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Effect of antisense oligonucleotide against mouse dentine matrix protein 1 on mineralization ability and calcium ions metabolism in odontoblast-like cell line MDPC-23

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Abstract

Pang JL, Wu BL, He WX, Zhang YQ, Zhao HP, Xie ZH. Effect of antisense oligonucleotide against mouse dentine matrix protein 1 on mineralization ability and calcium ions metabolism in odontoblast-like cell line MDPC-23. *International Endodontic Journal*, **39**, 527–537, 2006.

Aim To study the mineralization ability and the dynamic changes of intracellular and extracellular concentrations of calcium ions in the odontoblast-like cell line MDPC-23 affected by antisense oligonucleotide (AS-ODN) against mouse dentine matrix protein 1 (DMP1).

Methodology The expression of DMP1 in MDPC-23 cells was detected by an immunohistochemical method and its blocking outcome by the Western blot method. The alkaline phosphatase (ALP) activity, size and number of mineralized nodules, and the intracellular free ($[Ca^{2+}]_{if}$), total ($[Ca^{2+}]_{it}$) and the extracellular ($[Ca^{2+}]_e$) calcium ion concentrations in MDPC-23 cells in the experimental group affected with AS-ODN were compared with those in the control group (paired-samples *t*-test).

Results Dentine matrix protein 1 was stably expressed in a stable way in MDPC-23 cells; the expression was only just detectable at 12 h and became negative after 24 h affected by AS-ODN. Compared with the control groups, ALP activity of MDPC-23 cells in the AS-ODN group was decreased ($P < 0.05$), and both the number and size of mineralized nodules were smaller than those in the control group. $[Ca^{2+}]_{if}$ in the AS-ODN group increased and then decreased after 24 h. $[Ca^{2+}]_{it}$ dropped substantially to the lowest point at 24 h ($P < 0.01$). $[Ca^{2+}]_e$ increased before treatment for 24 h and then dropped, however, it was still higher than that of the control group.

Conclusions Antisense oligonucleotide against DMP1 could decrease mineralization ability and affect the intracellular and extracellular concentrations of calcium ions in MDPC-23 cells. This would indicate that DMP1 regulates the metabolism and transportation of calcium ions in odontoblasts, and thus boosts dentine mineralization.

Keywords: alkaline phosphatase, antisense oligonucleotide, calcium ions, dental dentine matrix protein 1, mineralization, odontoblast.

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Introduction

In dentinogenesis, dentine noncollagenous proteins (NCPs) play an important role in the induction and differentiation of odontoblasts and dentine mineralization. Dentine matrix protein 1 (DMP1) is one of the most important members of the mineralization tissue-specific proteins in dentine matrix and belongs to the SIBLING (small integrin binding ligand N-linked glycoprotein) family of cellular matrix proteins (Fisher & Fedarko 2003, Gluhak-Heinrich *et al.* 2003). DMP1 is mainly synthesized by odontoblasts and closely related to signal transduction, odontoblast differentiation and mineralization regulation in tooth germ development.

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The studies in this field mainly focus on the biological structure, physical and chemical characteristics of DMP1. The mechanisms of regulating the ontogeny and restricted tissue-specific expression of DMP1 are not clear. Cloning of DMP1 in various species has revealed the conservation of the acidic residues and serines that are strategically positioned for phosphorylation, and studies have shown that the extracellular matrix of bone contains fragments originating from DMP1 but it is intact in dentine (approx. 150–200 kDa) (Qin *et al.* 2001, Butler *et al.* 2002, Qin *et al.* 2003). The unique features of DMP1 include high acidity, serine richness, multiple glycosylation sites, and the presence of the RGD cell attachment tripeptide (Toyosawa *et al.* 1999, Kulkarni *et al.* 2000).

Dentine matrix protein 1 is detected mostly in dentine and odontoblasts (Septier *et al.* 2001), but it is present in other tissues or cells such as long bones, calvaria, ameloblasts (D'Souza *et al.* 1997, Kamiya & Takagi 2001, Toyosawa *et al.* 2001), cementum and pulp cells (Feng *et al.* 2003). Its spatio-temporal distribution during tooth germ development indicates that the expression of this gene is rigidly regulated in odontoblasts. *In situ* hybridization and immunohistochemical analyses showed that, DMP1 was detected in odontoblasts and dentinal tubules and their branches in mineralized root dentine and near the mineralization front (Toyosawa *et al.* 2004). It has been reported that DMP1 was mainly found in the secreting parts of odontoblast cytoplasm and pre-odontoblasts (Gao *et al.* 2002). It was expressed weakly in dental papilla and transiently in secreting ameloblasts when enamel matrix was initially secreted (Pang *et al.* 2003b). It was also detected mainly in cytoplasm and processes of odontoblasts and odontoblast-like cells in carious human permanent teeth (Pang *et al.* 2003a). These results indicated that DMP1 was involved in odontoblast differentiation and the mineralization process, as well as in the formation of reparative and reactive dentine.

