Short Communication

Recognition of Osteopontin by Rat Bone Marrow Derived Osteoblastic Primary Cells

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To study the role of osteopontin, we did cell adhesion and ALP assays of rat bone marrow osteoblastic cells (RBMO) on collagen Type I and osteopontin surfaces. The RBMO proved to adhere much more strongly to the osteopontin and to have higher ALP activity on the osteopontin, which suggests that pre-osteoblasts differentiate into osteoblasts that form bone by recognizing osteopontins.

Key words: osteopontin: osteoblast

Bone is a living tissue which undergoes matrix formation by osteoblasts and resorption by osteoclasts throughout its life; osteoblasts acting together in a small group excavate a tunnel through the old bone, and osteoclasts enter the tunnel behind them, line its walls and begin to form new bone. Typically about 5–10% of the bone in a healthy adult mammal is replaced every year by this remodelling. With respect to osteoblast differentiation through the remodelling process, it can be said that the adhesion of the pre-osteoblast to the bone surface is one of the major turning points in differentiation during which the pre-osteoblasts differentiate into mature osteoblasts that form bone. This association closely resembles the cell to substrate adhesion of an in vitro cell culture system. Taking this into account, our previous report have focused on the cell adhesion mechanism. In particular, the conserved sequence of the integrin β subunit (DLYYLMDLXYSMK: we call it β peptide) has proved to be a good probe to investigate the interaction between integrin β subunit and cell adhesion proteins (Y. K. Liu, A. Nemoto, Y. Feng, T. Uemura, J. Biochem., in press).

Rat bone marrow-derived differentiating osteoblastic primary cells (RBMO) have been reported to make large amounts of morphologically and biochemically identifiable matrices quickly. In vitro experiments have shown that the initial matrices made by differentiating osteogenic cells are analogous to the cement line found in vivo, which forms the natural interface between old and new bone. Therefore, RBMO cells are a good model system for investigation of the adhesion of osteoblasts to the bone surface. Previous reports have suggested that collagen Type I, osteopontin, and BSP in several kinds of bone protein are important in early biomineralization by RBMO. Because of the difficulty in isolating enough BSP proteins for our experiment, we focused on the role of collagen Type I and osteopontin in early mineralization by RBMO cells.

Chemicals-BSA, monoclonal anti-biotin alkaline phosphatase conjugate and N-succinimidyl-biotin were purchased from Sigma Co. The alkaline phosphatase substrate kit was from Bio-Rad Co., Richmond CA, U.S.A. The ALP IK (ALP assay kit) was from Wako Co. Ltd., Osaka, Japan.

The N-succinimidyl 3-(2-pyridyl)propionate (SPDP) and the BCA protein assay kit were from Pierce Co., Rockford, Illinois, U.S.A. The BCECF-AM (3′-O-acetyl-2,7-bis(carboxyethyl)-4 or 5-carboxyfluorescein, diacetoxymethyl ester) was from Dojindo Laboratories, Japan. Other reagents and chemicals were obtained commercially, with analytical purities. The Sephadex G-25 was from Pharmacia Co. Rat bone marrow-derived osteoblastic primary cells (RBMO) were obtained from young adult male Wistar rats by the method of Maniopoulous et al. For the primary culture, femora were removed and washed with z-Minimal Essential Medium (z-MEM) containing 1670 units/ml penicillin G, 500 μg/ml gentamicin, and 3.0 μg/ml amphotericin B. The epiphses of both sides were removed and the marrow cavity flushed out using z-MEM with 15% fetal bovine serum, 50 μg/ml of freshly-prepared ascorbic acid, 10 mM Na β-glycerophosphate and antibiotics at 1/10th of the concentration described above. 10−3 m dexamethasone (DEX) was added. The cells were incubated in a humidified atmosphere of 95% air with 5% CO2.

Osteopontins were isolated by the dissociative extraction procedure. After extracting proteins weakly bound to the bone tissues in the neonatal rat’s calvaria, using 4 m guanidine hydrochloride (GuHCl), 0.5 m tetrasodium ethylenediaminetetraacetic acid (EDTA) was used to extract the mineral-associated proteins (E-extract). Figure 1(a) shows the results of SDS–PAGE analysis of the E-extract. A single band (68 kDa) was observed and identified as osteopontins.

![Fig. 1. SDS-PAGE and Western Blot Immunoprecipitation Analysis of E-Extract Used in This Experiment.](image-url)

(a) The proteins in the E-extract were separated using SDS-PAGE on 12.5% gel and stained with "Stains-All". (b) Western blot immunoprecipitation analysis was performed according to the method recommended in the company protocol, using the ECL Western blotting detection kit (Amersham Life Science). Mouse monoclonal anti rat osteopontin (University of Iowa Bank) was used.

