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Research Article

Polyphosphate induces matrix metalloproteinase-3-mediated proliferation of odontoblast-like cells derived from induced pluripotent stem cells

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ABSTRACT

Inorganic polyphosphate [Poly(P)] may represent a physiological source of phosphate and has the ability to induce bone differentiation in osteoblasts. We previously reported that cytokine-induced matrix metalloproteinase (MMP)-3 accelerates the proliferation of purified odontoblast-like cells. In this study, MMP-3 small interfering RNA (siRNA) was transfected into odontoblast-like cells derived from induced pluripotent stem cells to investigate whether MMP-3 activity is induced by Poly(P) and/or is associated with cell proliferation and differentiation into odontoblast-like cells. Treatment with Poly(P) led to an increase in both cell proliferation and additional odontoblastic differentiation. Poly(P)-treated cells showed a small but significant increase in dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) mRNA expression, which are markers of mature odontoblasts. The cells also acquired additional odontoblast-specific properties including adoption of an odontoblastic phenotype typified by high alkaline phosphatase (ALP) activity and a calcification capacity. In addition, Poly(P) induced expression of MMP-3 mRNA and protein, and increased MMP-3 activity. MMP-3 siRNA-mediated disruption of the expression of these effectors potently suppressed the expression of odontoblastic biomarkers ALP, DSPP, and DMP-1, and blocked calcification. Interestingly, upon siRNA-mediated silencing of MMP-3, we noted a potent and significant decrease in cell proliferation. Using specific siRNAs, we revealed that a unique signaling cascade, Poly(P)→MMP-3→DSPP and/or DMP-1, was intimately involved in the proliferation of odontoblast-like cells.

Introduction

Inorganic polyphosphate [Poly(P)] is a linear polymer containing tens to hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds. In mammals, Poly(P) is located in erythrocytes and cells of the brain, heart, lung, and liver [1–4]. Poly(P) is known to promote intracellular calcification [5]. In addition to induction of alkaline phosphatase (ALP) activity in mouse osteoblastic MC3T3-E1 cells, Poly(P) up-regulates osteoblastic marker genes such as osteopontin (OP) and osteocalcin

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(OC) [6]. Therefore, Poly(P) is thought to play an important role in the maturation of bone-related immature cells, which might lead to the construction of bone tissues by osteoblasts. However, little is known about the functions of Poly(P) in other bone-related cells such as odontoblasts.

Matrix metalloproteinase (MMP)-3 is a protease capable of degrading the extracellular matrix (ECM) that contains proteoglycans, fibronectin, laminin, and type IV collagen, and activating procollagenase [7,8]. It is produced together with interstitial collagenase (MMP-1) by fibroblasts in response to increased levels of cytokines, including interleukin (IL)-1 and tumor necrosis factor, in inflammatory diseases such as dental pulp injury, periodontitis, and rheumatoid arthritis [9,10]. MMP-3 has been linked to joint and soft tissue destruction associated with these diseases by participating in inflammation [11–15]. Synthesis of MMP-3 is tightly controlled in vivo [14,16]. Although it is intuitive that dental pulp destruction may be a function of MMPs, our previous study reported that proinflammatory cytokine-induced MMP-3 actually accelerates wound healing of dental pulp injuries [17–19] and promotes the proliferation of odontoblastic cells derived from mouse embryonic stem (ES) and induced pluripotent stem (iPS) cells [18,20,21].

Several studies have examined the ability of dental pulp stem cells to regenerate dentin [22,23], and stem cell injection has been proposed as a putative replacement for traditional dental cavity treatment and pulp capping methods. In addition to blood vessels and nerves, odontoblasts are a predominant and unique component of dental pulp tissue [24] and might represent a novel therapeutic target for treatment of pulpitis. However, the specific effects of Poly(P) regulators have not been well defined in odontoblasts.

In addition to our previous studies [17–19], other reports have suggested the involvement of Poly(P) in apoptosis and modulation of the mineralization process in bone tissue [25,26]. Therefore, we speculated that Poly(P) has a unique physiological effect on odontoblast-like cells. Based on this hypothesis, we first studied the effect of Poly(P) on the cell proliferation of purified odontoblasts in vitro. We used an MMP-3 small interfering RNA (siRNA) to examine whether induction of MMP-3 by Poly(P) was associated with the proliferation of odontoblast-like cells derived from iPS cells (iPS-ODs). As a result, we revealed novel Poly(P) functions in the modulation of MMP-3 activity, leading to a potent increase in cell proliferation and further differentiation into odontoblastic cells.