Dentine matrix protein 1 is believed to have multiple functions *in vivo*, acting both as a signalling molecule and a regulator of biomineralization (Tartaix *et al.* 2004). It is now known that specific binding of DMP1 and possibly other NCPs on the collagen fibril might be a key step in collagen matrix organization and mineralization. DMP1 deposited into the extracellular matrix and nucleated the formation of hydroxyapatite (HA) *in vitro* in a multistep process that started with DMP1 binding calcium ions and initiating mineral deposition (He *et al.* 2003a,b). It is thought to have undergone proteolysis *in vivo* to generate functional cleavage fragments found in extracts of mineralized tissues (Narayanan *et al.* 2003, Steiglitz *et al.* 2004). Ye *et al.* (2004) reported that Dmp1-null mice displayed profound abnormalities in the skeleton and tooth, one of the most striking features being a severe abnormality in mineralization of bone, cartilage, dentine and enamel.

The alkaline phosphatase (ALP), which has been proposed to stimulate mineralization by supplying phosphate or by splitting away inorganic pyrophosphate, is regarded as a marker of odontoblast differentiation and dentine biomineralization (Woltgens *et al.* 1995). MDPC-23 cell, a spontaneously immortalised cell line, was derived from fetal mouse molar dental papillae and characterized as odontoblast-like cells. So it provides a valuable cell model for studies on functions and synthesis of various specific molecules involved in dentinogenesis. To understand the mechanisms of DMP1 during dentinogenesis, the functions of the DMP1 in dentine mineralization have been characterized. With the designed antisense oligonucleotide (AS-ODN) against DMP1, anti-mouse DMP1 monoclonal antibody that had been prepared (Gao *et al.* 2002) and MDPC-23 cell, the ALP activities and mineralized nodules were observed to reflect the functional changes of the cells affected by AS-ODN against DMP1. In addition to those, the $[Ca^{2+}]_{if}$, $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_{e}$ concentrations in MDPC-23 cells were measured dynamically after being treated by AS-ODN for different times. The ultimate goal was to identify the specific mechanisms of DMP1 during dentine mineralization.

Materials and methods

Design of AS-ODN against mouse DMP1

Antisense oligonucleotide against mouse DMP1 was designed according to the mouse DMP1 mRNA sequence retrieved from Genebank (serial number: U65020). The sense oligonucleotide (S-ODN) was also designed for negative control. The designed sequences are as follows: AS-ODN: 5′-AGTCTTCATATTGGGATG-3′; S-ODN: 5′-CATCCCAATATGAAGACT-3′, and they were synthesized and phosphorothioated by Shanghai Sangon Biological Engineering & Technology and Service Limited Co. (Bio Basic Inc., Shanghai, China).

Cell culture and preparation of cells coverslips

MDPC-23 cells, kindly provided by Prof. C.T. Hanks and Dr Jacques E. Nor (School of Dentistry, University of

Michigan, MI, USA), were cultured with α-MEM medium (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Biomeda Corporation, Foster City, CA, USA) 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2 mmol L^{-1} glutamine (Gibco BRL) in a humidified atmosphere containing 5% CO_2 at 37 °C. The 10 mm × 10 mm glass coverslips (with the thickness of 0.13–0.17 mm, Mingzhu Industrial Instrument Glass Factory, Ningbo, China) were cleansed after being soaked overnight with mixed acid solution (100 g L^{-1} dichromicum kalium and 1% hydrochloric acid) and ethanol for 4 h, and then they were toasted at 180 °C in a hot air drying oven for 5 h prior to use. The favourable MDPC-23 cells were inoculated into 24-well plates (Corning Costar, Rochester, NY, USA), which had been paved with sterile coverslips and continuously cultured. When the cells were spread out on the coverslips, the α-MEM medium and unattached cells were discarded and cells on coverslips were washed separately with the α-MEM medium and 0.01 mol L^{-1} phosphate buffer saline (PBS) three times. After fixing with 40 g L^{-1} paraformaldehyde for 30 min and washing with PBS, the cells were ready for immunohistochemistry.

Immunocytochemical assay

Expression of DMP1 in MDPC-23 cells was identified by immunohistochemistry. Streptavidin-peroxidase (SP) staining method (Chang *et al.* 2003) was used with anti-mouse DMP1 monoclonal antibody with a working concentration of 1 : 100. The staining procedure was performed according to the instructional manual of the kit (Boster Biological Technology Co., Ltd Wuhan, China). Peroxidase activity was revealed by incubation with DAB reaction solution. PBS, instead of the first antibody, was used as the negative control.

Identification of blockage of DMP1 expression in MDPC-23 cells

Well-growing MDPC-23 cells were inoculated into φ100 mm culture dishes (BD Falcon Primaria™, Franklin Lakes, NJ, USA) with the concentration of 2×10^5 cells mL^{-1} and then incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells in each group were treated with the α-MEM medium containing 10 μmol L^{-1} AS-ODN for 12, 24, 36, 48 h, and the normal medium and the medium containing 10 μmol L^{-1} S-ODN were taken as the control. When the time was up, the cells were harvested and washed with sterile ice-cold 0.01 mol L^{-1} PBS. 200 μL icecold modified NP-40 cell lysis buffer (Fukushi *et al.* 2004, de Pril *et al.* 2004) (1% NP-40, 20 mmol L^{-1} Tris–Cl, pH 7.8, 150 mmol L^{-1} NaCl, 0.2 g L^{-1} NaN_3, 1 mmol L^{-1} EDTA, 0.17 μg mL^{-1} pancreatic trypsin inhibitor aprotinin was added before use, 1 mmol L^{-1} phenyl methyl sulfonyl fluoride) was added to each dish under ice conditions for 30 min. The supernatant containing total protein was obtained and ready for use after cell collection and centrifugalization.

