ミトコンドリア分裂因子、ダイナミン関連タンパク質1 (Drp1) の抑制は象牙質形成を促進する

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Accelerated dentinogenesis by inhibiting the mitochondrial fission factor, dynamin related protein 1

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ABSTRACT

Undifferentiated odontogenic epithelium and dental papilla cells differentiate into ameloblasts and odontoblasts, respectively, both of which are essential for tooth development. These differentiation processes involve dramatic functional and morphological changes of the cells. For these changes to occur, activation of mitochondrial functions, including ATP production, is extremely important. In addition, these changes are closely related to mitochondrial fission and fusion, known as mitochondrial dynamics. However, few studies have focused on the role of mitochondrial dynamics in tooth development. The purpose of this study was to clarify this role. We used mouse tooth germ organ cultures and a mouse dental papilla cell line with the ability to differentiate into odontoblasts, in combination with knockdown of the mitochondrial fission factor, dynamin related protein (DRP)1. In organ cultures of the mouse first molar, tooth germ developed to the early bell stage. The amount of dentin formed under DRP1 inhibition was significantly larger than that of the control. In experiments using a mouse dental papilla cell line, differentiation into odontoblasts was enhanced by inhibiting DRP1. This was associated with increased mitochondrial elongation and ATP production compared to the control. These results suggest that DRP1 inhibition accelerates dentin formation through mitochondrial elongation and activation. This raises the possibility that DRP1 might be a therapeutic target for developmental disorders of teeth.

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1. Introduction

Mitochondria have various roles including ATP production, calcium metabolism, apoptosis regulation, and redox homeostasis. Mitochondria continue to divide and fuse throughout the life of a cell. The balance between division and fusion, known as mitochondrial dynamics, is closely linked to various cellular functions [1]. In mammals, dynamin related protein (DRP)1 is a major mitochondrial division factor, and MFN-1/2, and OPA1 are known as major fusion factors [2].

Because mitochondrial dynamics is involved in mitochondrial functions, its dysregulation is associated with various diseases [3]. Mitochondrial dynamics-related diseases are caused by both excessive fusion and excessive division of mitochondria. We have

shown that neuronal development and synapse formation are severely impaired due to excessive fusion and elongation of mitochondria in brain-specific DRP1-deficient mice [4]. Other groups have shown that mutations of genes encoding OPA1 and MFN2 are responsible for dominant optic atrophy and Charcot-Marie-Tooth neuropathy type 2A, respectively [5,6].

During tooth formation, ameloblasts differentiate from the odontogenic epithelium and form enamel [7]. Odontoblasts differentiate from undifferentiated dental papilla cells to form dentin [8]. Genes encoding signal transduction factors are involved in these processes, and mutations of these genes cause many kinds of tooth malformations [9–11]. In addition, mitochondria play an important role in functional differentiation of ameloblasts and odontoblasts [7,8]. However, the contribution of mitochondrial dynamics to odontogenesis has not been defined.

The purpose of this study was to clarify how mitochondrial dynamics is involved in odontogenesis by focusing on DRP1. It was difficult to analyze odontogenesis in DRP1 null mice because the

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embryos die by E10.5 due to severe developmental defects [4]. In this study, we used an organ culture system of mouse tooth germ and a mouse dental papilla cell line (mDP^{E6}) that can differentiate into odontoblasts, in combination with DRP1 gene knockdown technology. We found that the inhibition of DRP1 accelerated odontoblast differentiation and dentin formation.

2. Materials and methods

2.1. Organ culture and tissue preparation

Embryos were collected from C57BL/6Jcl female mice (CLEA Japan, Tokyo, Japan) at 18.5 days of gestation. Tooth germs of the lower first molars were dissected from mandibles of E18.5 embryos. Organ culture was performed according to the protocol of a previous study [12]. Briefly, dissected mandibles and tooth germs were cultured for eight days on nitrocellulose membranes (0.8 µm pore size, EMD Millipore, Hayward, CA, USA) in 24-well plates in BGJb medium (Thermo Fisher, Rockford, IL, USA) supplemented with 10 µg/mL ascorbic acid (Nakarai Tesque, Kyoto, Japan), 5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 units/mL penicillin (Thermo Fisher), and 100 µg/mL streptomycin (Thermo Fisher) in a humidified atmosphere of 5% CO₂ at 37 °C. All animal experiments were reviewed and approved by the animal ethics committee of Kyushu University and were conducted in accordance with Kyushu University guidelines.

