Reduction of connexin 43 expression in aged human dental pulp

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Abstract

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Aim To investigate the expression of connexin 43 (CX43) mRNA in young and old human dental pulp tissues to determine the characteristics of CX43 expression.

Methodology Samples were obtained from human dental pulp of healthy young (17–23 years) and aged (>50 years) subjects. CX43 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 value in the young pulp and that in the aged pulp was statistically analysed.

Results RT-PCR analysis of human dental pulp tissue detected CX43 mRNA in all the samples. CX43 was abundantly expressed in young adult dental pulp, but expression of CX43 mRNA was dramatically decreased in aged human dental pulp. QRT-PCR analysis also showed the reduced expression of CX43 in aged pulp, and expression of CX43 in young pulp was significantly higher (about 10-fold, $P < 0.01$, Mann–Whitney *U*-test).

Conclusion Reduction of CX43 expression may be associated with the loss of viability in human dental pulp, and is considered as one characteristic of aged pulp.

Keywords: ageing, connexin 43, dental pulp, gap junction, human, quantitative RT-PCR.

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Introduction

Aged dental pulp tissues have various characteristics including a reduction of pulp chamber size, fibrosis, atrophy, loss of cellularity, dyscalcification and degeneration of odontoblasts (Bernick & Nedelman 1975, Stanley 1978, Ketterl 1983). Reduction of chamber size is a common phenomenon in teeth from older patients, and it frequently leads to difficulty in clinical treatment of the pulp. Although cell viability deteriorates in aged tissues in general, dental pulp cells make tertiary dentine and the deposition of dystrophic dentine continues into old age at a diminishing rate (Woods *et al.* 1990). This implies that aged pulp still has the potential for odontogenesis. However, the condition of aged pulp has not been well characterized because markers to estimate its condition have not been established.

Connexins (CXs) comprise a family of gap junction proteins that form hexameric complexes in plasma membranes of adjacent cells, which in turn form intercellular channels. CXs are distinguished by reference to their molecular weights as predicted from their corresponding cDNAs (Beyer *et al.* 1987). CX43 is a 43 kDa-gap junction protein initially identified in rat cardiac myocytes (Beyer *et al.* 1987). Recently, it has been reported that CX43 expression in young rat bone marrow cells was higher than that in old ones (Haraguchi *et al.* 2003). Furthermore, examination of the expression and localization of CX43 in rat incisor odontoblasts and in pulp cells found that CX43 was

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highly expressed in young odontoblasts (Fried *et al.* 1996, Murakami *et al.* 2001). These data imply that CX43 is associated with the viability of odontoblasts and pulp cells, and that CX43 could be a useful marker of viability in pulp tissues.

In the present study, the expression of CX43 mRNA was examined in young and in aged human dental pulp tissue using RT-PCR and quantitative real-time RT-PCR (QRT-PCR) in order to determine cell viability in aged human 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 old, average 21.0 years). The teeth were extracted for orthodontic reasons. Thirteen samples of aged pulp tissue from third molars were obtained from individuals >50 years old (50–66 years old, average 56.0 years). The teeth were extracted because of marginal periodontal disease, but they did not have any signs of symptoms of pulpitis. The teeth were extracted at the Tokyo Dental College Hospital after the patients' informed consent had been obtained. The extracted teeth were immediately frozen in liquid nitrogen, and then stored at −80 °C until RT-PCR examination. 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

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

RT-PCR and QRT-PCR

For RNA extraction, dental pulp tissues were carefully removed from the teeth by mechanical means. Total RNA was extracted from the tissues using the acid guanidinium-thiocyanate-phenol-chloroform (AGPC) method with TRIzol (Invitrogen, Grand Island, NY, USA) and cDNA synthesis was performed as described previously (Murakami *et al.* 2001). For analysis of CX43 gene expression by RT-PCR, RNA was reverse-transcribed into complementary DNA (cDNA) using a Takara RNA PCR kit (Takara, Tokyo, Japan). The sequences of the primers were specific, as confirmed by a computer-assisted search of an updated version of GenBank. Primer sequences used to detect human CX43 and β-actin (as an internal control) are given 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, 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. Reactions were carried out in a DNA thermal cycler (MJ Research Inc., Watertown, MA, USA) under the following conditions: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The amplified products were then analysed by 1.7% agarose gel electrophoresis and visualized by ultraviolet illumination after staining with ethidium bromide.