The concentration of total protein extracted was estimated with the Bradford DC Protein Quantitating kit (Bio-Rad, Hercules, CA, USA). Twenty-five milligrams of total protein was resolved on a 10% sodium dodecyl sulphate polyacrylamide electrophoresis (SDS–PAGE) gel, transferred electrophoretically to nitrocellulose membrane, and subsequently probed with the appropriate antibody. Immunoprecipitation was performed by incubating the total protein with anti-mouse DMP1 monoclonal antibody overnight at 4 °C. The antigen-antibody complex was precipitated with the use of protein G agarose (Sigma-Aldrich Corporation, St Louis, MO, USA) and subsequently resolved on a 10% SDS–PAGE gel. Using β-actin as inner control, the difference of DMP1 expression at different times was analysed by the UVP AutoChemi™ Imaging and Analysis System (UVP, Upland, CA, USA).

Detection of ALP activity

MDPC-23 cells were inoculated into four 96-well plates (Corning Costar) with a concentration of 5×10^4 cells mL^{-1} and continued to culture. Sixteen wells in each plate were randomly selected for α-MEM medium containing 10 μmol L^{-1} S-ODN, 5, 10 and 20 μmol L^{-1} AS-ODN and the normal medium group ($n = 64$). After each four of 16 wells in each group had been treated with different medium for 1, 3, 5, 7 days respectively, they were washed with 0.01 mol L^{-1} PBS and blotted. The detection procedure was performed according to the manual of the ALP detection kit (Biosino Bio-Technology & Science Inc., Peking, China). Fifty microlitres 0.1% (v/v) Triton X-100 was added to every well overnight in 4 °C environment. The supernatant was mixed with 100 μL the substrate solution containing 10 mmol L^{-1} disodium p-nitrophenyl phosphate in 100 mmol L^{-1} diethanolamine buffer (pH 10.5) with 0.5 mmol L^{-1} $MgCl_2$ in every well, 37 °C for 30 min. The reaction was stopped by adding 1 mL

0.2 mol L^{-1} NaOH. OD values were measured at 410 nm wavelength using a Dynatech MR7000 microplate reader (Dynatech, Billinghurst, UK) and the data were illustrated in Fig. 3.

von Kossa staining and mineralized nodules counting

The inoculated cells on coverslips had been continuously cultured for 10 days with α-MEM medium containing 10 μmol L^{-1} AS-ODN or S-ODN. von Kossa staining was performed according to following procedures: the fixed cells on the coverslips were immerged into freshly prepared 50 g L^{-1} argent nitrate solution after open-air drying. Solarization was carried out under ultraviolet light for 60 min, 50 g L^{-1} natrium thiosulfuricum was added to the coverslips and then washed with distilled water. Then the coverslips were sealed after re-staining with nuclear fast red. To eliminate false positives during staining, 20 g L^{-1} alizarin bordeaux was used. Five samples in each group and five fields of vision in each sample were selected randomly. The stained cells were then put on an acetic acid resin network of an anatomical microscope, and the mineralized nodules whose areas exceeded 4 mm^2 were counted at 40-fold magnification.

Detection of $[Ca^{2+}]_{if}$

The intracellular free calcium ion concentrations ($[Ca^{2+}]_{if}$) in MDPC-23 cells affected with AS-ODN were detected by laser scanning confocal microscope (LSCM, MRC-1024; Bio-Rad Laboratories, 2000 Alfred Nobel Drive, Hercules, CA, USA). The well-growing cells in φ60 mm culture dishes (BD Falcon Primaria^{TM}, Franklin Lakes, NJ, USA) with the concentration of 4×10^4 cells mL^{-1} were treated with α-MEM medium containing 10 μmol L^{-1} AS-ODN for 12, 24, 36, 48 h, with the normal medium at 12 h as the control group. Four samples at each time were selected in each group. They were loaded with Fluo-3 AM (Molecular Probes, Eugene, OR, USA; Final concentration 2.2 μmol L^{-1}) and incubated away from light at 37 °C for 30 min after washed with D-Hank's solution. Then the intracellular fluorescence intensity relatively representing $[Ca^{2+}]_{if}$ was dynamically scanned with LSCM three times. The mean relative fluorescence value (RFV) was obtained from the change of fluorescence value by subtracting background value from experimental fluorescence value and demonstrated in Fig. 5.

Detection of $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_e$

The prepared cells were treated with α-MEM medium containing 10 μmol L^{-1} AS-ODN or S-ODN for 12, 24, 36, 48 h, with the normal medium as the control group. The culture medium was obtained at each time in every group and subpackaged 1 mL into a centrifuge tube before cell harvesting for detection of extracellular calcium ion concentrations ($[Ca^{2+}]_e$). Then the cells were harvested into a centrifuge tube with the concentration of 1×10^6 cells mL^{-1} after trypsinization and counting. After being washed with PBS and then centrifuged, the cells were lysed with NP-40 cell lysis buffer. Then intracellular total calcium ion concentrations ($[Ca^{2+}]_{it}$) were detected in the cell lysate prepared by freezing and thawing repeatedly, sonic oscillation for 30 min and centrifugalization. There were 10 samples prepared at each time in every group ($n = 40$).

