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Osteoblast-Related Gene Expression of Bone Marrow Cells during the Osteoblastic Differentiation Induced by Type I Collagen

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Bone marrow contains multipotent cells that differentiate into fibroblasts, adipocytes, and osteoblasts. Recently we found that type I collagen matrix induced the osteoblastic differentiation of bone marrow cells. Three weeks after cells were cultured with type I collagen, they formed mineralized tissues. In this study, we investigated the expression of osteoblast-related genes (alkaline phosphatase, osteocalcin, bone sialoprotein, osteopontin, and cbfa-1) during the osteoblastic differentiation. The expression of alkaline phosphatase and osteopontin genes increased time-dependently during the osteoblastic differentiation. Osteocalcin and bone sialoprotein genes were expressed in cells that formed mineralized tissues, and both were expressed only after cells reached the mineralized tissue-formation stage. On the other hand, the cbfa-1 gene was expressed from the early differentiation stage. The Asp-Gly-Glu-Ala (DGEA) amino acid domain of type I collagen interacts with the α2β1 integrin receptor on the cell membrane and mediates extracellular signals into cells. When the collagen-integrin interaction was interrupted by the addition of DGEA peptide to the culture, the expression of osteoblastic phenotypes of bone marrow cells was inhibited. These findings imply that the collagen-α2β1 integrin interaction is an important signal for the osteoblastic differentiation of bone marrow cells.

Key words: gene expression, osteoblastic differentiation.

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Bone marrow is composed of multipotent cells that differentiate into fibroblasts, adipocytes, and osteoblasts (*1*, *2*). They form bone in a diffusion chamber or ceramic blocks when implanted in ectopic sites *in vivo* (*3*, *4*). They also differentiate into osteoblasts in the presence of vitamin C and β-glycerophosphate (*5*, *6*) and in the presence of dexamethasone *in vitro* (*7*). These findings indicate that bone marrow cells could differentiate into osteoblasts in an appropriate environment, and that they may participate in the recruitment of bone-forming cells.

Collagen matrices and other extracellular proteins have been used to support phenotypes and tissue-specific functions *in vitro* (*8*, *9*), which demonstrates that extracellular matrices maintain cell functions. The major organic component of bone is type I collagen. It has been shown that type I collagen affects the expression of bone cell phenotypes (*10*, *11*).

Recently we found that bone marrow stromal cells differentiate into osteoblasts and express osteoblastic phenotypes when cultured with type I collagen matrix. In this study, we investigated the expression of osteoblast-related genes, including cbfa-1, of bone marrow cells during the osteoblastic differentiation induced by type I collagen. Cbfa-1 is one of the transcriptional factors that regulates osteoblastic differentiation and bone formation (*12*), and BMPs induce or stimulate the expression of cbfa-1 mRNA (*13*, *14*). These findings indicated that cbfa-1 was a crucial factor for osteoblastic differentiation; however, our findings demonstrated that cbfa-1 gene expression was independent of the differentiation lineage.

We also found that the interaction of the Asp-Gly-Glu-Ala (DGEA) amino acid domain in the type I collagen molecule and the α2β1 integrin receptor of cells is a crucial signal for the induction of osteoblastic differentiation of bone marrow cells.

MATERIALS AND METHODS

1. Cell Culture—Bone marrow stromal fibroblastic cells were harvested from rat femur by the method of Maniatopoulos (*7*). Briefly, bone marrow cells (5×10^5 cells) were harvested from the femurs of 4-week-old rats and cultured in α-MEM medium containing 15% fetal calf serum. When cells reached confluence, they were covered with 1 ml of neutralized collagen solution (0.35% concentration) and maintained at 37°C for 2 h in a humidified atmosphere. After gelation of the collagen solution, medium was added to the dish and changed every two days thereafter. The neutralized collagen solution was prepared as described previously (*15*). Briefly, type I collagen (Koken, Tokyo) was mixed with HEPES buffer and a fivefold concentration of α-MEM to neutralize the pH of the mixed solution.

