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Small GTPase Rho signaling is involved in β1 integrin-mediated up-regulation of intercellular adhesion molecule 1 and receptor activator of nuclear factor κB ligand on osteoblasts and osteoclast maturation

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Abstract

We assessed the characteristics of human osteoblasts, focusing on small GTPase Rho signaling. β1 Integrin were highly expressed on osteoblasts. Engagement of β1 integrins by type I collagen augmented expression of intercellular adhesion molecule 1 (ICAM-1) and receptor activator of nuclear factor κB ligand (RANKL) on osteoblasts. Rho was activated by β1 stimulation in osteoblasts. β1 Integrin-induced up-regulation of ICAM-1 and RANKL was inhibited by transfection with adenoviruses encoding C3 transferase or pretreated with Y-27632, specific Rho and Rho-kinase inhibitors. Engagement of β1 integrin on osteoblasts induced formation of tartrate-resistant acid phosphatase (TRAP)-positive multinuclear cells (MNC) in a coculture system of osteoblasts and peripheral monocytes, but this action was completely abrogated by transfection of C3 transferase. Our results indicate the direct involvement of Rho-mediated signaling in β1 integrin-induced up-regulation of ICAM-1 and RANKL and RANKL-dependent osteoclast maturation. Thus, Rho-mediated signaling in osteoblasts seems to introduce major biases to bone resorption.

Keywords: Rho; Osteoblasts; β1 Integrin; ICAM-1; RANKL

Rho is a major small GTP-binding protein and acts as molecular switch that control a large variety of signal transduction pathways, many of which regulate actin cytoskeleton remodeling in various types of cells. In addition, Rho participates in the regulation of cytokinesis, gene transcription and G1 cell cycle progression [1,2]. Like other GTPases, Rho proteins act as binary switches by cycling between an inactive (GDP-binding) and active (GTP-binding) conformation state [3,4]. In the active (GTP-bound) state, Rho relays extracellular signals to a large number of downstream effectors. Therefore, Rho is a very important signaling element that cooperates with other small GTP-binding proteins to regulate various cell functions, such as cell proliferation and apoptosis [5].

Bone metabolism is based on a self-regulating cellular event. The two major processes of bone remodeling, bone formation and resorption, are closely regulated by intercellular signaling involving soluble factors, systemic hormones, and cellular adhesion [6]. The small GTPase is involved in bone resorption by regulating cytoskeletal organization in osteoclasts and osteoclast functions [7]. It has been reported that cytoskeletal rearrangements are under the control of Rho family GTPases and Rho-dependent Rho kinase activation increases bone resorption in

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osteoclasts [8]. However, the relevance of Rho-mediated signaling to osteoblast function is largely unknown.

Osteoblasts play a central role in bone formation by synthesizing multiple bone matrix proteins and by differentiation into osteocytes. However, osteoblasts also regulate osteoclast maturation by producing bone-resorbing cytokines and by direct cell attachment, resulting in bone resorption [9,10]. Cell adhesion of osteoblasts and osteoclastic precursors of hematopoietic origin is a prerequisite for osteoclast maturation. Several studies have demonstrated that interaction of RANKL on osteoblast and RANK on osteoclast precursor provides an essential signal to osteoclast precursors for their maturation into resorbing cells [11]. We have previously reported that human osteoblasts express ICAM-1 and that interaction between ICAM-1 expressed on osteoblasts with integrins, leukocyte function-associated antigen (LFA)-1, expressed on monocytes, is required for osteoclast maturation by RANKL on osteoblasts [9].

Integrins are a superfamily of cell surface receptors involved in cell–cell and cell–matrix adhesion. In addition to providing a direct link between the extracellular matrix (ECM) and cytoskeleton, integrins also regulate the production of second messengers within the cell [12,13]. Osteoblasts are always surrounded by and encounter ECMs including type I collagen and fibronectin mainly through β1 integrin. We have recently reported that β1 integrin-dependent adhesion of osteoblasts to bone matrices induces ICAM-1 and RANKL expression and RANKL-dependent osteoclast maturation via tyrosine kinase, especially focal adhesion kinesis (FAK) [14]. However, it is yet unclear the signaling pathways involved in β1 integrin on osteoblasts. Because Rho activity is essential for focal adhesion formation when cells are plated on a matrix, it is assumed that Rho is involved in signaling pathway from β1 integrin [15].