According to the manual of the calcium ion detection kit calcium reagent (Sigma-Aldrich Corporation), 10 μL out of each sample of AS-ODN, S-ODN, standard (2.5 mmol L^{-1} standard calcium ion solution), normal (the control group) and deionized water (the blank group) groups was mixed gently with 990 μL freshly prepared calcium reagent working solution by combining equal proportion of calcium binding reagent and calcium buffer. The absorbance (A) of all tested tubes were read and recorded at 575 nm wavelength at least 3 min after mixing the working solution and the samples. The changes in absorbance (ΔA) were obtained by subtracting absorbance of blank from absorbance of standard, controls and samples to represent calcium concentrations. Calcium concentrations in samples were determined as follows:

$$[Ca^{2+}]_{it}(\text{mmol}\,L^{-1})\,\text{of samples} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{standard concentration.}$$

The $[Ca^{2+}]_e$ of 10 samples of collected culture medium at each time in every group ($n = 40$) were detected by TAS-986-type atomic absorption spectrometer (Purkinje General Instrument Co., Ltd, Peking, China) with a negative high voltage 320 V, working electric current 3.0 mA, and wavelength 422.7 nm. All data were demonstrated with mean ± SD for statistical analysis.

Statistical analysis

The normality of distributions of the response variables was tested with the Kolmogorov–Smirnov test, and the

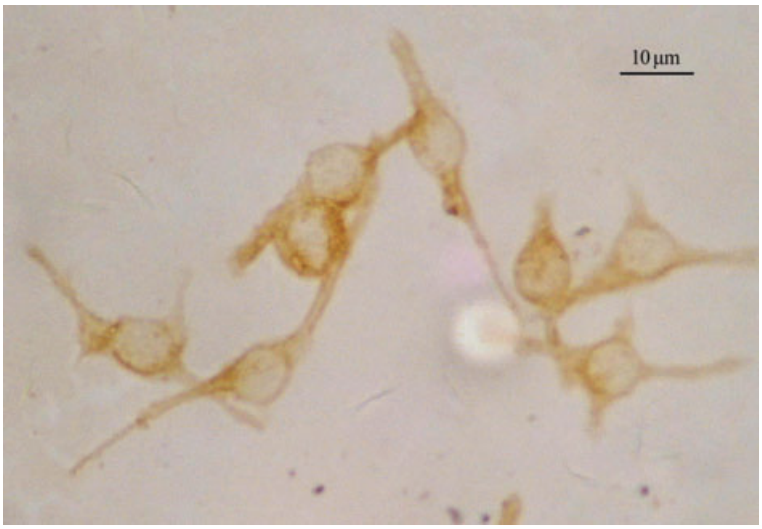


Figure 1 Figure showing positive staining of DMP1 in cytoplasm of MDPC-23 cells, and the standard appearance of MDPC-23 cells with multiple polar cell membrane process and large nuclei. SP method, ×200.

results showed that all parameters obeyed normal distribution. Paired-samples *t*-test was used for relationship comparison of ALP activities, $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_e$ concentration groups from their normal groups. Results with a $P < 0.05$ were regarded as statistically significant. SigmaStat statistical software package (SPSS10.0, Chicago, IL, USA) was used to process the data.

Results

Expression of DMP1 in MDPC-23 cells

The appearance of yellow–brown peroxidase staining in the cytoplasm or nuclei was regarded as positive immunohistochemical result. Figure 1 shows strong positive straining in the cytoplasm of the MDPC-23 cells showing morphological features of plasma cells. The control group yielded a negative immunohistochemistry result, which indicated this monoclonal antibody was specific to mouse DMP1. This demonstrated that the MDPC-23 cell line could express DMP1 stably and this protein could be regarded as one of the specific markers of MDPC-23 cells.

Western blot result

Western blot results (Fig. 2) showed that the normal and the S-ODN group cells had a specific staining band (DMP1, about 150–180 kDa, the top panel of Fig. 2a). After AS-ODN against DMP1 treatment for 12 h, the staining intensity decreased, and after 24 h this staining band could not be detected (Fig. 2b). This result indicated that the expression of DMP1 in MDPC-23 cell line was blocked absolutely after treatment for 24 h with α-MEM medium containing 10 μmol L^{-1} AS-ODN.

Result of ALP activity detection

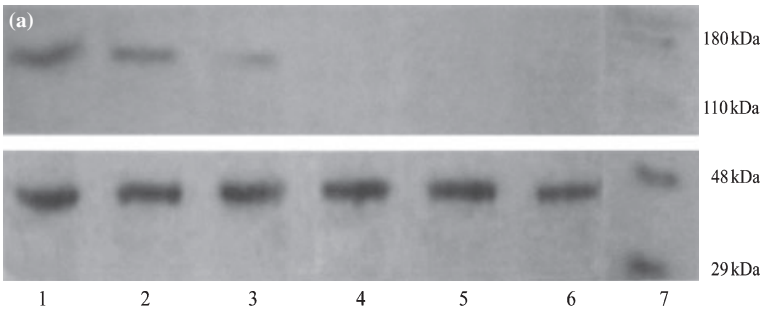
As shown in Fig. 3, ALP activities of MDPC-23 cells in the normal and S-ODN groups had no notable changes at different times. While compared with those two groups the activities in the AS-ODN group with different concentrations markedly decreased. Especially in the groups treated with 10 μmol L^{-1} AS-ODN, the differences were more distinguished.

