ORIGINAL

Soluble matrix from osteoblastic cells induces mineralization by dental pulp cells

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Abstract : Dental pulp cells have a capacity to differentiate into mineralization-inducing cells. To clarify the molecular mechanism, we established an *in vitro* mineralization-inducing system by rat clonal dental pulp cell line, RPC-C2A, and tried to purify a mineralization-inducing factor in conditioned medium (CM) from preosteoblastic MC3T3-E1 cells. The active factor was impermeable to an ultrafiltrating membrane, and sedimented by ultra-centrifugation. The sedimented factor was found as a needle-like structure about 1.3 μm in average length as observed by transmission electron microscopy. The factor contained type I collagen, suggesting not a matrix vesicle, but a soluble matrix. The mineralization-inducing activity was also detected in CM from primary culture of rat calvaria (RC) cells. These results suggested that the soluble matrices from osteoblastic cells serve, at least in part, as differentiation-inducing agents. J. Med. Invest. 53 : 297-302, August, 2006

Keywords : *dental pulp cells ; osteoblast ; extracellular matrix ; mineralization*

INTRODUCTION

Although both dental pulp and dentin originate from mesenchymal dental papilla, the former remains unmineralized while the latter is highly mineralized (1). Dental pulp cells are responsible for homeostasis of dental mineralized tissue. For example, pulp tissue forms reparative dentin in protective response to external stimuli including caries, chemicals, and trauma (2, 3). In addition, the size of the dental pulp cavity is reduced as a result of peritubular dentin deposition with advancing age (4). On the other hand, the incidence of pulp stone formation that is also a mineralized product is not age-dependent (5). The regulatory mechanisms of

both mineralization-inducing activity and maintaining activity in the unmineralized state remain to be clarified.

Matrix mineralization is observed in primary culture of pulp cells. In contrast, clonal pulp cells do not easily produce mineralized nodules even though osteogenic agents such as bone morphogenetic proteins (BMPs) are added to the culture as shown previously (6). On the other hand, mouse calvaria-derived preosteoblastic cell line MC3T3-E1 not only forms mineralized nodules steadily (7) but also secretes a variety of cytokines including BMP-2 and BMP-4 (8, 9). No other established cell line induces matrix mineralization easily. In addition, conditioned medium (CM) from MC3T3-

E1 cells induce mineralization by clonal rat dental pulp cells, whereas CM from rat osteosarcoma cells ROS 17/2.8, normal human articular chondrocytes, or normal human dermal fibroblasts do not induce mineralization by them *in vitro* ((6), and unpublished observation by Ueno *et al*.). There are several diverse mechanisms of mineral deposition during the formation of hard tissues. Acellular mineralization in biodegradable three-dimensional structures occurs in cell-free culture media (10) as well as at the implantation sites (11) and in the early stage of cementum formation in molars (12). On the other hand, it is generally accepted that cellular mineralization is initiated by release of matrix vesicles from living cells and by apoptotic bodies from dying cells (13, 14). It is well known that matrix vesicles contain actin, annexins II and V, carbonic anhydrase, phosphatases (pyrophosphatase, alkaline phosphatase (ALP), 5′ AMPase, Ca-ATPase), proteoglycan link protein, type X collagen, calbindin, lactate dehydrogenase, membrane-derived phospholipids, but not type I collagen (13, 14). In contrast, the constituents of media vesicles and soluble matrix remain unknown. However, vesicle-mediated mineralization is not observed in many invertebrates and enamel tissues (15, 16). Thus the mechanism of initiation of matrix mineral deposition still remains to be determined.

In this study to investigate both mineralization-inducing and maintaining mechanisms at a molecular level, we partially purified and characterized the active substance from CM of preosteoblastic MC3T3-E1 cells, applying von Kossa staining as well as performing the transmission electron microscopic analysis.