The aim of the present study was to determine the roles of Rho in β1 integrin-mediated regulation of functional molecules in osteoblasts. Our results demonstrate that Rho-mediated signaling was directly involved in β1 integrin-induced up-regulation of ICAM-1 and RANKL, and RANKL-dependent osteoclast maturation.

Materials and methods

Cells and purification of human osteoblastic cells. Primary osteoblast-like cells were purified from metaphyseal trabecular bone in the proximal femur of five patients with osteoarthritis during total hip arthroplasty by the established procedures of Russell and colleagues [16,17]. After removing pieces of cortical bone, the fragments were cut into small pieces and washed extensively. The bone explants were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS; Gibco) in 25 cm^2 culture flasks (Falcon, Lincoln Park, NJ) at a humidified 5% CO_2 atmosphere. The obtained cells showed a flat polygonal shape with multiple spindle legs and exhibited characteristics of osteoblast-like phenotype including osteocalcin (OC), bone sialoprotein, type I collagen, and bone alkaline phosphatase (ALP) as described previously [17]. Human osteoblast-like cell line MG63 (ATCC, Rockville, MD) was cultured in 10% FCS-containing DMEM.

Antibodies and materials. The following monoclonal antibodies (mAbs) were used as purified immunoglobulin (Ig)s in preparation of staining and analysis of cell surface or cytoplasmic molecules: control mAb thy1.2 (Becton–Dickinson, San Jose, CA), human CD29 (β1 integrin) mAb MAB13, human CD18 (β2) mAb TS1/18 (kindly provided by Dr. K.M. Yamada, National Institutes of Health [NIH], Bethesda, MD), human CD61 (β3) mAb, human CD49a (α1) mAb TS2/7, human CD49b (α2) mAb, human CD49c (α3) mAb, human CD49d (α4) mAb NIH49d-1, human CD49e (α5) mAb MAB16, human CD49f (α6) mAb NIH49f-1, human CD51 (αv) mAb 23C6, human CD54 (ICAM-1) mAb 84H10 (kindly provided by Dr. S. Shaw, NIH, Bethesda, MD), anti-human RANKL mAb (Sigma Chemical Co., St. Louis, MO), human CD106 (VCAM-1) mAb 2G7 (kindly provided by Dr. W. Newman, Otsuka America, Rockville, MD). Rho-kinase inhibitor Y-27632 was obtained from Biomol (Plymouth Meeting, PA).

Stimulation of osteoblasts by β1 integrin using mAbs and substrates. Purified collagen type I (10 μg/ml), and control bovine serum albumin (BSA; 10 μg/ml, Wako) were applied to plates in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) at 4 °C overnight. Subsequently, the plates were washed with PBS, and the cells were added to each well and then incubated in FCS-free DMEM at 37 °C for the indicated time. After harvesting from the wells, the obtained cells were settled in media suitable for the following experiments.

Adenoviral infection. Recombinant adenoviruses encoding green-fluorescent protein (GFP), the Rho inhibitor C3 transferase, the exoenzyme from *Clostridium botulinum* that specifically ribosylates and inhibits the function Rho gene [18,19], and the regulator of G-protein signaling domain of p115RhoGEF (p115-RGS) were produced as described previously [20]. The cells were infected with recombinant adenoviruses at a multiplicity of infection of 30 for 1 h at 37 °C. Cells were then starved in 10% FCS-containing DMEM and cultured for an additional 48 h before treatment. Under these conditions, infection with adenoviruses coding for GFP made almost 100% of cells GFP-positive.