Result of von Kossa staining and counting

The result of the von Kossa staining is described as shown in Fig. 4. Compared with the control group (Fig. 4a), the size of the mineralized nodules in the experimental group (Fig. 4b) was smaller and the degree of mineralization was weaker. The number of mineralized nodules bigger than 4 mm^2 was 231 in all tested fields of vision of the control group while only 68 in the experiment group. This demonstrated that the mineralization ability of MDPC-23 cells treated by AS-ODN against DMP1 degraded substantially.

$[Ca^{2+}]_{if}$ dynamic change in the cell

The LSCM result is shown in Figs 5 and 6, the RFV standing for $[Ca^{2+}]_{if}$ of MDPC-23 cells in the control group change a little at different times with the mean RFV 87 (±40). But in the AS-ODN group RFV rose to the maximum with the mean RFV 104 (±27) when the



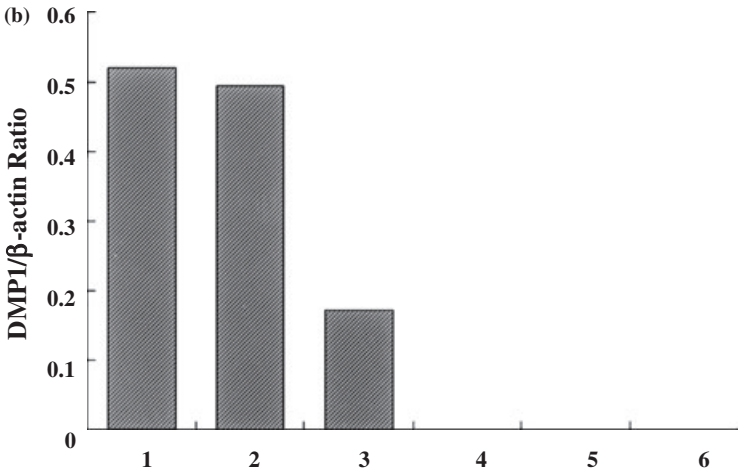


Figure 2 Western blot showing specific staining bands (DMP1 was about 150-180 kDa, the top panel of Fig.2a; β-actin was about 43 kDa the bottom panel of Fig.2a) in the normal and the S-ODN group cells. Western blot analysis showing the staining intensity became weaker when treated by AS-ODN against DMP1 for 12h and no detection after affected for 24h (Fig.2b).
1. Normal group
2. S-ODN group after affected for 12h
3-6. AS-ODN group after affected for 12,24,36,48h
7. Standard protein marker.

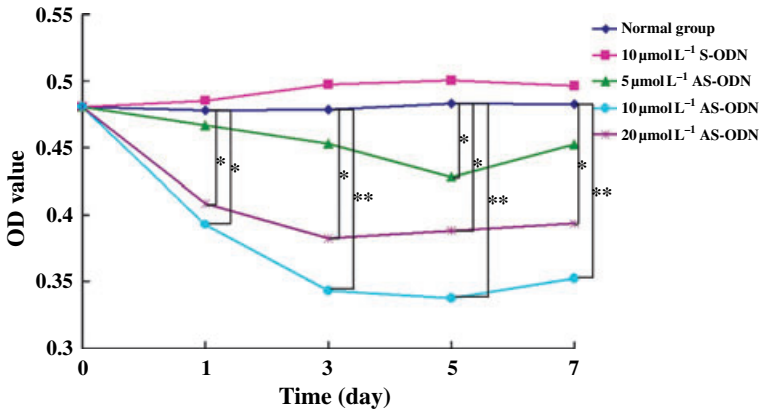


Figure 3 ALP activities markedly decreased in MDPC-23 cells treated with AS-ODN against DMP1 with different concentrations at different times (n = 64 in each group; Paired *t* test, compared with normal group, $^{*}P < 0.05$, $^{**}P < 0.01$).

cells had been treated by 10 μmol L^{-1} AS-ODN for 12 h, then it reduced to the minimum with the mean RFV 68 (±22) at 48 h. Those results indicated that $[Ca^{2+}]_{if}$ of MDPC-23 cells degraded gradually under the effect of AS-ODN against DMP1.

$[Ca^{2+}]_{it}$ and $[Ca^{2+}]_e$ dynamic change in the cell

Table 1 shows the $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_e$ concentrations in MDPC-23 cells changed little at different times in the S-ODN and normal groups. The $[Ca^{2+}]_{it}$ value was lower in the AS-ODN group than the other groups at different times. $[Ca^{2+}]_{it}$ reached the minimum value 0.14 (±0.02) mmol L^{-1} when the cells had been treated with AS-ODN for 24 h ($P < 0.01$), after that it increased slightly when treated up to 48 h, but it was still lower than in the control group. Moreover, $[Ca^{2+}]_e$ of the cells in the AS-ODN group increased before treatment for 24 h and then reduced, although it was still higher than that of the control group.