MATERIALS AND METHODS

RT-PCR analysis

Total RNA was isolated with TRI REAGENT (Molecular Research Center) from RDP4-1, RPC-C2A, AtT20, Balb3T3, and C3H10T1/2 cells, reverse transcribed with oligo(dT), and subjected to PCR within the log-linear range of amplification for either ALP, BMP-2, BMP-4 (30 cycles), or GAPDH (20 cycles). *ALP*, *BMP-2*, *BMP-4*, and *GAPDH* - specific primers were as follows : ALP forward, 5′-CTGGACCTCATCAGGATTTG-3′ ; ALP reverse, 5′-GCTGTGAAGGGCTTCTTGTC-3′; BMP-2 forward, 5′ TGAACACAGCTGGTCTCAGG - 3′; BMP-2 reverse, 5′-GCTAAGCTCAGTGGGGACAC-3′; BMP-4 forward,

5′-CAGAGCCAACACTGTGAGG - 3′; BMP-4 reverse, 5′-TCCACTCCCTTGAGGTAACG-3′; GAPDH forward, 5′-CATTGACCTCAACTACATGG-3′; GAPDH reverse, 5′-CTCAGTGTAGCCCAGGATGC-3′.

Cell culture

Confluent RPC-C2A cells and RDP 4-1 cells were cultured for 10 days in α-MEM medium supplemented with 0.22%NaHCO₃, antibiotics, 10%FBS, 2mMβ-glycerophsphate, 50μg/ml L-ascorbic acid, and 10^8M dexamethasone with or without 50%CM as described previously (6). The cells were subjected to phase-contrast microscopy in a Nikon TM-D microscope (Nikon).

Transmission electron micrographs of sedimented active matrices

Culture fluid from day 1 to 4 after confluency was centrifuged to remove cells and cell debris for 5 min at 800 xg and CM was collected from the supernatant. After the CM was centrifuged for 30 min at 10,000 xg, the supernatant was subjected to ultracentrifugation for 3h at 150,000 xg in a Beckman TLA 100.3 rotor. For transmission electron microscopy, the fixed and ethanol-dehydrated pellets were embedded in Epon 812 (TAAB Laboratories) and sections were cut on an LKB Ultratome V (LKB). Ultrathin sections were double-stained with uranyl acetate in 50% ethanol and Reynolds solution and examined in a Hitachi H-800 electron microscope (Hitachi).

Western blot analysis

Samples (40μg) were separated by 7.5% SDS-polyacrylamide gel electrophoresis as described previously (17). Proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Immobilon-P ; Millipore). The membranes were blocked with TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.5) containing 5% skim milk (Difco) and labeled with rabbit anti-mouse type I collagen antibody (Calbiochem) (1 : 500 diluted) and then with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences). Immunoreactive bands were located using an ECL Western blotting detection kit (Amersham Biosciences) and RX-U X-ray film (Fujifilm). The relative intensity of the signals measured by JOYCE-LOEBL Microdensitometer 3CS (Joyce-Loebl).

Mineralization-inducing assay

Confluent RPC-C2A cells were cultured for 10 days with or without CM from either RC cells or MC3T3-E1 cells. For staining of mineralized nodules, the cells were fixed with 10% neutral buffered formalin and stained by the von Kossa technique with 3% silver nitrate as described previously (18).

Cell growth

CM from RC cells or MC3T3-E1 cells was added to the culture of confluent RPC-C2A cells. Cell growth was measured with the metabolic indicator Alamar Blue (Biosource International) (10% of incubation volume), which was added at 24h after CM treatment (19). After a 4-hour incubation, part of the medium was collected and the extent of proliferation was determined by measuring each plate at 570 nm and 600 nm for each plate in a Beckman DU 8 spectrophotometer. To obtain the specific absorbance for the level of the reduced state, the absorbance at 600 nm was subtracted from that at 570 nm. The cell-free blank value was then subtracted from each value. Data obtained from the mean of two separate experiments in triplicate are expressed as percentage of control (0% CM).