Flow microfluorometry. Staining and flow-cytometric analysis of osteoblasts were conducted by standard procedures, as described previously, using a FACScalibur (Becton–Dickinson, Mountain View, CA). Briefly, 2×10^5 cells were incubated with negative control mAb thy-1.2, and several mAbs in FACS media consisting of HBSS (Nissui, Tokyo, Japan), 0.5% human serum albumin (HSA; Yoshitomi, Osaka, Japan) and 0.2% NaN3 (Sigma–Aldrich, Tokyo, Japan) for 30 min at 4 °C. After washing the cells with FACS medium, they were further incubated with phycoerythrin (PE)-conjugated goat anti-mouse IgG Ab for 30 min at 4 °C. The staining of cells with mAbs was detected using FACScalibur. Quantification of cell surface antigen on a single cell was calculated using standard beads (QIFKIT; DAKO Japan, Kyoto, Japan).

Rho activation assay. Rho activation was determined by a pull-down assay using GST–Rhotekin-Rho-binding domain (GST–RBD). Forty-eight hours after adenovirus infection, osteoblasts were cross-linked with or without anti-CD29 (β1) mAb MAB13 at concentration of 10 mg/ml for 20 min, quickly washed with ice-cold Tris-buffered saline, and lysed in 500 μl of lysis buffer. Cell lysates were immediately centrifuged at 8000 rpm at 4 °C for 5 min and equal volumes of lysates were incubated with 30 μg GST–RBD beads for 1 h at 4 °C. The beads were washed with wash buffer, and bound Rho was eluted by boiling each sample in Laemmli sample buffer. Eluted samples from the beads and total cell lysate were then electrophoresed on 12% SDS–polyacrylamide gel electrophoresis gels, transferred to nitrocellulose, blocked with 5% nonfat milk, and analyzed by Western blotting using a polyclonal anti-Rho antibody.

Coculture of osteoblasts and monocytes and subsequent staining of tartrate-resistant acid phosphatase. Osteoblasts were seeded onto 48-well multi-well dishes (5×10^3 cells/well). Then osteoblasts, with or without transfection of several adenoviral vectors, were reseeded onto BSA- or type I collagen-precoated 48-well multi-well dishes. Purified peripheral blood $CD14^+$ monocytes from healthy donors (4×10^5 cells/well) were added to osteoblasts, and they were cocultured for 9 days in DMEM containing 10% heat-inactivated FCS in the presence of 10^{-7} M $1\alpha,25(OH)_2D_3$. Osteoclasts were cytochemically stained for TRAP (Sigma–Aldrich) as described previously [21]. The number of TRAP-positive MNC that contained more than three nuclei was counted under a light microscope.

Statistical analysis. All data were analyzed by ANOVA, followed by post hoc Scheffe's *F*-test. All values are reported as means ± standard deviation (SD).

Results

β1 Integrin is highly expressed on osteoblasts

First, we assessed the expression of various integrin subunits on primary human osteoblasts using FACScalibur. Fig. 1A shows the number of 10 representative subunits of integrins on osteoblasts. Among the screened molecules, β1 integrin was highly expressed on osteoblasts. Among α subunits of integrins, α2, α3, α4, and α5 were expressed on osteoblasts. A histogram implied that the vast majority of osteoblasts expressed β1. We therefore assumed that β1 integrin, which is consistently highly expressed on osteoblasts, might play a functional role in primary osteoblasts.