Tissue preparation was according to the protocol of a previous study [12]. Briefly, cultured organs were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) for 12 h at 4 °C, followed by embedding in paraffin wax. Serial sections were cut at 5 µm thick in the anterior-posterior direction to obtain the largest part of the tooth germ, and were stained with hematoxylin and eosin as described previously [12].

2.2. Culture of a mouse dental papilla cell line

The mDP^{E6} mouse dentate papilla cell line was used for analysis of differentiation into odontoblasts as described previously [13]. Briefly, mDP^{E6} cells were cultured in alpha minimum essential medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 units/mL penicillin (Thermo Fisher), and 100 µg/mL streptomycin (Thermo Fisher) in a humidified atmosphere of 5% CO₂ at 37 °C. Differentiation into odontoblasts was induced by culturing for five days in the presence of 10 nM dexamethasone (Sigma-Aldrich), 1.8 mM potassium hydrogen phosphate (Nakarai Tesque), 100 µM ascorbic acid, and 10 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA).

2.3. Inhibition of DRP1

In organ culture, we used an antisense oligodeoxynucleotide (5'-GCGCTCCATGACCCCGAA-3') against mouse DRP1 mRNA, and a corresponding sense oligodeoxynucleotide (5'-TTCGGGGGTCATGGAGGCGC-3') as the control. These oligodeoxynucleotides were transfected using an HVJ Envelope Transfection kit (GenomeONE™-Neo, Ishihara Sangyo, Osaka, Japan) according to the manufacturer's protocol. The culture medium containing oligodeoxynucleotides was changed every 24 h as described previously [12].

In cell culture, mDP^{E6} cells were seeded in 6-well plates at 9.6×10^3 cells per well. After 24 h, ON-TARGETplus Mouse Dnm11 siRNA (No. 74006; GE Healthcare, Buckinghamshire, UK) against DRP1 mRNA, and ON-TARGETplus non-targeting siRNA (#1; GE Healthcare) as control siRNA were transfected using Lipofectamine RNAi Max (Thermo Fisher). After 48 h, siRNA against DRP1 mRNA

and control siRNA were transfected and cultured for a further 72 h.

2.4. Analysis of predentin formation by tooth germ in organ culture

Two different 50 µm wide areas including predentin were selected around the starting point of dentin formation in each tooth germ. This was followed by measuring the areas of predentin using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The average values of four different areas were calculated in the antisense DRP1 and control groups.

2.5. Immunohistochemical staining of DRP1 in tooth germs in organ culture

For immunohistochemical analysis of DRP1 expression in cultured tooth germs, we used monoclonal mouse anti-DRP1 (clone: 8/DLP1, BD Biosciences, NJ, USA) antibody according to the manufacturer's protocol (EnVision + HRP kit, Agilent, Santa Clara, CA, USA). After blocking peroxidases, the primary antibody were added for 60 min at room temperature followed by reaction with the horseradish peroxidase (HRP)-labeled secondary antibodies for 30 min. 3,3'-Diaminobenzidine was applied to detect a positive signal and samples were counterstained with hematoxylin.

2.6. Western blot analysis

mDP^{E6} cells were washed three times with phosphate-buffered saline, suspended in Laemmli sample buffer (62.5 mM Tris HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, and 10% glycerol) and incubated at 95 °C for 10 min for whole cell lysate preparation. Protein concentrations were quantified using the BradfordUltra assay (Expedeon, Cambridge, UK). Whole cell lysates containing 10 µg protein were separated by SDS polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore). For detecting DRP1, monoclonal mouse anti-DRP1 (clone: 8/DLP1, BD Biosciences) and HRP-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as primary and secondary antibodies, respectively. For control, primary rabbit anti-Hsp90 and secondary HRP-labeled goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology) were used. The primary and secondary antibodies were added for 60 min at room temperature, followed by membrane development with the ECL Prime kit (GE Healthcare). Immunoreactive bands were analyzed using LAS-1000 Pro (Fuji-film, Tokyo, Japan). The relative expression level of DRP1 was calculated using the expression level of Hsp90 as an internal control.

