental pulp has not been clarified, and in particular it is not known whether osteocalcin is associated with aging in pulp tissue.

In the present study, the expression of osteocalcin mRNA was examined in young and in aged human pulp tissue using RT-PCR and quantitative real-time RT-PCR (QRT-PCR) in order to evaluate cell viability and to determine the characteristics of osteocalcin expression in aged human dental pulp.

Materials and methods

Materials and tissue preparation

Young dental pulp tissue was obtained from the third molars of 11 healthy young subjects (17–23 years of age, average 21 years). The teeth were extracted for orthodontic reasons. Aged dental pulp tissue was obtained from the third molars of 13 older subjects (50–66 years of age, average 56 years). The teeth were extracted due to periodontal disease, but they did not have any symptoms of pulpitis. The teeth were extracted at the Tokyo Dental College Chiba Hospital, Japan, after informed consent had been obtained. The extracted teeth were immediately frozen in liquid nitrogen, and were then stored at −80 °C until analysed by RT-PCR. A summary of the clinical information is given in Table 1.

Table 1 Clinical data of the subjects

Case	Age	Gender	Site
Young subjects			
1	17	M	18
2	17	M	28
3	18	M	48
4	21	F	18
5	21	F	28
6	22	F	28
7	23	F	18
8	23	F	28
9	23	F	38
10	23	F	48
11	23	F	28
Aged subjects			
1	50	F	28
2	50	M	48
3	51	M	48
4	52	F	28
5	53	F	28
6	56	M	18
7	56	M	18
8	57	F	28
9	57	F	28
10	57	M	48
11	59	M	18
12	64	F	48
13	66	F	18

Pulp samples used in this study were obtained from 11 young (17–23 years) and 13 aged (50–66 years) subjects.

RT-PCR

Total RNA from the dental pulp tissue of young and aged adults was extracted by a modified acid-guanidinium-thiocyanate-phenol-chloroform (AGPC) method using the TRIzol RNA extracting mixture, according to the manufacturer's recommendations. For analysis of osteocalcin gene expression by RT-PCR, RNA was reverse-transcribed into complementary DNA (cDNA) using a Takara RNA PCR Kit (Takara, Otsu, Japan). The sequences of the primers used to detect rat specific osteocalcin and β-actin (as an internal control) were specific, as confirmed by a computer-assisted search of an updated version of GenBank, and are shown in Table 2. In each tissue sample, 100 ng total RNA was reverse transcribed using random primers and the products were subjected to PCR amplification under the same conditions described above. The reaction mixture was added to the RNA solution, and was incubated at 42 °C for 1 h, heated at 94 °C for 5 min, and then chilled at 4 °C. For PCR, the cDNA reaction mixture was diluted with 40 μl PCR buffer and mixed with 50 pmol of the 5′ and 3′ primers. The reactions were carried out in a DNA thermal cycler (MJ Research Inc., Watertown, MA, USA) under the following conditions: 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min. After the reaction, the amplified products were analysed by 1.7% agarose gel electrophoresis and visualized by ultraviolet illumination after staining with ethidium bromide.

Table 2 Primer sequences for the detection of specific osteocalcin and β-actin

	Primer sequences	Product size
Osteocalcin	F: 5′-AAAGGCGTTAAGGATCGCGTG-3′ R: 5′-GTCATCAGGCCGAGGCCT-3′	293 bp
β-actin	F: 5′-GTCATGGGTGTGAACCATGAGAA-3′ R: 5′-TGGTCATGAGTCCTTCCACGAT-3′	804 bp

QRT-PCR

QRT-PCR using the TaqMan MGB probe (Applied Biosystems, Foster City, CA, USA) was carried out as

previously reported with modifications (Ohtsuka *et al.* 2000, Muramatsu *et al.* 2004). Human beta glucuronidase (GUSB) was used as an internal control. The TaqMan MGB probes of human osteocalcin and of GUSB were purchased from Applied Biosystems (cat. no. Hs01587813_g1, 4326320E respectively). Amplification was then performed in duplicate using the primer sets in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) with denaturation for 10 min at 95 °C followed by 40 PCR cycles of denaturation at 95 °C for 15 s and annealing or extension at 60 °C for 1 min. ABI 7700 system software was used to monitor the changes in FAM and VIC dye in each cycle. The threshold cycle (Ct) value for each reaction, which reflects the amount of PCR needed to identify a target gene, and the relative level of osteocalcin for each sample, was calculated as outlined by the manufacturer. Briefly, GUSB was used to normalize the amount of osteocalcin mRNA. Its Ct value was subtracted from that of the osteocalcin gene to obtain a ΔCt value. The difference (ΔΔCt) between the ΔCt value of each sample for the gene target and the ΔCt value of the calibrator was determined. Specimens were analysed three times and the values were averaged. To confirm the specificity of the PCR reaction, PCR products were electrophoresed on 2% agarose gels.

Statistical analysis

Differences between young and aged subjects were analysed statistically using the Mann–Whitney *U*-test ($P < 0.01$).

Results

Representative RT-PCR products examined in this study are shown in Fig. 1. Expression of osteocalcin and β-actin mRNAs in young and in aged human pulp was observed in all pulp tissue examined, regardless of age. Beta-actin gene expression used as an internal control was positive at 804 bp and was equal in all samples. The RT-PCR products of osteocalcin were also found at 293 bp in both the young and the aged tissues. The identity of each product was confirmed by Southern hybridization with nested probes (data not shown).

To validate the expression of osteocalcin in young and in aged human pulp tissue, QRT-PCR was carried out. Based on the ΔΔCt relative to young human pulp, the relative expression levels of osteocalcin mRNA in aged pulp were calculated. The reference used was young pulp, considered to have a value of 100. QRT-PCR demonstrated a reduced expression of osteocalcin in aged pulp tissue and it was lower (16.8 ± 8.2) than in the young pulp tissue (Fig. 2) at a statistically significant level ($P < 0.01$, Mann–Whitney *U*-test).