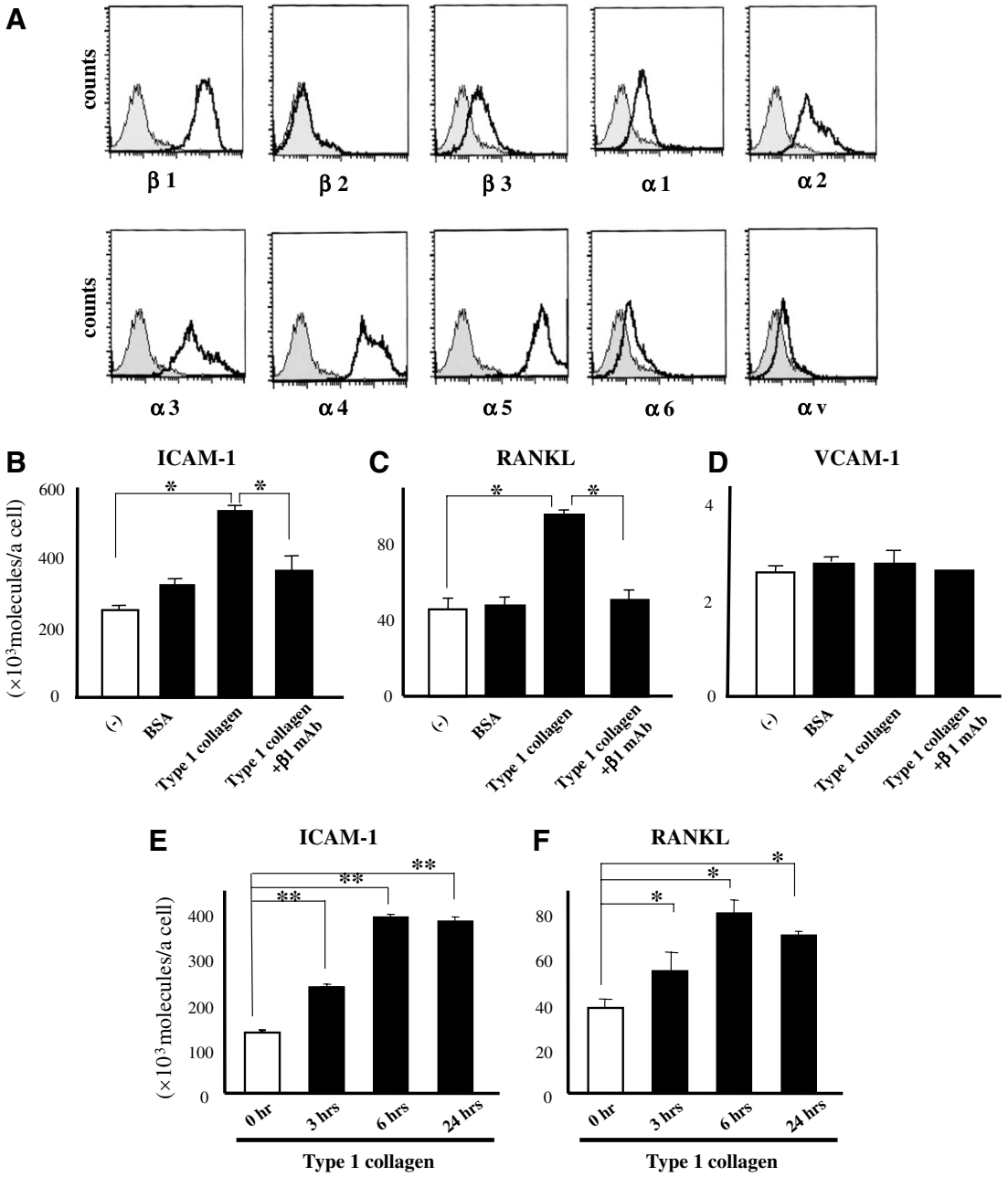

Fig. 1. High expression of β1 integrin on osteoblasts and engagement of β1 integrin by type I collagen augments ICAM-1 and RANKL expression on osteoblasts. (1) Osteoblasts were stained with several mAbs. Flow-cytometric analyses were performed using FACScalibur. (A) Open histograms: number of cells stained with mAb in each logarithmic scale on a fluorescence amplifier. Shaded histograms: profiles of Thy1.2 mAb as a negative control. (2) Osteoblasts with or without pretreatment with anti-β1 integrin blocking mAb were incubated on plastic plates precoated with BSA and type I collagen (10 μg/ml) at 37 °C for 6 h. ICAM-1 (B), RANKL (C) and VCAM-1 (D) expression levels were determined by FACScalibur. Similarly, osteoblasts were incubated on plastic plates precoated with type I collagen (10 μg/ml) for several hours (E,F). Each value represents the number of molecules expressed per one cell, calculated using standard QIFKIT beads from five different experiments. Data are presented as means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

Engagement of β1 integrin by type I collagen augments ICAM-1 and RANKL expression on osteoblasts