2. Histological Staining of Alkaline Phosphatase Activity and Calcium Precipitation—Bone marrow cells were fixed with paraformaldehyde, then washed with distilled water several times. For the detection of alkaline phosphatase activity, a solution of 0.25% naphthol AS-BI phosphate and

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0.75% Fast Blue BB dissolved in 0.1 M Tris buffer (pH 9.3) was poured into dishes. For the detection of calcium precipitation by Von Kossa staining, a solution of 5% silver nitrate in distilled water was poured into dishes.

3. *Measurement of Alkaline Phosphatase (ALP) Activity and DNA Content*—ALP activity and DNA content were measured as described previously (*16*). Briefly, alkaline phosphatase activity was measured by the liberation of *p*-nitrophenol from *p*-nitro-phenylphosphate at 37°C, and one unit was defined as the amount that liberated 1.4 mg of *p*-nitrophenol. The DNA content was determined using bis-benzimidazole (Hoechst 33258). The intensity of fluorescence was measured at an excitation wavelength of 356 nm and emission wavelength of 458 nm.

4. *Measurement of Collagen Synthesis in Cell-Matrix*—The content of newly synthesized collagen in the cell-matrix was shown as the radioactivity of collagenase-digestible protein (CDP), which was measured by the bacterial collagenase digestion assay (*17*). Briefly, bone marrow cells were cultured in medium containing 10 μCi of ^{3}H-proline and 15% fetal calf serum (FCS) for 4 h, and cell-matrix layers were digested with highly purified bacterial collagenase (Advance Biofactures Corporation, Form III). Then 10% trichloroacetic acid and 1% tannic acid were added to the reaction mixture, and it was maintained at 4°C overnight. After centrifugation at 12,000 rpm for 10 min at 4°C, the radioactivity of the supernatant was determined using a liquid scintillation counter.

5. *Preparation of Plasmids and cDNA Templates for Riboprobe Synthesis*—Templates for the osteoblast-related genes of alkaline phosphatase (ALP), osteocalcin (OCN), bone sialoprotein (BSP), osteopontin (OPN), and cbfa-1 were obtained by RT-PCR using RNA extracted from bone marrow cells that expressed osteoblastic phenotypes. PCR products were ligated into pGEM-T vector (Promega, Tokyo) for generating antisense riboprobes from the T7 promoter. Primer sequences from 5′ to 3′ for PCR were: osteocalcin (forward, ATGAGGACCCTCTCTCTGCTC; reverse, CTAAACGGTGGTGCCATAGAT), which sequence was referred to in a previous report (*18*); bone sialoprotein (forward, AAGCAGAGGATTCTGAAG; reverse, TCAGCTGTGGTCTCTACC), the sequence of which has been reported (*19*); osteopontin (forward, ATGAGACTGGCAGTGGTT; reverse, GCTTTCATTGGAGTTGCT) (*20*); and cbfa-1 (forward, ATGCTTCATTCGCCTCACAAAC; reverse, CTACAACCTTGAAGGCCACG) (*21*).

6. *Northern Blotting*—Total RNA was extracted from rat bone marrow cells using the acid guanidine-phenol-chloroform method (*22*). Twenty micrograms of RNA was separated by 1.4% denaturing agarose gel electrophoresis in MOPS buffer. Then RNAs were transferred onto a nylon membrane. Hybridization to RNA immobilized on the nylon membrane was performed for 15 h at 50°C with RNA probe-labeled DIG-11-dUTP in a buffer (50% formamide, 5× SSC, 50 mM $NaPO_4$ [pH 7.0], 7% SDS, 2% blocking buffer, 0.1% lauroylsarcosine, and 100 μg/ml of salmon sperm DNA) after prehybridization at 50°C for 1 h in the same buffer without the probe. Membranes were washed in 2× SSC containing 0.1% SDS for 10 min at room temperature, followed by 0.1× SSC containing 0.1% SDS for 10 min at 68°C. Signals were detected by exposing the membrane to X-P film for 5 h.