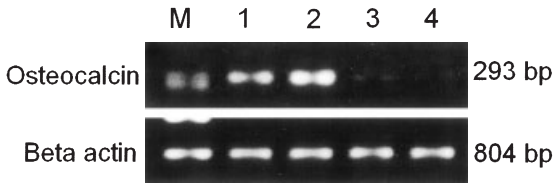

Figure 1 Expression of osteocalcin and β-actin from young and aged human pulp. PCR products were run at 35 cycles. 1–2: young pulp; 3–4: aged pulp. Expression of osteocalcin and β-actin is observed in all pulp examined in this study, regardless of age. Osteocalcin gene expression is recognizable at 293 bp in young and in aged pulp. The expression level of osteocalcin is more intensive in the young pulp compared with aged pulp.

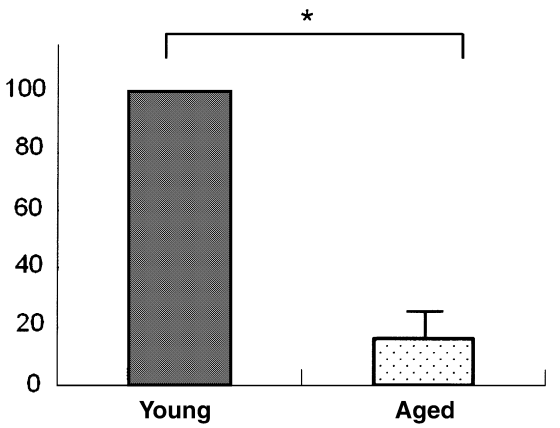

Figure 2 Gene expression of osteocalcin was normalized to that of human GUSB. Each value is expressed as the mean of duplicates. The reference was young pulp, considered to have a value of 100. QRT-PCR shows a definite reduction of osteocalcin in old pulp that is significantly lower (16.8 ± 8.2) than that in young pulp ($P < 0.01$, Mann–Whitney *U*-test).

Discussion

Although many studies dealing with aged dental pulp have been reported, few biochemical analyses have evaluated the viability of aging human pulp (Uitto & Ranta 1973, van Amerongen *et al.* 1983). Recently it has been shown that the gap junction protein, connexin 43 (CX43), was decreased substantially in aged

human dental pulp, and suggested that CX43 is a candidate to evaluate viability in aged pulp (Muramatsu *et al.* 2004). There have been many reports that osteogenic cells express CX43 and osteocalcin (Lecanda *et al.* 1998, Li *et al.* 1999, Adamo *et al.* 2001), and Lecanda *et al.* (1998) showed that CX43 deficiency caused delayed ossification, craniofacial abnormalities and osteoblast dysfunction, and elicited a decrease in osteocalcin expression. Therefore, it was hypothesized that osteocalcin could serve as a useful marker in addition to CX43 (Muramatsu *et al.* 2004). The expression of osteocalcin was therefore characterized in the present study. There was a decline of osteocalcin in aged, compared with young, pulp tissues using RT-PCR and QRT-PCR. These results suggest that the decrease in the expression of osteocalcin may be a useful marker of the viability of aged pulp.

Reduction of chamber size is a common phenomenon in aged teeth (Oi *et al.* 2004), and it frequently leads to difficulties in root canal treatment. Although cell viability deteriorates in aged tissues in general, these findings imply that aged dental pulp still has the ability to create dentine at a diminishing rate (Woods *et al.* 1990). In the present study, it was hypothesized that aged human pulp creates dentine by reducing osteocalcin expression, as one study showed that osteocalcin-knockout mice had lost osteocalcin but had increased bone formation (Ducy *et al.* 1996). Furthermore, experiments using the vitamin K antagonist warfarin revealed excessive mineralization with growth plate closure in rats when treated chronically (Price *et al.* 1982). The results of the present study showed a distinct decline of osteocalcin mRNA in aged human dental pulp. These results suggest that reduction of osteocalcin may cause reduction of chamber size and should be considered a characteristic of aged pulp tissues.

Ranly *et al.* (1997) showed expression of osteocalcin at a high level, while the present study showed reduction of osteocalcin expression. The difference in results might be due to the methodologies used. The other group used a 35-cycle-amplification with 56 °C annealing temperature and a semiquantitative method (osteocalcin/GAPDH) using a densitometer. However, in preliminary studies, conventional RT-PCR was used to examine osteocalcin expression using the same primer sequences, and many conditions for determining appropriate amplification cycles were tested. Preliminary results showed that the 35-cycle amplification used in the study of Ranly *et al.* (1997) was too high for osteocalcin detection, because PCR products reached a plateau at less than 30 cycles, suggesting that the 35-cycle amplification used by the other group was not appropriate for quantitation. Furthermore, many bands were found when the 56 °C annealing temperature was used, and therefore in this study QRT-PCR with a 62 °C annealing temperature was employed.

Decreased osteocalcin mRNA expression in aged pulp was demonstrated in the main and preliminary experiments, and so the expression of cbfa-1, a transcription and regulation factor in osteoblast maturation (Sato *et al.* 1998), was also evaluated in young and aged human pulp tissues. Preliminary data showed that the expression of cbfa-1 in aged pulp was equal to that in young pulp (data not shown). These results imply that osteocalcin expression is normal in aged people and the reduction may be caused by a disturbance in its translation.

Conclusion

Reduction of osteocalcin expression may be associated with the loss of viability in human dental pulp, and may be a characteristic of aged human pulp.

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