To characterize the function of β1 integrin on osteoblasts, we assayed the biological activities of type I collagen on osteoblasts. Type I collagen is a major ligand for cell surface β1 integrin. Expression of ICAM-1 and RANKL were markedly induced by incubation of these cells on collagen-coated plastic plates (Fig. 1B and C). In contrast, type I collagen did not induce VCAM-1 expression (Fig. 1D). No change was noted when these cells were incubated on BSA-coated plates. ICAM-1 and RANKL expression induced by type I collagen was completely inhibited by pretreatment of cells with anti-β1 mAb (Fig. 1B and C). Furthermore, time-dependent expression of ICAM-1 and RANKL were induced by type I collagen, both expression reaching the maximum about 6 h (Fig. 1E and F). These results suggest that β1 integrin appears to play a pivotal role in ICAM-1 and RANKL up-regulation on osteoblasts and type I collagen is a possible ligand involved in β1 integrin-induced ICAM-1 and RANKL expression on osteoblasts.

Involvement of small GTP-binding protein Rho activation in β1 integrin-mediated signaling in osteoblasts

We measured Rho activation and its inhibition in osteoblasts using the GST-Rhotekin fusion protein. We used the Rho inhibitor C3 transferase and the regulator of G-protein signaling domain of p115RhoGEF (p115-RGS), which inhibits endogenous p115RhoGEF function by blocking the interaction of p115RhoGEF with Gα12/13 and by its GAP activity on Gα12/13. After adenoviral infection, cells were reseeded onto plastic plates precoated with BSA and type I collagen (10 μg/ml) at 37 °C for 6 h, and the cell lysates were incubated with Rhotekin-bound GST beads to determine Rho activation. β1 Integrin stimulation increased the amount of activated Rho in osteoblasts infected with or without adenoviruses encoding p115-RGS (Fig. 2). However, the expression of C3 transferase completely prevented β1 integrin-mediated Rho activation (Fig. 2). These results suggest that β1 integrin induces Rho activation in osteoblasts. On the other hand, Rho activation induced by β1 integrin stimulation was not inhibited in osteoblasts infected with adenoviruses encoding p115-RGS. Therefore, p115RhoGEF does not seem to be involved in β1 integrin-mediated Rho signaling pathway in osteoblasts. In other words, adenovirus encoding p115-RGS can be considered a control vector.

Rho-mediated signaling induces ICAM-1 and RANKL expression on osteoblasts and MG63

ICAM-1 and RANKL expression on osteoblasts and MG63 with or without infection with adenoviruses encoding control vector (GFP or p115-RGS) were induced by

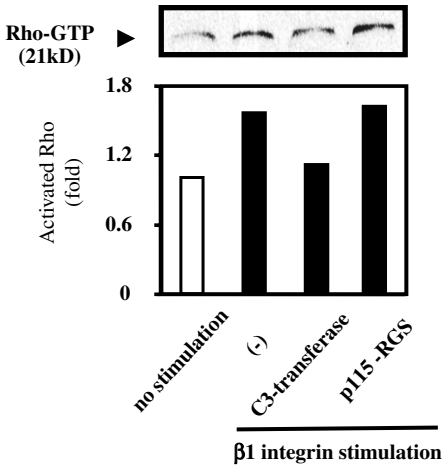

Fig. 2. Rho was activated by β1 integrin stimulation on osteoblasts. Osteoblasts were infected with or without adenoviruses encoding GFP (control vector), the Rho inhibitor C3 transferase, or p115-RGS. Cells were cultured for 48 h in DMEM containing 1% FCS, and then reseeded onto plastic plates precoated with BSA and type I collagen (10 μg/ml) at 37 °C for 6 h. Rho activity is indicated by the amount of RBD-bound Rho (top), and is expressed as fold increase relative to unstimulated control (bar graph). Results are representative of five different experiments. Western blot analysis confirmed that equal amounts of total Rho were used for pull-down assay under each condition (data not shown).

adhesion to type I collagen (Fig. 3). In contrast, type I collagen-induced up-regulation of ICAM-1 and RANKL on osteoblasts infected with adenoviruses encoding C3 transferase was completely inhibited (Fig. 3A and B). And we confirmed similarly changes on human osteoblast-like cell line MG63 (Fig. 3C and D). Next, we pretreated MG63 with or without several concentrations of Rho-kinase inhibitor Y-27632, and then determined ICAM-1 (Fig. 3E) and RANKL (Fig. 3F) expressions by FACScalibur. Y-27632 showed a dose-dependent inhibition of both ICAM-1 and RANKL expression. These data suggest that small GTPase Rho signaling augments ICAM-1 and RANKL expression on primary osteoblasts and MG63.