QRT-PCR was carried out according to Ohtsuka *et al.* (2000) with modification. A total of 2 μL cDNA was used in a 23-μL PCR mixture containing 1X SYBR PCR buffer, 3 mmol L^{-1} $MgCl_2$, 0.8 mmol L^{-1} dNTP and 0.025 U μL^{-1} AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA, USA). Primers for human CX43 were used and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The primer sequences are given in Table 2. Amplification was then performed in duplicate using the primer sets in an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA, USA) with denaturation for 10 min at 95 °C followed by 40 PCR cycles

	Primer sequences	Product size (bp)
For RT-PCR		
Connexin 43	F: 5′-AAAGGCGTTAAGGATCGCGTG-3′ R: 5′-GTCATCAGGCCGAGGCCT-3′	438
β-Actin	F: 5′-TGTATGCCTCTGGTCGTACCAC-3′ R: 5′-CTCCTGAGCGCAAGTACTCTGT-3′	804
For QRT-PCR		
Connexin 43	F: 5′-ACTTGCCTTTTCATTTTACTTC-3′ R: 5′-CCTGGGCACCACTCTTTT-3′	88
GAPDH	F: 5′-GTCATGGGTGTGAACCATGAGAA-3′ R: 5′-TGGTCATGAGTCCTTCCACGAT-3′	136

Table 2 Primer sequences for the detection of CX43 and β-actin in RT-PCR and of CX43 and GAPDH in QRT-PCR

of denaturation at 95 °C for 15 s and annealing or extension at 60 °C for 1 min. The ABI 7700 system software monitored the changes in fluorescence of SYBR Green I 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 CX43 for each sample, was calculated as outlined by the manufacturer. Briefly, GAPDH was used to normalize the amount of CX43 mRNA. Its Ct value was subtracted from that of the CX43 gene to obtain a ΔCt value. The difference (ΔΔCt) between the ΔCt values of the samples 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 statistically analysed using Mann–Whitney *U*-test ($P < 0.01$).

Results

A representative example of RT-PCR products examined in this study is shown in Fig. 1. Expression of CX43 mRNA from the young and from the aged subjects was observed in all the pulp tissues examined, regardless of age. β-Actin gene expression used as an internal control was positive at 804 bp and was equivalent in all samples. CX43 gene expression was recognizable at 438 bp both in young and in aged pulp. However, the expression level of CX43 was more intense in the young pulp compared with that in aged pulp. The identity of the products was confirmed by Southern hybridization with nested probes (data not shown).

To validate the expression of CX43 in young and in old human pulp tissue, QRT-PCR was carried out.

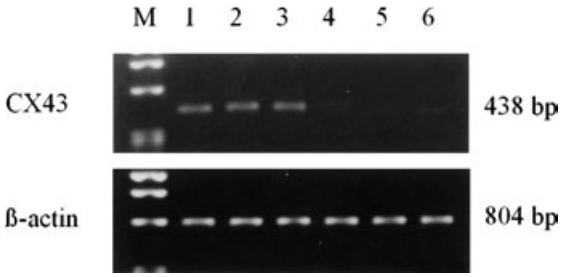

Figure 1 Expression of connexin 43 (CX43) and β-actin from young and aged pulp. PCR products are shown at 35 cycles. 1–3: young pulp; 4–6: aged pulp. Expression of CX43 and β-actin from the young and the aged pulp is observed in all pulp tissues examined in this study, regardless of age. CX43 gene expression is recognizable at 438 bp both in young and aged pulp. The expression level of CX43 is more intense in young pulp compared with that in aged pulp.