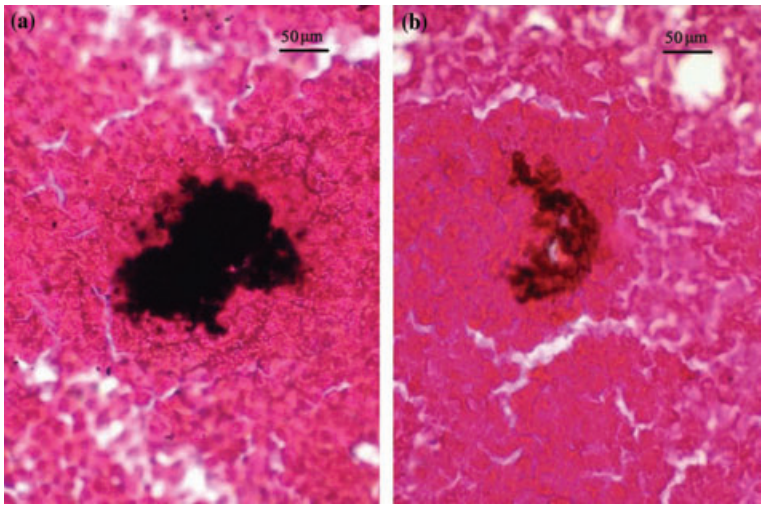


Figure 4 Figures showing the mineralized nodules in MDPC-23 cells. Compared with the control group (Fig.4a, n = 25), the size of the mineralized nodules in experiment group (Fig.4b, n = 25) was smaller and the degree of mineralization was weaker. von Kossa staining, ×40.

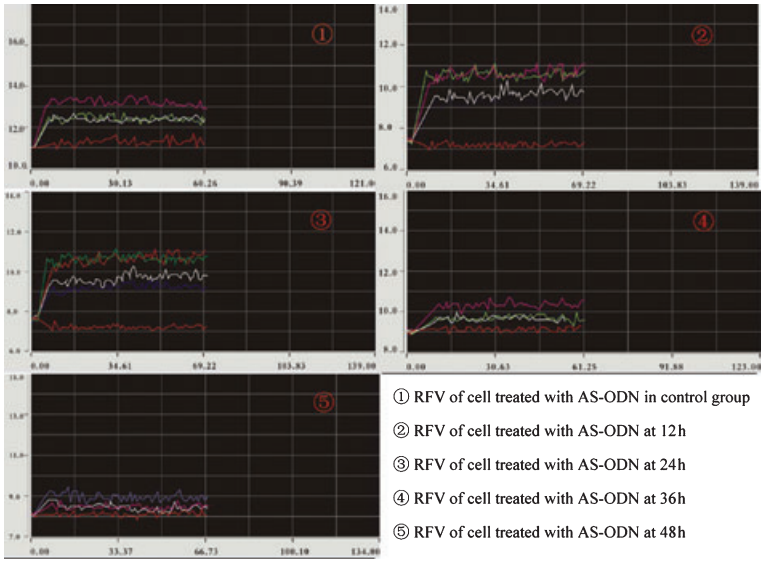


Figure 5 RFV of MDPC-23 cells treated with AS-ODN against DMP1 at different times (X-axis: Time (sec); Y-axis: Fluorescence intensity; the bottom red line: background fluorescence value; the other upper coloured lines: experimental fluorescence value).

The difference with the control group was not significant ($P = 0.08$) but there was a tendency for $[Ca^{2+}]_e$ concentrations to increase. Their dynamic changes are as shown in Figs 7 and 8.

Discussion

Dentine matrix protein 1, the first dentine-related gene to be cloned, originally cloned from rat odontoblast cDNA library, is reported as a dentine specific protein and has been believed to play important roles in dentine mineralization (George *et al.* 1993). DMP1 gene expression is not specific to dentine but observed in other tissues such as bone, ameloblasts and brain. Studies have shown that overexpression of DMP1 in pluripotent mesenchymal cells induces the odontoblast-like phenotype (Narayanan *et al.* 2001). Those results indicate that this protein probably plays an important role in odontoblast differentiation and dentine mineralization.

Odontoblast differentiation is known to be limited to mesenchymal-derived cells only (Sawada & Inoue 1998, Kikuchi *et al.* 2004). Functional differentiation of odontoblasts is characterized by synthesis of type I collagen and NCPs. This orchestration of multiple events during dentinogenesis leads odontoblasts to

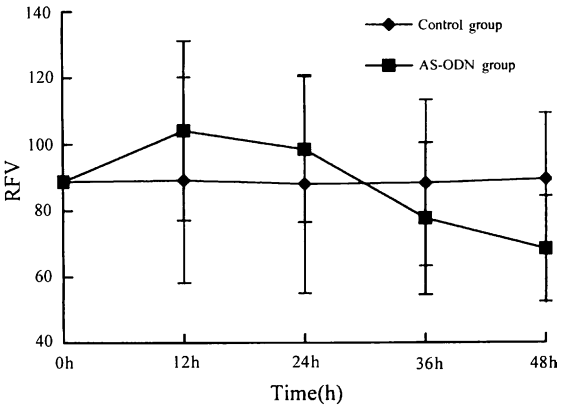


Figure 6 The dynamic change of $[Ca^{2+}]_{if}$ of MDPC-23 cells treated with 10 μmol L^{-1} AS-ODN against DMP1 at different times.