2.7. Analysis of mitochondrial morphology

After induction of odontoblast differentiation, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, followed by permeabilization for 5 min with 0.1% Triton-X 100 (Nakarai Tesque). A rabbit anti-Tom20 antibody (Santa Cruz Biotechnology) was used as the primary antibody at room temperature for 90 min, and an Alexa Fluor 594-labeled goat anti-rabbit IgG antibody (Thermo Fisher) was reacted as the secondary antibody at room temperature for 60 min. Fluorescent images were captured with an Axio Imager M2 using Apotome 2 (Zeiss, Oberkochen, Germany).

2.8. RNA extraction and mRNA expression analysis

Total RNA was recovered using the RNeasy Mini kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized using the

Super Script IV VILO Master Mix (Thermo Fisher) according to the manufacturer's instructions. For the quantitative polymerase chain reaction (qPCR), specific primers for dentin matrix protein (DMP)-1 mRNA (forward: 5'-ACCACAATACTGAATCTGAAAGCTC-3', and reverse: 5'-TGCTGTCCGTGTGGTCACTA-3') and 18s rRNA (forward: 5'-CGGCTACCACATCCAAGGAA-3', and reverse: 5'-GCTGGAAT-TACCGCGGCT-3') were used. qPCR reactions were analyzed on an Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher) using GoTaq qPCR Master Mix (Promega, Madison, WI, USA) for 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The relative expression level of Dmp1 mRNA was calculated using the expression level of 18s rRNA as the internal control.

2.9. Analysis of dentin sialophosphoprotein (DSPP) secretion

After induction of odontoblast differentiation of mDP^{E6} cells, culture medium was collected and the amount of DSPP was analyzed using an enzyme-linked immunoassay kit (Cusabio, Wuhan, China) and Infinite 200 plate reader (Tecan, Männedorf, Switzerland).

2.10. Measurement of ATP levels

After induction of odontoblast differentiation, cells were recovered by enzymatic treatment with TrypLE Express (Thermo Fisher). The number of cells was counted with a hemocytometer and the amount of ATP in 2.5×10^3 cells was measured using CellTiter-Glo (Promega) and an Infinite 200 plate reader.

2.11. Statistical analyses

Statistical analyses were performed by Student's t-test. P values less than 0.05 were considered to be significant.

3. Results

3.1. Development of tooth germ in mouse embryo-derived organ cultures under DRP1 inhibition

In organ cultures of the first molar tooth germ derived from E18.5 embryos, the tooth germ grew up to the early bell stage, and ameloblasts showed columnar morphology and nuclear

Fig. 1. Analysis of mouse tooth germ using organ culture under dynamin related protein (DRP)1 inhibition.

A) Morphology of tooth germ under DRP1 inhibition (hematoxylin and eosin staining). a, b) control and c, d) DRP1 inhibition (antisense (AS)-DRP1). Scale bars show 100 μm. Magnified views of frames 1 and 2 in panel a, 3 and 4 in panel b, 1 and 2 in panel c, and 3 and 4 in panel d are shown along the right side. Black arrows: ameloblasts; asterisks: predentin; white arrows: odontoblasts.

B) The areas of predentin in two places in two different tooth germ tissues (total of four different areas) of control and DRP1 inhibition (AS-DRP1) were measured (black boxes). Scale bars show 100 μm. Quantitative analysis of the amounts of predentin. Data are expressed as means ± SD. *P < 0.05.

polarization in both control and DRP1 inhibited cultures on the eighth day (Fig. 1Aa ~ d, black arrows). Enamel formation was not observed.