Based on the ΔΔCt relative to young human pulp, the relative expression levels of CX43 mRNA in old pulps were calculated. The reference was young pulp, considered to have a value of 100. QRT-PCR demonstrated a reduced expression of CX43 in old pulp tissue and that it was lower (8.7 ± 2.2) than in young pulp tissue (Fig. 2) at a statistically significant level ($P < 0.01$).

Discussion

Although many morphological studies on aged pulp tissue have been carried out, only a few biochemical analytical studies have evaluated the viability of the human pulp during ageing (Uitto & Ranta 1973, van Amerongen *et al.* 1983). Previously, it has been demonstrated that CX43 expression was remarkably lower in old rat osteoblast-like cells (Haraguchi *et al.* 2003) and in the short odontoblasts of rat incisors (Murakami *et al.* 2001). It has been suggested that CX43 might be a candidate factor influencing the viability of pulp cells. Furthermore, a loss of junctional

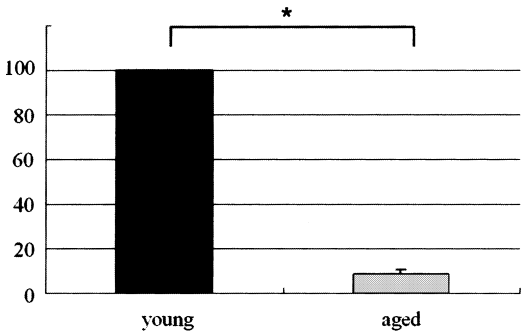

Figure 2 Gene expression of CX43 was normalized against GAPDH. Each value is expressed as the mean of duplicates. The referent was young pulp, considered to have a value of 100. QRT-PCR shows that reduction of CX43 mRNA is observed in old pulp and that it is significantly lower (8.7 ± 2.2) than that in young pulp.

attachment of odontoblasts occurred in aged rat molar pulp morphologically has been demonstrated (Lovschall *et al.* 2002). Therefore, the present study focused on CX43, a gap junction protein, as a determinant of pulp viability. The results demonstrated a significant decline of CX43 expression in aged human pulp using RT-PCR and QRT-PCR. The results suggest that the decline of CX43 expression may be a useful marker of pulp health, and is considered as one characteristic of aged pulp tissues.

Schirrmacher *et al.* (1992) considered that the function of gap junctions is important for stem cells to differentiate into osteoblastic cells. Schwab *et al.* (1998) reported that the number of CX43-immunoreactive osteogenic cells was higher than in fibroblastic cells *in vitro*, and that this higher incidence of CX43 expression was probably related to the growth and cytodifferentiation programmes in developing tissues. In the present study, CX43 mRNA expression in young pulp was higher than that in aged pulp. These results indicate that young pulp has a high level of dentineogenic potential and differentiation ability compared with aged pulp.

A decrease of CX43 expression in aged pulp tissues compared with young pulp was demonstrated in this study. There have been many reports that osteogenic cells express both CX43 and osteogenic markers including osteocalcin, and their relationship has been clarified (Lecanda *et al.* 1998, Li *et al.* 1999, Adamo *et al.* 2001). CX43 deficiencies cause delayed ossification, craniofacial abnormalities and osteoblast dysfunction, and show a decline in osteocalcin expression (Lecanda *et al.* 2000). Furthermore, it has also been reported that a loss of osteocalcin expression induces an increase in bone formation (Ducy *et al.* 1996). Preliminary examination also showed a decline of osteocalcin expression in aged pulp tissues (data not shown). The sum of these reports implies that the loss of CX43 expression may be associated with dystrophic calcification in aged pulp. It can be postulated that a decline of CX43 function induces the deposition of tertiary dentine and the reduction of chamber size, which is commonly observed in the pulp of older people.

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