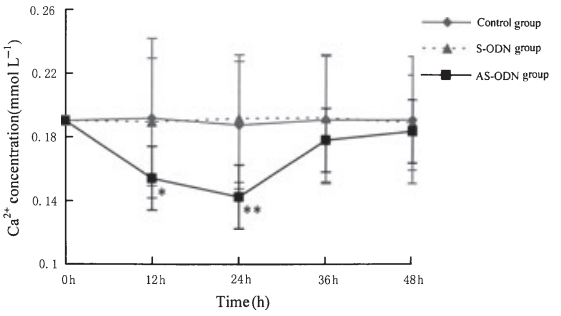


Figure 7 The dynamic change of $[Ca^{2+}]_{it}$ in MDPC-23 cells treated with 10 μmol L^{-1} AS-ODN against DMP1 at different times.

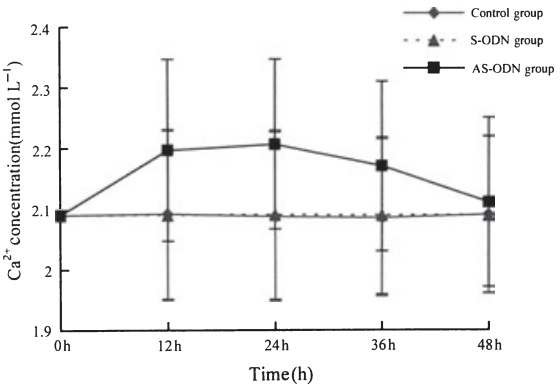


Figure 8 The dynamic change of $[Ca^{2+}]_{e}$ in MDPC-23 cells treated with 10 μmol L^{-1} AS-ODN against DMP1 at different times.

synthesize and secrete a matrix conducive to the controlled growth of HA crystals. In this study, immortalized odontoblast-like cell line MDPC-23 derived from 18 to 19 days CD-I fetal mouse molar dental papillae was used. This cell line exhibited several unique features and some were characteristics of odontoblast *in vivo*: epithelioid morphology of all cells with multiple cell membrane processes, high alkaline phosphatase activities in all cells, formation of multilayered nodules and multilayered cultures when maintained in ascorbic acid and beta-glycerophosphate, and expression of two markers for odontoblast differentiation, i.e. dentine phosphoprotein and dentine sialoprotein (Hanks *et al.* 1998, Sun *et al.* 1998). DMP1 and other extracellular matrix proteins may help to characterize genes specifying the odontoblast phenotype and the signal pathways underlying odontoblast differentiation (Priam *et al.* 2005). In the present study, this cell line expressed DMP1 in a stable manner and demonstrated a standard appearance with multiple polar cell membrane processes and large nuclei; these could be regarded as the unique phenotypic markers of odontoblast activity. Although this immortalized odontoblast-like cell line displays biological variability including growing faster and changing in phenotype over time, it is still a useful tool to study the biological function of DMP1 in dentine calcification.

The role of AS-ODN includes binding specifically to target gene and preventing the gene out of transcription and protein translation. In the study 18 bp

Table 1 The $[Ca^{2+}]_{it}$ and $[Ca^{2+}]_{e}$ in MDPC-23 cells treated with 10 μmol L^{-1} As-ODN against DMP1 at different times (Paired *t* test, compared with Control group, $^{*}P < 0.05$, $^{**}P < 0.01$)

	$[Ca^{2+}]_{it}$ (mmol L^{-1}): mean ± SD			$[Ca^{2+}]_{e}$ (mmol L^{-1}): mean ± SD		
Time (h)	Control group ($n = 40$)	S-ODN group ($n = 40$)	AS-ODN group ($n = 40$)	Control group ($n = 40$)	S-ODN group ($n = 40$)	AS-ODN group ($n = 40$)
12	0.19 ± 0.05	0.19 ± 0.04	0.15 ± 0.02*	2.09 ± 0.14	2.09 ± 0.14	2.20 ± 0.15
24	0.19 ± 0.04	0.19 ± 0.04	0.14 ± 0.02**	2.09 ± 0.14	2.09 ± 0.14	2.21 ± 0.14
36	0.19 ± 0.04	0.19 ± 0.04	0.18 ± 0.02	2.09 ± 0.13	2.09 ± 0.13	2.17 ± 0.14
48	0.19 ± 0.04	0.19 ± 0.03	0.18 ± 0.02	2.09 ± 0.13	2.09 ± 0.13	2.11 ± 0.14

AS-ODN against mouse DMP1 was designed which could inhibit coding initial segment of DMP1 efficiently by blocking leader peptide mRNA. Computer analysis showed that this sequence was not homologous with other mouse genes, and its blocking efficiency greatly increased after the sequence was phosphorothioated. The experimental result indicated that the inhibition outcome of AS-ODN against mouse DMP1 was specific to MDPC-23 cell. The staining intensity of the 150–180 kDa band became weak when the cells were treated by AS-ODN against DMP1 for 12 h, which meant AS-ODN had started working. After 24 h AS-DON treatment, the band around 150–180 kDa was not detected, which meant the expression of DMP1 in MDPC-23 cells was blocked absolutely.