Rho inhibitor C3 transferase completely inhibits TRAP+MNC formation in osteoblast from peripheral monocytes

Osteoblasts were transfected with or without adenoviruses vector encoding GFP (control vector) or C3 transferase. In a coculture system using osteoblasts with peripheral monocytes, osteoblasts reseeded onto BSA- or type I collagen-precoated dishes, were evaluated for the formation of TRAP+MNC from the monocytes. The formation of TRAP+MNC from the monocytes was markedly induced by adhesion to type I collagen. However, Rho inhibitor C3 transferase completely inhibited TRAP+MNC formation in osteoblasts from peripheral monocytes (Fig. 4). These results suggest that Rho is involved in β1 stimulation, and this helps commit hematopoietic precursors toward osteoclast development.

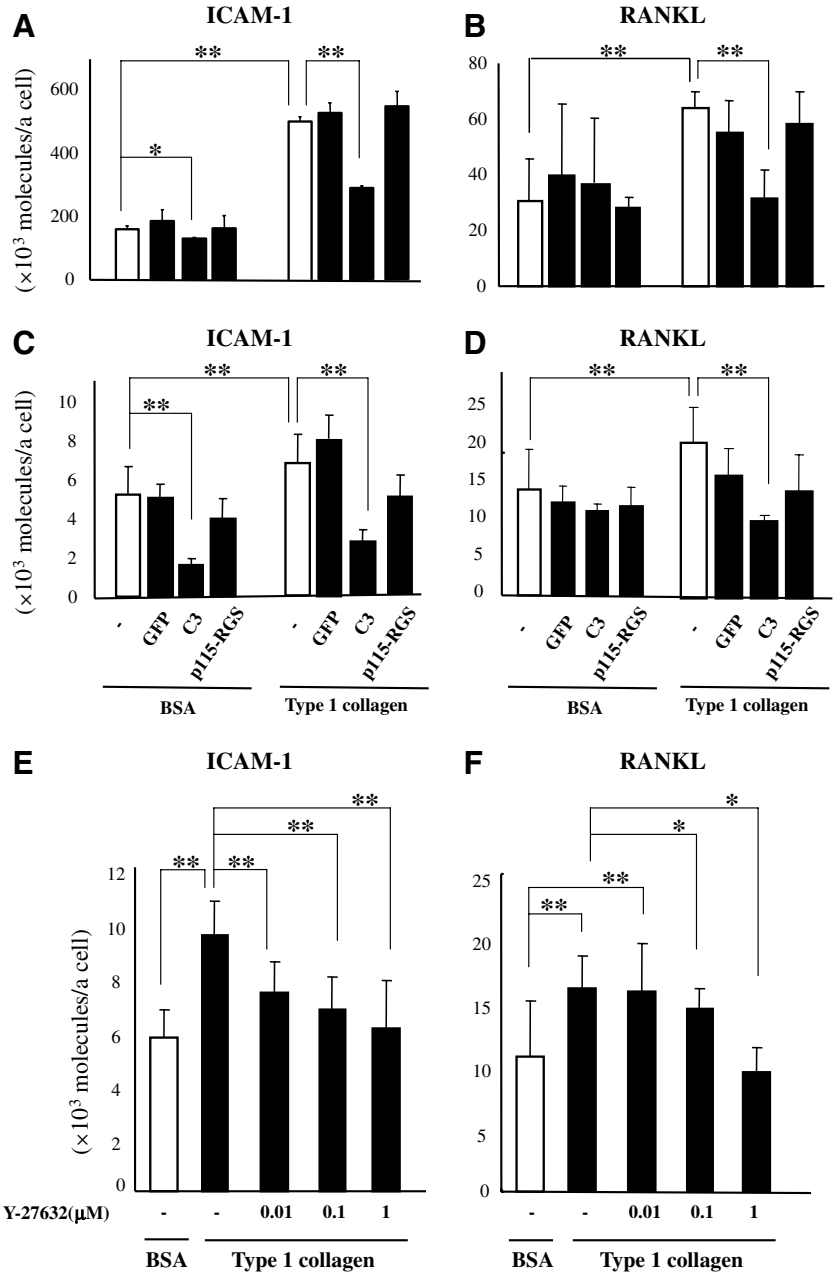

Fig. 3. Involvement of Rho-mediated signaling in β1 integrin-induced ICAM-1 and RANKL expression on osteoblasts. Primary osteoblasts (A,B) and MG63 (C,D) infected with or without adenoviruses encoding GFP, C3 transferase or p115-RGS were cultured for 48 h in DMEM containing 1% FCS, and MG63 (E,F) were pretreated with or without the indicated concentration of Rho-kinase inhibitor Y-27632 for 30 min. Next, all cells were reseeded onto plastic plates precoated with BSA and type I collagen (10 μg/ml) at 37 °C. At 6 h after osteoblasts and MG63 were reseeded, ICAM-1 and RANKL expression levels on primary osteoblasts (A,B) and MG63 (C–F) were determined by FACScalibur from five independent experiments. Each value represents the number of molecules expressed per one cell, calculated using standard QIFKIT beads from five independent experiments. Data are expressed as means ± SD. $^{*}P < 0.05$,$^{**}P < 0.01$.

Discussion

In this study, we observed the relevance of small GTPase Rho to β1 integrin-mediated signaling in osteoblasts. β1 Integrins were highly expressed on osteoblasts and engagement of β1 integrins could induce Rho activation, expression of ICAM-1 and RANKL, and subsequent osteoclast maturation.

β1 Integrins are major adhesion receptors that mediate interactions between osteoblasts and ECMs found in bone, such as collagen, fibronectin, osteopontin, thrombospondin, and vitronectin. Several studies indicate that α2β1 and α1β1 integrins play a role in the osteoblastic differentiation induced by bone morphogenetic protein (BMP-2) and matrix mineralization [22]. However, we have previously reported that β1 integrin/FAK-mediated signaling is