RESULTS

We first investigated the morphology of bone marrow cells cultured with type I collagen matrix or in the conventional culture dish, and carried out alkaline phosphatase (ALP) and Von Kossa staining of bone marrow cells. ALP activity was detected from two weeks, and the magnitude of ALP staining increased time-dependently; however, cells did not express ALP activity at week one (Fig. 1, A, B, and C). On the other hand, bone marrow cells cultured in the conventional culture dishes for three weeks showed weak ALP activity (Fig. 1D). The situation with Von Kossa staining was similar to that with ALP staining (Fig. 1, E, F, and G). Distinct nodules on dishes cultured for three weeks with type I collagen matrix were revealed by Von Kossa staining, indicating the progress of calcium precipitation, and the numbers of nodules increased time-dependently. However, cells showed no Von Kossa staining when they were cul-

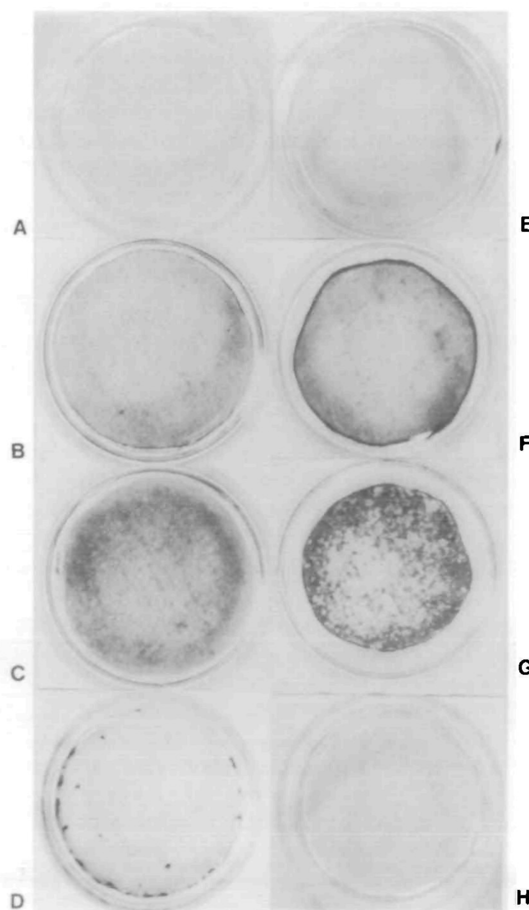

Fig. 1. **Alkaline phosphatase staining of bone marrow cells cultured with type I collagen matrix for one week (panel A), two weeks (panel B), and three weeks (panel C).** The intensity of enzyme staining increased time-dependently. When bone marrow cells were cultured in the conventional culture dish for three weeks, only slight alkaline phosphatase activity was detected (panel D). Calcium stained by Von Kossa staining of cells cultured with type I collagen for one week (panel E), two weeks (panel F), and three weeks (panel G). Calcium staining increased time-dependently. Bone marrow cells cultured in the conventional dishes did not form mineralized nodules (panel H).

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tured in the conventional culture dishes for three weeks (Fig. 1H). These observations indicated that bone marrow cells formed mineralized tissues. Mineralization proceeds by the precipitation of hydroxyapatite, and a high content of calcium indicates a high degree of mineralization. At week three, high calcium content was detected in the matrix, indicating that mineralization had occurred in the cell-layers (Fig. 2). However, at weeks one and two, calcium content was low (Fig. 2) and mineralized tissues were not formed. Cells in the plastic dishes showed consistently low calcium content (Fig. 2)

Mineralized tissue formation is a typical osteoblastic phenotype. Next we measured ALP activity of cells. High ALP activity is an osteoblastic phenotype. ALP activity of cells cultured on collagen matrix was low at one week, and increased time-dependently. At week three, the enzyme activity was 12-fold higher than that of cells at week one (Fig. 3). Cells in the plastic dishes showed a low level of ALP activity for three weeks (Fig. 3).