During dentine mineralization, crystal nucleation and growth processes are considered to be matrix regulated. The nature of the HA crystals deposited is under the precise control of the collagen template and NCPs. Acidic proteins like osteopontin, bone sialoprotein, osteonectin, osteocalcin, bone acidic glycoprotein 75, and DMP1, expressed by both odontoblasts and osteoblasts, have been implicated to have an important role in matrix mineralization through the morphological regulation of crystals (Linde & Goldberg 1993). The unique features of DMP1 are that it is highly hydrophilic, rich in glutamic acid, aspartic acid, and serines. The serine residues are embedded in acidic sequences that make them good substrates for phosphorylation by casein kinase I and II. DMP1 could accelerate the assembly of the collagen fibrils *in vitro* and also increase the diameter of the reconstituted collagen fibrils. In mineralization studies *in vitro*, He & George (2004) demonstrated that in the presence of calcium and phosphate ions, apatite deposition was only found at the collagen-bound DMP1 sites. The exact biochemical function of DMP1 in either bone or dentine is not known, but functional motifs for phosphorylation have offered clues. It could be postulated that DMP1, because of its high acidic nature, could bind to calcium, thereby initiating the nucleation process and, furthermore, turning on the entire cascade of regulated HA crystal growth (Tartaix *et al.* 2004).

Alkaline phosphatase is a marker for odontoblast-like differentiation, because odontoblasts show much higher ALP activity than undifferentiated dental mesenchymal cells (Shiba *et al.* 2003, Nakashima *et al.* 2004). A finding in this study is that blockage of DMP1 expression could decrease ALP activity levels which was regarded as an important marker of mineralization ability in MDPC-23 cells. Subsequently an *in vitro* nodule formation assay was carried out to determine whether the lack of participation of DMP1 could affect mineralized matrix formation. Nodule formation, because of secretion of extracellular matrix proteins with the presence of phosphate ions and ascorbic acid, has been considered to precede mineralization and to be an important feature for mineralization. von Kossa staining showed that the size and degree of mineralization of the mineralized nodules decreased in the AS-ODN group compared with the control group, and the number of mineralized nodules reduced in the AS-ODN group (68 mineralized nodules in the AS-ODN group versus 231 in the control group). It was indicated that the inhibition of DMP1 expression affected the onset of mineralization in MDPC-23 cells, and the size of the nodules significantly reduced and the number decreased. Consistent with the hypothesis, the mineralization ability was weakened because of the lack of DMP1 in this cell line. Thus it seems reasonable to conclude that DMP1 could participate in the initiation of the process of dentine mineralization.

The participation of Ca^{2+} is indispensable to dentine mineralization. It is regarded as the second messenger for information transfer, the synthesis and releasing of transmitter in vital movement of a cell. Meanwhile, it is a basal component of sclerous tissues such as bone and dentine. The deposit of Ca^{2+} and onset of mineralization in dentine are precisely controlled and concerned with NCPs such as DMP1. DMP1 is predicted to bind to Ca^{2+} based on its primary structure, having a high affinity for HA. Other studies in the field had shown that recombinant DMP1 (rhDMP1) made in *Escherichia coli* without post-translational modification (Srinivasan *et al.* 1999) could nucleate HA *in vitro*. Thus it would be useful to study the change of Ca^{2+} concentration in odontoblast-like cell lines, in order to understand the mechanisms of dentine calcification. The present study showed that the inhibition of DMP1 expression in MDPC-23 cell could increase $[Ca^{2+}]_{if}$ before treatment for 24 h and reached a maximum (the mean RFV 104 ± 27) following 12 h with 10 μmol L^{-1} AS-ODN against DMP1. The level of DMP1 in the cells was reduced and the bindings of DMP1 and free Ca^{2+} were decreased, which resulted in an increase of free Ca^{2+} in the cells. After that $[Ca^{2+}]_{if}$ decreased, probably due to the compensating action of intracellular Ca^{2+} metabolism. Then $[Ca^{2+}]_{it}$ decreased to a minimum 0.14 ± 0.02 mmol L^{-1} after treatment for 24 h because of the changes of $[Ca^{2+}]_{if}$, Ca^{2+} metabolism and transportation. The Ca^{2+} released from the cells

into the extracellular culture fluid was infinitely diluted and led to the $[Ca^{2+}]_e$ of each group being similar and not significantly different ($P = 0.08$), but there was a tendency for $[Ca^{2+}]_e$ concentrations in the AS-ODN group to increase. The results indicate that DMP1 can bind Ca^{2+} and then take part in the regulation process of dentine mineralization. Little is known regarding the mechanisms by which DMP1 regulates this process. It is hoped that the present findings can help to pave the way for future studies pertaining to the identification of whether DMP1 can affect the functions of Ca^{2+} binding proteins and Ca^{2+} transport system in odontoblasts.

Conclusions

Antisense oligonucleotide against DMP1 can decrease the ALP activity and the number and size of mineralized nodules in the odontoblast-like cell line MDPC-23. AS-ODN increases $[Ca^{2+}]_{if}$ concentrations and then decreases them after treatment for 24 h, decreases $[Ca^{2+}]_{it}$ concentrations to the lowest point at 24 h and increases $[Ca^{2+}]_e$ concentrations of MDPC-23 cells compared with their control groups. In addition, this study establishes new molecular links between the metabolism and transportation of calcium ions in odontoblasts and the important dentine mineralization regulator DMP1.

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