RESULTS

Expressions of osteogenic related genes

Extracellular matrix (ECM) mineralization is a physiological process in bones and teeth while a pathological process in anywhere else. Although the molecular mechanisms for ECM mineralization are not fully understood, ALPs such as tissue-nonspecific alkaline phosphatase and BMPs are possibly involved in the formation of mineralized matrix made up of hydroxyapatite and type I collagen (20). At first, RT-PCR analysis was performed to examine the expressions of osteogenic related genes including *BMP2, BMP4, and alkaline phosphatase,* in clonal cell lines without exogenous factors (Fig. 1A). *BMP-2* mRNA was detected in RDP4-1, another clonal rat dental pulp-derived cell line, and C3H10T1/2 cells which also differentiate to osteoblastic cells. The only cell line that simultaneously expressed three osteogenic mRNA (BMP-2, BMP-4, and alkaline phosphatase) was RPC-C2A, suggesting that this may be is the best target dental pulp-derived cell line for the induction of mineralization among clonal cell lines examined.

Mineralization-inducing activity

Addition of CM from MC3T3-E1 cells has a significant effect upon bone nodule formation by RPC-C2A cells, but not by RDP4-1 cells (Fig. 1B). The active factor(s) in CM was not filtered through an ultrafiltration membrane (MW cutoff: 1,000,000), suggesting a large complex. Thus, CM was subjected to ultracentrifugation at 150,000 xg for 3h after centrifugation at 10,000 xg for 30 min. Mineralization-inducing activity was sedimented as approximately 1.3 μm-long needle-like complexes as revealed by TEM (Fig. 2). These sedimentable matrices exhib-
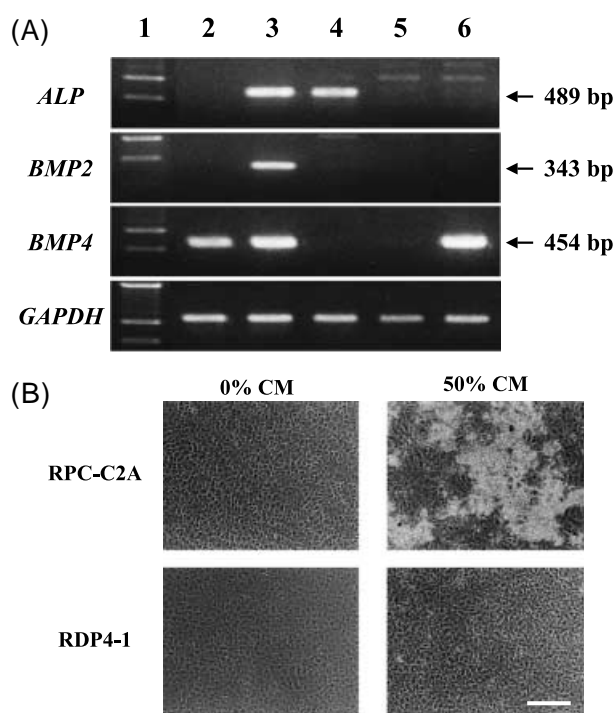

Fig. 1   RPC-C2A cells express osteogenic genes and produce bone nodules after treatment with conditioned medium from MC 3T3-E1 cells. (A) RT-PCR analysis of *ALP*, *BMP-2*, *BMP-4*, and *GAPDH* mRNA. Lane 1, size marker; lane 2, RDP-4-1 cells; lane 3, RPC-C2A cells; lane 4, AtT 20 cells, mouse pituitary tumor-derived cell line; lane 5, Balb/c3T3 cells; lane 6, C3H 10T1/2 cells. (B) Phase-contrast micrograph of rat dental pulp cells treated with conditioned medium (CM) from MC3T3-E1 cells. Bar, 200 μm.
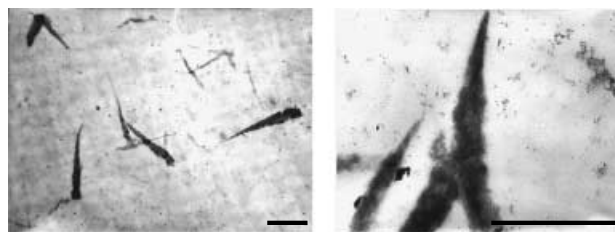

Fig. 2   Transmission electron micrographs of sedimented active matrices. After ultracentrifugation of CM, the fixed and ethanol-dehydrated pellets were embedded in Epon 812 and sections were prepared with an LKB Ultratome V. Ultra thin sections were examined in a Hitachi H-800 electron microscope. Bar, 1 μm.

ited mineralization-inducing activity in RPC-C2A cells. The mineralizing-inducing activity exclusively resided in the sedimented matrices not in the supernatant. In addition, we found that collagenase treatment lost both structure and activity of sedimented matrices (data not shown).