Next we investigated the expression of osteoblast-related genes of bone marrow cells during the osteoblastic differentiation by Northern blot analysis. ALP gene expression increased time-dependently and was stronger when cells were cultured with type I collagen matrix (Fig. 4). The expression of OCN and BSP genes, which are typical osteoblastic phenotypes, was clearly observed at week three after cells were cultured with type I collagen (Fig. 4). These findings indicated that osteoblastic-specific genes were expressed in bone marrow cells cultured with type I collagen matrix. OPN gene expression increased from two weeks in cells cultured with collagen matrix; however, it also increased slightly in cells cultured in conventional dishes (Fig. 4). Cbfa-1 gene expression was observed both in cells cultured in conventional dishes and in cells cultured with collagen matrix, and it was slightly higher in the latter (Fig. 4).

Our results indicate that type I collagen induced the osteoblastic differentiation of bone marrow cells. Type I collagen interacts with α2β1 integrin receptors, and the Asp-Gly-Glu-Ala (DGEA) amino acid sequence in the collagen molecule acts as the binding domain (*23*). We examined the effect on osteoblastic differentiation of interrupting the collagen-integrin interaction with DGEA peptide. Bone marrow cells were cultured with collagen matrix and media

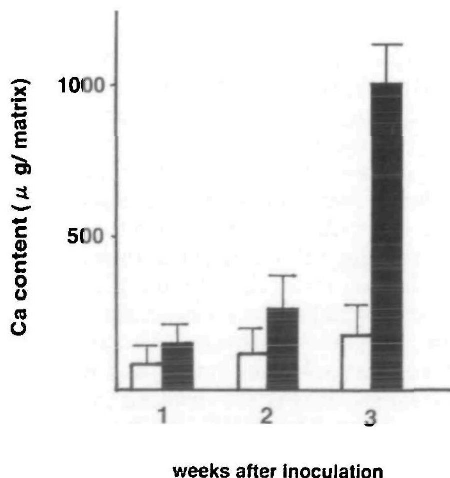

Fig. 2. **Calcium contents in the cell-matrix.** Bone marrow cells were cultured in conventional culture dishes (□) or type I collagen matrix (■). At week three, matrices of cells cultured with collagen matrices showed high calcium content. Data are shown as means ± SD, evaluated from five dishes.

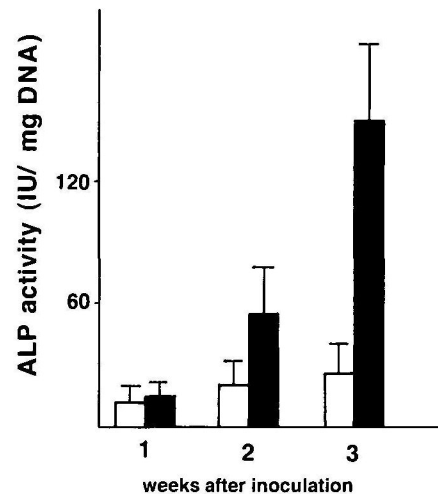

Fig. 3. **Alkaline phosphatase activity of bone marrow cells in plastic dishes (□) or with collagen matrices (■).** The enzyme activity of cells that were cultured with collagen matrix increased time-dependently. However, cells in the plastic dishes showed weak activity during the culture period. Values are shown as means ± SD, evaluated from five dishes.