In dentinogenesis, in addition to columnar morphology and nuclear polarization of odontoblasts, predentin formation was observed in both control and DRP1 inhibited cultures (Fig. 1Aa ~ d, white arrows and asterisks). Quantitative analysis showed that the amount of predentin was increased significantly in DRP1 inhibited compared to control cultures (Fig. 1B). Immunohistochemical analysis showed that the expression of DRP1 in organ cultures was reduced by DRP1 antisense (Fig. 2). These results indicate that differentiation of undifferentiated dental papilla cells to odontoblasts, and dentin formation, are enhanced by inhibiting DRP1.

3.2. Effect of DRP1 siRNA in mDP^{E6} cells

To further analyze the effect of inhibiting DRP1 on odontoblast differentiation, we used mDP^{E6} cells that have the ability to differentiate into odontoblasts. Expression of DRP1 protein was downregulated significantly in the presence of DRP1 siRNA (Fig. 3A and B). Microscopically, mitochondria showed elongation and a tubular shape in DRP1 inhibited compared to control cells (Fig. 3C–H). These results show that decreased expression of DRP1 protein by siRNA is associated with mitochondrial elongation in mDP^{E6} cells.

3.3. Differentiation of mDP^{E6} cells into odontoblasts under DRP1 inhibition

DSPP and DMP-1 are dentin matrix proteins produced and secreted by odontoblasts, and are also used as differentiation markers of these cells. We analyzed the differentiation potential of mDP^{E6} cells into odontoblasts by measuring these molecules under DRP1 inhibition. Expression of DMP-1 mRNA (Fig. 4A) and the amount of secreted DSPP protein (Fig. 4B) were increased significantly under DRP1 inhibition. Similar to observations in the mouse tooth germ organ culture, these results suggest that inhibiting DRP1 accelerates odontoblast differentiation and dentin formation.

Fig. 2. Immunohistochemical staining of dynamin related protein (DRP)1 of tooth germs in organ culture.

Tissues were exposed to mouse anti-DRP1 antibody. Diaminobenzidine was applied to detect a positive signal and samples were counterstained with hematoxylin. The results show that the expression of DRP1 in organ cultures is reduced by DRP1 antisense (AS-DRP1). Magnified views of frames in upper panels are shown in lower panels.

3.4. ATP production under DRP1 inhibition

In order to investigate the relationship between the activity of mitochondria and the differentiation potential of mDP^{E6} cells to odontoblasts under DRP1 inhibition, the amount of ATP produced was measured. ATP production by mDP^{E6} cells was increased significantly under DRP1 inhibition compared to control cells (Fig. 4C). Mitochondria are the most important source of ATP in cells and ATP production correlates directly with mitochondrial activity. It is likely, therefore, that the acceleration of odontoblast differentiation and dentin formation caused by inhibiting DRP1 are associated with enhanced mitochondrial activity and ATP production.

4. Discussion

The purpose of this study was to clarify the involvement of mitochondrial dynamics in tooth formation by focusing on DRP1 which is essential for mitochondrial division. We found that differentiation into odontoblasts and dentin formation were accelerated by mitochondrial fusion in response to inhibition of DRP1. These results also suggest that DRP1 may be a therapeutic target for developmental disorders of teeth.

Mitochondrial activity increases during the differentiation of various stem cells [14,15]. We showed previously that dihydroorotate dehydrogenase, which interacts with the mitochondrial respiratory chain, is important for the differentiation of an osteoblast precursor cell line into osteoblasts [16]. In addition, we showed that the differentiation of stem cells from human exfoliated deciduous teeth into osteoblasts is attenuated in a patient with Leigh encephalopathy, a syndrome in which ATP production is decreased due to a mitochondrial respiratory chain abnormality [17].

Differentiation from stem cells to somatic cells involves complicated changes in cell morphology and acquisition of various functions, requiring more energy and metabolic conversion. More specifically, differentiation of stem cells into odontoblasts is associated with dramatic morphological and structural changes for dentin formation, such as cell elongation, nuclear polarization, and organelle localization. Therefore, it is important to activate various mitochondrial functions such as increasing the supply of ATP and metabolic regulation, including calcium [18–20].