Western blot analysis

In general, matrix vesicles do not contain type I collagen (13, 14). The CBB-stained SDS-PAGE pattern of sedimented matrices showed that the sample was not easily dissociated with a reducing agent and that the molecular weight seemed too high to run into a separation gel, suggesting that the sedimented matrices contain collagens. In order to verify whether the complex was a matrix vesicle or soluble matrix, SDS-PAGE and immunoblot analyses with anti-type I collagen antibody were performed. CBB-stained gel showed that most of the large MW sample did not move into the separation gel, suggesting that it did not dissociate easily with the SDS sample buffer (21) once after sedimented (Fig. 3A). After immunoblotting, type I collagen molecules containing $\alpha 1$ and $\alpha 2$ chains were detected at about 2 : 1 ratio in the dissociated sample in addition to the undenatured large MW collagen (lane 2 in Fig. 3B). In whole cell extract (WCE), the possible degradation products were also observed (lane 1 in Fig. 3B). These results suggest that the factor secreted is not a matrix vesicle but a soluble matrix.

von Kossa staining

Next, in order to determine if a matrix such as this is restricted and specific to MC3T3-E1 cells, CM from primary-cultured rat calvaria (RC) cells (22) was examined for the activity of matrix mineralization of RPC-C2A cells. The multilayered RPC-C2A cells and the mineralized nodules stained strongly by von Kossa technique (18) in their ECM were observed (Fig 4A).

Alamar Blue assay

ince the cell proliferation-promoting activity with CM from RC cells was observed by phase-contrast microscopy, Alamar Blue assay was performed to quantify the reduction ability by mitochondria (18) (Fig. 4B). CM from RC cells exhibited stimulation of the RPC-C2A cell growth by 1.8-fold, whereas CM from MC3T3-E1 cells was without effect, indicating that the RC CM includes a growth factor in addition to a differentiation factor.

(A)

(B)
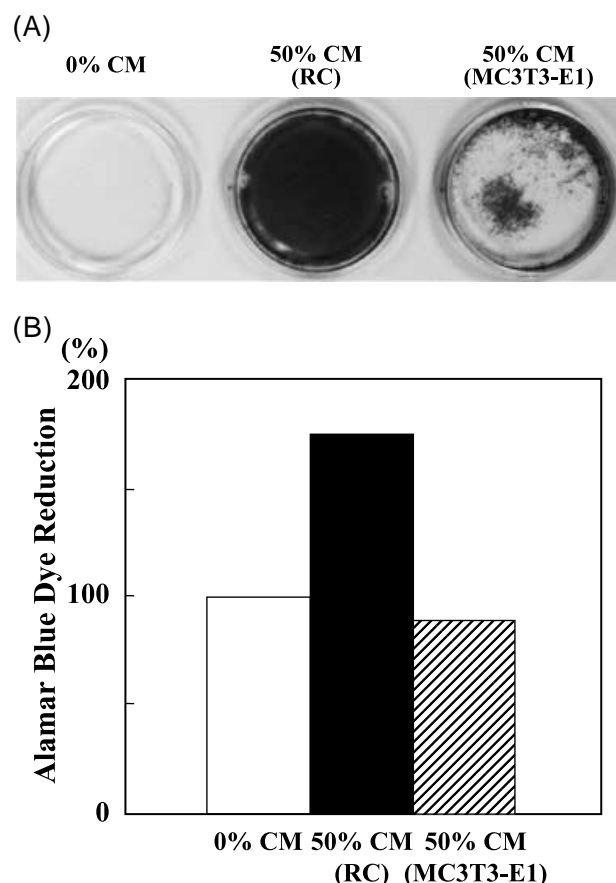
Fig. 4 Effect of CM from primary-cultured rat calvaria (RC) cells on mineralization and proliferation of RPC-C2A cells. (A) Confluent RPC-C2A cells were cultured with or without CM from either RC cells or MC3T3-E1 cells. In order to detect mineralized nodules, the cells were stained by the von Kossa technique. (B) CM from RC cells or MC3T3-E1 cells was added to the culture of confluent RPC-C2A cells. Cell growth was evaluated with Alamar Blue assay.
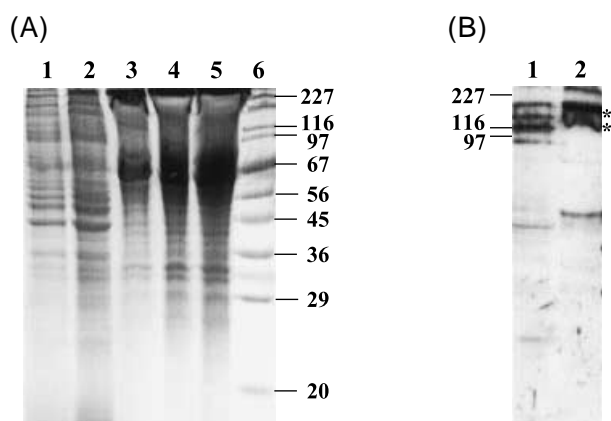
(A)