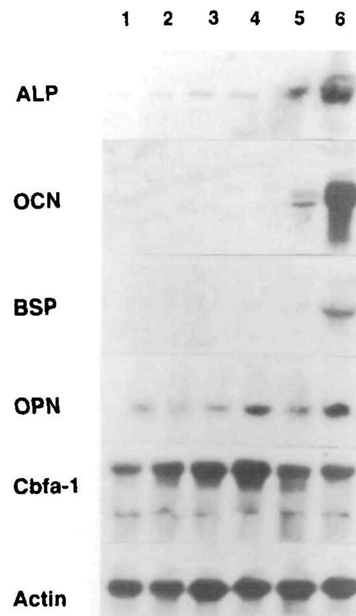

Fig. 4. **Gene expression of alkaline phosphatase (ALP), osteocalcin (OCN), bone sialoprotein (BSP), osteopontin (OPN), and cbfa-1 in bone marrow cells cultured in a conventional culture dish for 1 week (lane 1), 2 weeks (lane 3), and 3 weeks (lane 5), or cultured with type I collagen matrix for 1 week (lane 2), 2 weeks (lane 4), and 3 weeks (lane 6).** Bone marrow cells cultured with type I collagen matrix for 3 weeks formed mineralized tissues, and OCN and BSP gene expression was restricted to cells of the mineralized tissue-forming stage. However, the cbfa-1 gene was expressed from the early differentiation stage.

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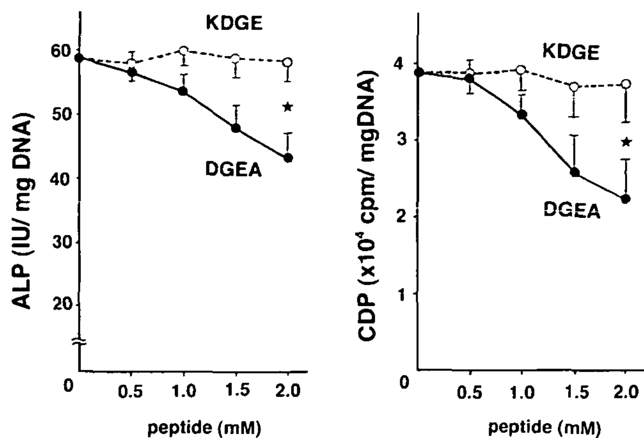

Fig. 5. **The effect of Asp-Gly-Glu-Ala (DGEA) and Lys-Asp-Gly-Glu (KDGE) peptides on ALP activity and collagen synthesis of bone marrow cells cultured with type I collagen matrix.** Both parameters were down-regulated dose-dependently by the presence of DGEA peptide (●), while KDGE peptide (○) showed no effects. Values are evaluated from four dishes and shown as mean ± SD. ⋆ $p < 0.01$ by Student's t-test.

containing various amounts of DGEA peptide for 6 days. Then we measured ALP activity and collagen synthesis. These parameters were down-regulated dose-dependently in the presence of DGEA peptide (Fig. 5). However, KDGE, a related peptide without binding affinity for α2β1 integrin, did not influence ALP activity and collagen synthesis (Fig. 5). These findings imply that the collagen-integrin receptor interaction was a crucial signal for the osteoblastic differentiation of bone marrow cells.

DISCUSSION

The histological observations (ALP staining and Von Kossa staining) indicated that cells cultured with collagen matrix showed osteoblastic phenotypes at week three after plating the collagen matrix. On the other hand, bone marrow cells cultured in the conventional culture dishes did not express osteoblastic phenotypes. The osteoblastic differentiation of bone marrow cells by type I collagen matrix was confirmed by the investigation of time-dependent change of calcium content in the matrix, alkaline phosphatase activity of cells and osteoblast-related gene expression.