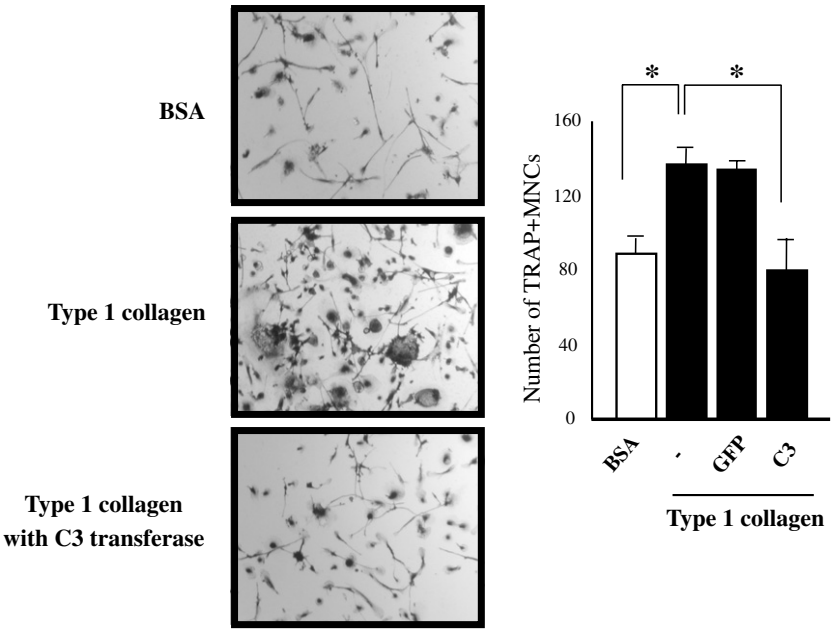

Fig. 4. Rho inhibitor C3 transferase completely inhibits TRAP+MNC formation in osteoblasts from peripheral monocytes. Osteoblasts were transfected with or without adenovirus vector encoding GFP (control vector), C3 transferase. Then, in a coculture system using osteoblasts with peripheral monocytes, osteoblasts reseeded onto dishes precoated with BSA or type I collagen, were evaluated for the formation of TRAP+MNC from the monocytes. Data are representative of five independent experiments. Data represent the means ± SD number of TRAP+MNC in one well. $^{*}P < 0.01$.

involved in ICAM-1 and RANKL induction of osteoblasts, and affects cellular adhesion between osteoblasts and osteoclast precursors through the ICAM-1/LFA-1 and RANKL/RANK pathways and leads to differentiation of osteoclast progenitors to osteoclasts [14]. Thus, β1 integrin-mediated signaling on osteoblasts could control two paradoxical processes; bone formation and bone resorption.

Concerning bone metabolism, small GTPases play important roles because they are molecular switches that act as ''hub'', which radiates multiple signaling pathways critical for diverse cellular functions. We previously reported the relevance of H-Ras and its downstream effectors to functions of osteoblasts and proposed that H-Ras signals, especially those followed by the Raf-1/mitogen-activated protein kinase (MAPK) pathway, but not PI 3-K, induce cell-cycle arrest and subsequent apoptosis presumably via the reciprocal regulation of Fas/Bcl-2 expression [23]. Among several small GTPases, Rho proteins are essential for focal adhesion formation when cells are plated on a matrix. Furthermore, integrins regulate signaling pathways to members of Rho GTPases, Rho, Rac and Cdc42, which are molecular switches that control the dynamics and structure of actin-based processes, such as filopodia, lamellipodia, and stress fiber formation [2]. It has been reported that cytoskeletal rearrangements are under the control of Rho family GTPases and require functional integrin αvβ3 in osteoclasts, and Rho-dependent Rho kinase activation increases bone resorption in osteoclasts [7]. However, the relevance of Rho-mediated signaling to osteoblast function is largely unknown.

In the present study, we found that β1 integrin stimulation increased the amount of activated Rho in osteoblasts infected with or without adenoviruses encoding control vector. Since β1 integrin-induced Rho activation was completely blocked by transfection of C3 transferase, Rho signaling is involved in β1 integrin-mediated signaling in osteoblasts. We also observed a complete inhibition of β1 integrin-mediated up-regulation of ICAM-1 and RANKL on C3 transferase-expressing primary osteoblasts and osteoblastic cell line MG63. Similarly, Rho-kinase inhibitor Y-27632 completely inhibited β1 integrin-mediated up-regulation of both ICAM-1 and RANKL expression on MG63. Furthermore, up-regulation of TRAP+MNC formation by β1 integrin stimulation was completely abrogated by infection with adenoviruses encoding C3 transferase. These findings suggest that Rho-mediated signaling followed by β1 integrin stimulation positively controls ICAM-1 and RANKL expression on osteoblasts and subsequent osteoclast maturation. On the other hand, several studies have indicated that Rho signaling inhibits proliferation and differentiation of osteoblasts. *Pasuteurella multocida* toxin (PMT), a bacterial toxin that activates Rho, inhibits osteoblast differentiation through by the down-regulation of BMP-2 and BMP-4 expression [24]. Moreover, the ROK inhibitor Y-27632 stimulates the expression of BMP-4 and osteoblast marker, ALP and OC [24]. These results suggest that Rho and its effecter protein ROK pathway is an important negative regulator of osteoblast differentiation. Thus, it seems that Rho-mediated signaling in osteoblasts may introduce major biases to bone resorption.

In conclusion, our novel findings suggest a pivotal relevance of Rho-mediated signaling to osteoblast function, and a pivotal role in osteoclastogenesis. A rational design of future therapeutic strategies for osteoporosis could per-

haps include the exploitation of Rho pathway to modulate osteoblast function directly.

Acknowledgments

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