(B)

Fig. 3 Detection of type I collagen in the sedimented matrix. (A) Samples were separated by 7.5% SDS-polyacrylamide gel electrophoresis and the gel was stained with Coomassie brilliant blue R250. Lanes 1 and 2, WCE of MC3T3-E1(25 and 50 µg, respectively) ; lanes 3-5, 150,000xg pellet (25, 50, and 75 µg, respectively) ; lane 6, broad-range molecular weight (MW) marker (Apro Science). MWs (x10³) are shown at the right margin. (B) Western blot analysis was performed with rabbit anti-mouse type I collagen antibody and horseradish peroxidase-conjugated donkey anti-rabbit IgG. Lane 1, MC3T3-E1 WCE ; lane 2, 150,000xg pellet. Star ( ) indicates immunoreactive bands.

DISCUSSION

In the present study, the mineralization-inducing factor was found to be sedimented by ultracentrifugation and composed of at least type I collagen (Figs. 2 and 3B). The matrix vesicle does not contain type I collagen and shows a round shape with approximately 200 nm in diameter at maximum (13, 14). Therefore, this mineralization-inducing factor is thought to be a kind of soluble matrix that possibly contains a differentiation factor and other ECM molecules besides type I collagen. To further examine both the potency and generality of induction of matrix mineralization by CM from osteoblastic cells, we analyzed for CM from RC cells (Fig. 4A). The CM from RC cells has an extremely strong effect on matrix mineralization of RPC-C2A cells with the maximal concentration at 50% in medium, demonstrating a stronger activity than that of CM from MC3T3-E1 cells. This may be because MC3T3-E1 cells lost the capacity to produce a certain growth factor and a differentiation factor during the course of subculture by a number of generations (7). A growth factor increases the number of precursor cells differentiating to osteoblasts, resulting in enhanced matrix mineralization.

In conclusion, we found that not only MC3T3-E1 cells but also RC cells produce the sedimentable matrices containing at least type I collagen (Fig. 2) that have an ECM mineralization inducing activity. The both structure and activity of sedimented matrices were lost by collagenase treatment, suggesting the importance and an involvement of type I in mineralization inducing activity. It is well known that extracellular matrix vesicles are not always the site of initial mineral deposition (13-16). In certain cell culture systems (23) and in turkey tendon (24), matrix vesicle and collagen mineralization start simultaneously but distinct in site. In addition, there are no extracellular matrix vesicles in many invertebrates and in enamel (15, 16). Therefore, there must be other means of initiating mineralization besides accumulating ions in a protected environment as matrix vesicles do. For example, as one of the new paradigms for the morphogenesis of calcium-based biominerals, Olszta *et al* proposed the mineralization of type I collagen via a polymer-induced amorphous liquid-precursor process (25, 26). We believe that the results obtained from the present study provide another important issues for the new basic mechanism of

inducing matrix mineralization other than well-established matrix vesicles. Further molecular characterization and identification of the mineralization inducing activity in the CM is required to apply for medical purpose in future, including therapy for osteoporosis.

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