ALP gene expression was coincident with the expression of the enzyme activity. At week three, cells cultured with collagen matrix showed a high level of ALP mRNA. Osteocalcin appears immediately before the onset of mineralization. Its expression increases rapidly as mineralization increases, and the synthesis of osteocalcin is recognized at the late stage in osteoblastic differentiation (*24, 25*). Therefore, osteocalcin is highly specific for the mineralization and is a good marker of osteoblastic phenotypes. The presence of bone sialoprotein (BSP) is highly restricted to bone and mineralized tissues, as is the distribution of osteocalcin (*26, 27*). Expression of BSP by bone marrow stromal cells correlates with the formation of mineralized tissues, and strong mRNA expression of BSP is observed in fully differentiated osteoblasts associated with the initial formation of bone (*28, 29*). Next we investigated the expression of the osteopontin (OPN) gene, and found that in cells cultured with collagen matrix it increased time-dependently. OPN is a major non-collagenous protein synthesized by differentiated osteoblasts and deposited into the mineralizing matrix (*30, 31*). However, OPN does not have a role in the initial formation of mineral crystals in bone-like tissue, because it does not bind collagenous matrix of bone; and it accumulates even though mineral formation is absent (*30, 31*). Furthermore, rat bone marrow cells express the OPN gene in conventional culture, and its expression is enhanced as the osteoblastic differentiation proceeds after treatment with dexamethasone (*32*). Thus OPN might not be a typical phenotype of osteoblasts. Cbfa-1 has been identified as an essential factor for osteoblastic differentiation, and bone disappears in cbfa-1-knockout mice (*13, 33*). Our results showed that cbfa-1 gene expression occurred in bone marrow cells that did not differentiate to osteoblasts, and the change of the mRNA level was independent of osteoblastic differentiation. These findings do not conflict with the results of Xiao *et al.* (*34*). They showed that the message level of cbfa-1 did not change, but that the binding activity of the cbfa-1 product to DNA increased in the osteoblastic differentiation lineage. They speculated that this phenomenon was due to the phosphorylation of cbfa-1 protein by MAP kinase (*35*).

We also demonstrated that the interaction of collagen with α2β1 integrin, was a crucial event for the expression of osteoblastic phenotypes of bone marrow cells. We used a DGEA peptide that shows action similar to that of the cell-binding domain of type I collagen, and showed that blocking of the interaction suppressed the expression of the osteoblastic phenotype of bone marrow cells. It was reported that the extracellular matrix supports the expression of tissue-specific genes during osteoblast differentiation and suppresses the proliferation of cells (*36*). Lynch *et al.* (*11*) reported that collagen matrix induced the osteoblastic differentiation of osteoblasts, and speculated that this induction might be due to the interaction of the collagen matrix with integrin receptors of cells. Recently Takeuchi *et al.* (*37*) reported that the interaction of type I collagen with cell-surface α2β1 integrin receptors on MC3T3-E1 cells is required for osteoblastic differentiation of these cells. Xiao *et al.* (*34*) also demonstrated that an α2 integrin–collagen interaction is necessary for the expression of osteoblast-specific genes. Our demonstration that collagen–α2β1 integrin interaction was an important signal for the osteoblastic differentiation of bone marrow cells is consistent with these findings.

Integrins are considered to mediate information about the extracellular environment by serving as a direct link between the extracellular matrix and the actin cytoskeleton *via* the proteins talin and vincullin into cells. In cells, signal transduction molecules stimulate tyrosine phosphorylation, which is linked to mitogen-activated protein kinase (MAPK) and other pathways (*38, 39*). Recently it was reported that focal adhesion kinase and extracellular signals-related kinase/MAPK are involved in the induction of ALP activity in osteoblasts (*40*). Inhibition of tyrosine kinase, destruction of focal adhesion, or overexpression of antisense focal adhesion kinase mRNA inhibited activation of extracellular signal-regulated kinase and MAPK, and inhibition of MAPK prevented the increase of ALP activity (*40*). These findings suggest that integrin activation of MAPK or focal adhesion kinase is an important factor in

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the cellular response to the extracellular matrix.

In the near future, we intend to clarify the precise mechanism of osteoblastic differentiation by the extracellular matrix and the pathway of signal transduction in osteoblasts.

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