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Figure 2. (a) Inhibition of RBMO Cell Attachment to Collagen Type I by the β Peptide. (b) Inhibition of RBMO Cell Attachment to Osteopontin by the β Peptide.

by the Western blot immunoprecipitation analysis, as shown in Fig. 1(b).

For the cell adhesion assays, 96-well microtiter plates were coated with collagen Type I diluted in HCl solution, pH 3.0 (500 μg/ml) and osteopontin diluted in the buffer A (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂). Each plate was washed once with 200 μl of the adhesion buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂). The non-specific binding sites were blocked by incubating with 100 μl of the adhesion buffer with 30 mg/ml BSA for 1 h at 30°C. Then the plate was washed twice with 200 μl of the adhesion buffer. Up to 3 μl/ml of BPECF-AM was added to the cells suspended in the medium and they were then incubated for 15 min at 37°C. The supernatant was removed after centrifugation at 1200 rpm for 10 min, and the cells were resuspended in fresh medium and adjusted to a cell density of 6 x 10⁵/ml. Then 100 μl of the mixture of the cell suspension and an appropriate concentration of the β peptide were incubated in each well at 37°C for 1 h. After these were washed with 330 μl of PBS, and 100 μl of 1% Triton X-100 in PBS was added, the plates were incubated at room temperature for 2 h. Then fluorescence at 530 nm was measured with an excitation wavelength of 490 nm.

Figure 2 shows the inhibitory effect of the β peptide on RBMO binding to the collagen Type I and osteopontin surfaces. The inhibition rate of RBMO attachment to collagen Type I and osteopontin by the β peptide (100 μM) was 34% and 30%, respectively. This indicates that the RBMO attaches to the collagen Type I and osteopontin surfaces via integrin. However, the inhibition does not reflect the binding ability of matrix proteins to integrin, because the integrin α subunit also contributes to this binding. To compare the adhesion ability of the RBMO to these two proteins, we measured the inhibition by osteopontin of the adhesion of the RBMO to the collagen Type I coated surface, as shown in Fig. 3. The results suggested that the osteopontins strongly inhibited RBMO adhesion. With a low concentration of osteopontin (about 180 nM), the inhibition rate was almost 100%. This means that the RBMO cells specifically recognize the osteopontins and adhere to their surface.

To discover the role of the osteopontin, we measured the ALP (alkaline phosphatase) activity of the RBMO cells cultured on collagen Type I and osteopontin surfaces, according to the following procedure. Six well microtiter plates were coated with collagen Type I in HCl solution and osteopontin in buffer A (25 μg/ml) for 1 h incubation at 37°C. After they were washed twice with buffer A, the wells were incubated with a blocking buffer (1% BSA in buffer A). After these were rinsed twice with PBS, RBMO cell suspensions were added to each well and incubated for 2, 3, 5, and 7 days. Then ALP activity was measured by the method recommended in the company protocol. Figure
Fig. 4. ALP Activity of RBMO Cells Cultured for 3 Days after Subculture on Collagen Type I and Osteopontin Surfaces.

4 shows the ALP activity of the RBMO cells cultured for 3 days on the collagen Type I and osteopontin surfaces. The ALP activity of the cells on the osteopontin surface was 40% higher than that on the collagen Type I surface. Three sets of experiments and the courses of development of activity (2-7 days) consistently showed over 40% enhancement on the osteopontin surface. This indicates that differentiation of RBMO cells can be stimulated by culture on an osteopontin surface.

The differentiation of osteoblasts has not yet been fully described. There exist several kinds of factors which can affect the differentiation of osteoblasts—for example PTH, TGF-β, BMP, and extracellular bone matrix proteins. In particular, the recognition of bone matrix proteins by osteoblasts provides local information to the cells. Therefore, osteoblast adhesion to specific extracellular bone matrix proteins can modulate various aspects of cell behavior, including growth, differentiation and protein production. The course of development of ALP activity shows maximum activity in a 3 day culture. Thus, the RBMO cells used in this study can be said to be at a very early differentiating stage compared to the matured osteoblasts. The results obtained in this study with respect to the recognition of osteoponins by the RBMO cells suggest that pre-osteoblasts attach to the bone surface and differentiate into matured osteoblasts with bone formation ability by recognizing osteoponins. This is a natural idea, considering the osteogenic lineage. To confirm this hypothesis, it is necessary to further investigate individual osteoblast responses to osteopontin and intracellular signal transduction stimulated by the osteopontin integrin binding, especially with regard to autophosphorylation of the FAK (focal adhesion kinase) in osteoblasts.

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