In the current study, focusing on odontogenesis, we showed that inhibiting DRP1 promoted differentiation of undifferentiated dentate papilla cells to odontoblasts. This change was associated with mitochondrial fusion and increased ATP production. Mitochondrial activation and an increase of ATP production are correlated with mitochondrial elongation [21,22]. Thus, mitochondrial dynamics play an important role in controlling the mitochondrial activity required for cell differentiation. Our results suggest that these changes are accelerated by inhibiting DRP1 through elongated and activated mitochondria. Further analysis of odontoblast ultrastructure will be required to confirm this hypothesis.

In the current study, we showed that the secretion of DSPP from odontoblasts was increased due to DRP1 inhibition. DSPP is a matrix protein synthesized and secreted by odontoblasts, and is essential for dentin formation [23]. Matrix proteins are secreted generally by exocytosis. In various kinds of cells, exocytosis occurs via a common mechanism involving the SNARE family of proteins, calcium, and ATP [24–27]. Mitochondria have an important role in the process of exocytosis. For example, in pancreatic acinar cells, mitochondria are involved in the secretion of digestive enzymes through the metabolic regulation of calcium and ATP [28]. Odontoblasts express SNAP-25, one of the SNARE proteins [29]. Therefore, in odontoblasts, increased mitochondrial fusion and activity due to DRP1 inhibition may be related to the increased secretion of

Fig. 3. Effect of DRP1 siRNA on DRP1 protein expression and mitochondrial morphology of cultured mDP^{E6} cells.

A) Western blot analysis for DRP1. B) Quantitative analysis of immunoreactive bands. C–H) Mitochondrial morphology: C, D, E) control; F, G, H) DRP1 inhibition by siRNA (si-DRP1). D) Magnified view of a frame in panel C. E) Magnified view of a frame in panel D. G) Magnified view of a frame in panel F. H) Magnified view of a frame in panel G.

Fig. 4. Effect of DRP1 siRNA on the induction of odontoblast differentiation of cultured mDP^{E6} cells.

A) Relative expression levels of dentin matrix protein (DMP)-1 mRNA. B) Relative amounts of dentin sialophosphoprotein (DSPP) secreted by cultured mDP^{E6} cells. C) Relative amounts of ATP in cultured mDP^{E6} cells. Data are expressed as means ± SD from three experiments. *P < 0.05.

DSPP. This result is similar to a previous report in osteoblasts, that have the same mesenchymal lineage as odontoblasts, that osteoblast functions including secretion are improved by mitochondrial fusion in response to DRP1 inhibition under oxidative stress [30].

Mineralization of the dentin matrix is indispensable for the maturation of dentin. This process involves calcium vesicles secreted by odontoblasts. The mechanism is similar to the mineralization mechanism in osteogenesis. Recently, using stem cells from human exfoliated deciduous teeth derived from a Leigh encephalopathy patient, we showed that mineralization of bone matrix is linked to the regulation of calcium metabolism associated with osteoblast mitochondrial activity [17]. Overall, the secretion of dentin matrix proteins and mineralization of dentin matrix may be accelerated by mitochondrial elongation and activation during dentin formation.

Mitochondrial dysfunction and hyper-fission have been found in various damaged cells [31,32]. These changes are ameliorated by inhibiting division and promoting mitochondrial fusion [33]. Amelogenesis imperfecta and dentinogenesis imperfecta are the most common tooth anomalies. The mutation of many genes encoding various signaling molecules has been found in these tooth malformations. These anomalies are also caused by maternal factors during pregnancy and as a result of side effects of various drugs [34,35]. Treatment and preventive methods for these developmental tooth anomalies from the fetal to childhood periods have not yet been developed. In the current study, we did not evaluate enamel formation by DRP1 inhibition. However, it was shown that mitochondria contribute to differentiation into ameloblasts and protein secretion from these cells [36]. Therefore, it is possible that mitochondrial dynamics may be involved in amelogenesis.

Although it is necessary to verify this hypothesis by further experiments, our study raises the possibility that DRP1, or other regulators of mitochondrial dynamics, may be a new therapeutic target for treating developmental tooth defects.

Conflicts of interest

The authors have no conflicts of interest to declare.

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