Materials and methods

Materials

Type 65 Poly(P) with an average chain length of 65 phosphate residues was prepared from sodium tripolyphosphate (Taihei Chemical Industrial Co., Ltd., Osaka, Japan). Concentrations of Poly(P) are described in terms of phosphate residues [27]. We dissolved 20 g sodium tripolyphosphate in 200 mL distilled water, added 32 mL of 96% ethanol, and collected the precipitates as Poly (P). As a control, sodium phosphate buffer (pH 6.9) was used instead of Poly(P).

Cell culture

The mouse iPS cell line iPS-MEF-Ng-20D-17 [28] was a kind gift from Prof. Shinya Yamanaka (Center for iPS cell Research and Application, Kyoto University, Kyoto, Japan) and maintained as described previously [28,29]. Rat odontoblast-like cells (KN-3 [30]; kindly provided by Dr. Chiaki Kitamura, Kyushu Dental College, Kitakyushu, Japan) were maintained as described previously [30] and used as an authentic control. Purified iPS-ODs were prepared as reported previously [31].

A monoclonal anti-α2 integrin antibody is known to potently suppress the expression of odontoblastic markers in these culture systems. We have confirmed that α2 integrin expression in iPS cells triggers their differentiation into iPS-ODs [31]. The proportion of α2 integrin-positive cells in the total population of differentiated iPS-ODs is a measure of the purity of iPS-OD preparations. Flow cytometric analysis estimated the proportion of α2 integrin-positive cells to be $98.64 \pm 0.54\%$ ($n=3$). Up to day 21 of culture, iPS-ODs displayed odontoblast-like physiological characteristics including a calcification activity and ALP activation. For Poly(P) treatment, the culture medium was replaced with alpha-minimal essential medium supplemented with 10% fetal bovine serum and Poly(P). The cells were then cultured for 7 days. Culture medium was changed every 3 days.

Functional assay for assessment of the odontogenic phenotype

To assess the phenotype of cultured cells, we measured ALP activity and calcification as markers of differentiation. ALP activity was determined using an ALP Staining Kit (Primary Cell Co., Ltd., Hokkaido, Japan). Mineralization of Poly(P)-treated cells was quantified using Alizarin red S (ARS) (Sigma-Aldrich). ARS staining was observed and photographed under a BZ-9000 microscope (Keyence, Osaka, Japan) and quantified by a previously reported method [32].

Real-time quantitative polymerase chain reaction (qPCR) analysis

qPCR was performed in triplicate with approximately 25 ng RNA, 0.25 mL Quantitect RT Mix (Qiagen Inc., Valencia, CA, USA), 1.25 mL of 20× Primer/Probe Mix (Assays-on-demand™; Applied Biosystems, Carlsbad, CA, USA), and 12.5 μL Mastermix (Quantitect RT-PCR kit; Qiagen) in a 25 μL reaction volume. The following primer/probe sets were used: mouse alkaline phosphatase [*ALPL*]; Mm00475834_m1, human osteocalcin [*BGLAP*] (mouse available); Hs01587814_g1, mouse osteopontin [*SPP1*]; Mm00436767_m1, mouse dentin sialophosphoprotein [*DSPP*]; Mm00515667_g1, mouse dentin matrix protein-1 [*DMP-1*]; Mm01208363_m1, mouse MMP-3; Mm00440295_m1, human *MMP-1* (mouse available); Hs00899658_m1, mouse *MMP-2*; Mm00439498_m1, mouse *MMP-9*; Mm00442991_m1, mouse *MMP-13* Mm00439491_m1, rat alkaline phosphatase [*ALPL*]; Rn00575319_g1, rat osteocalcin [*BGLAP*]; Rn00566386_g1, rat osteopontin [*SPP1*]; Rn01449972_m1, rat dentin sialophosphoprotein [*DSPP*]; Rn02132391_s1, rat dentin matrix protein-1 [*DMP-1*]; Rn01450120_m1, rat MMP-3; Rn00591740_m1, human *MMP-1* (rat available); Hs00899658_m1, rat *MMP-2*; Rn01538170_m1, rat *MMP-9*; Rn00579162_m1, rat *MMP-13*; Rn01448194_m1. Thermal cycling conditions were 30 min at 50 °C, 15 min at 95 °C, and then 40 cycles of 15 s at

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