Chick osteocyte-derived protein inhibits osteoclastic bone resorption

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In order to investigate the role of osteocytes in bone resorption, we examined the homogenate and conditioned medium from purified chick calvarial osteocytes in a pit-formation assay using unfractionated bone cells from mice. The osteocyte homogenate markedly inhibited pit formation, whereas the conditioned medium of osteocytes had no effect. This inhibitory activity was not the result of cytotoxicity of the homogenate. A novel bone-resorption-inhibitory protein was purified from collagenase-digested chick calvarial fragments enriched in osteocytes. The inhibitory protein, of molecular mass 18.5 kDa, showed signifi-

cant dose-dependent inhibition of pit formation by unfractionated bone cells from mice and rabbits, and by human giant tumour cells. This protein also inhibited the bone-resorbing activity of purified osteoclasts in the pit-formation assay in the absence of other effector cells. Microinjection of the protein into osteoclasts caused disruption of the podosomes in the cells. The N-terminal 25-amino-acid sequence of the protein showed 68 % identity to a part of Rho-GTP-dissociation inhibitor. Thus chick calvarial osteocytes may be involved in the regulation of bone resorption by osteoclasts.

INTRODUCTION

Bone tissue contains a variety of cell types, including osteoclasts, osteoblasts and osteocytes. Osteoclasts and osteoblasts exist on the bone surface, and work functionally in the respective degradation and formation of bone constituents [1–3]. Bone is constantly remodelled by these cells through cycles of resorption/formation, and the bone mass is maintained by coupling of their functions [4,5]. On the other hand, osteocytes, which originate from osteoblasts, are the only type of bone cell that is enclosed within bone tissue [6–9]. More than 90 % of the bone cells in normal bone tissue are osteocytes [10]. However, the function of these cells in bone metabolism is not clearly understood. Osteocytes in calcified bone matrix contact each other or surface bone cells through a large number of cytoplasmic processes via gap junctions [6–8,11–13], and are suggested to control the differentiation of osteoblasts into osteocytes [8,11,12]. Osteocytes are also reported to express the mRNA of the bone-formation-stimulatory proteins insulin-like growth factor-1 [14] and bone morphogenetic proteins BMP-6 and -7 [15]. These findings suggest that osteocytes are involved in the modulation of osteoblast generation and bone formation. However, little is known about the role of osteocytes in osteoclastic bone resorption.

In the resorption phase of bone remodelling, mature osteoclasts degrade bone constituents by secreting H^+ and cysteine proteases into the osteoclast–bone interspace [2,16], and finally osteoclasts stop their function and leave the bone surface [2]. In this process, osteoclasts are thought to excavate a large number of osteocytes from the bone collagen matrix. Some of these excavated osteocytes and/or residual collagen fibril fragments containing osteocytes are incorporated into osteoclasts by phagocytosis [2,17–20]. In electron microscopic studies, osteocytes are shown to be engulfed and destroyed by osteoclasts during bone degradation [17,20]. Therefore it is speculated that osteocytes may transmit some signals to osteoclasts after the osteoclast–osteocyte

encounter or after their incorporation into osteoclasts. In the present study, in order to investigate the role of osteocytes in bone resorption, we examined the effects of purified chick osteocyte homogenate and an osteocyte-derived protein on osteoclastic bone resorption in a pit-formation assay, an *in vitro* model of osteoclastic bone resorption [21].

MATERIALS AND METHODS

Materials

The materials used in this study and their sources were as follows: α -minimal essential medium (α -MEM), from Flow Laboratories (McLean, VA, U.S.A.); collagenase (type I; from *Clostridium histolyticum*) and acrylamide, from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Cellmatrix (1-A) and collagenase (S-1) for collagen gel matrix culture, from Wako Pure Chemical Co. (Osaka, Japan); salmon calcitonin, from Peninsula Laboratories Inc. (Belmont, CA, U.S.A.); Q-Sepharose fast flow, Sephadex G-50, Mono Q and Superose 12, from Pharmacia-LKB (Piscataway, NJ, U.S.A.). Fluorescein isothiocyanate (FITC)-labelled α -actinin was generously supplied by Dr. S.-S. Lim and Dr. D. R. Swartz (Indiana University, Indianapolis, IN, U.S.A.). The dye/protein ratio used was 2.6:1 (as the dimer).

Chick embryos (16-day) and 5-day-old white rabbits were purchased from Ohmiya Poultry Experiment Station (Ohmiya, Saitama, Japan); 13-day-old ICR mice were from Nippon SLC (Shizuoka, Japan). Human giant tumour cells were prepared and generously provided by Dr. Y. Mori (University of Tokyo School of Medicine, Tokyo, Japan).

Preparation of cell populations for the pit-formation assay

Unfractionated bone cells were prepared from 13-day-old ICR mice or 5-day-old white rabbits. In brief, the femurs and tibiae of

Abbreviations used: α -MEM, α -minimal essential medium; FBS, fetal bovine serum; PBS(–), Ca- and Mg-free PBS; TRAP, tartrate-resistant acid phosphatase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; FITC, fluorescein isothiocyanate; GDI, GTP-dissociation inhibitor. § To whom all correspondence should be addressed.

mice or rabbits were minced and shaken gently in 5% (v/v) fetal bovine serum (FBS) in α -MEM for 30 s. After removal of bone particles, this suspension of unfractionated bone cells was used for the pit-formation assay.

Human giant tumour cells, stored in liquid nitrogen, were thawed and suspended in 5% FBS/ α -MEM and then centrifuged at 1400 rev./min for 5 min. The supernatant was discarded, and the pellet was resuspended in the medium and centrifuged again. The pelleted cells were resuspended in the same medium and used for the pit-formation assay.

Purified osteoclasts were prepared from unfractionated bone cells from rabbits by the method of Kakudo [21]. Briefly, crude cells were inoculated on to Cellmatrix (type-I collagen gel matrix), and cultured for 4 h. After having been washed with Ca- and Mg-free PBS [PBS(–)], cells were sequentially treated with 0.001% Pronase E/0.02% EDTA in PBS(–) at 37 °C for 15 min and 0.02% collagenase in PBS(–) at room temperature for 7 min. Cells released from Cellmatrix by each enzyme treatment were removed step by step. The cells still attached to Cellmatrix were retreated with 0.1% collagenase in PBS(–) at room temperature for 1 min, and the released cells were collected and washed with 5% FBS/ α -MEM by centrifugation at 500 rev./min for 3 min. The pelleted cells were suspended in the medium, inoculated on to dentine slices and used for the pit-formation assay. To examine the purity of the isolated cells, the cells on dentine slices were stained for tartrate-resistant acid phosphatase (TRAP) by the method of Burstone [22], and the number of TRAP-positive and multinucleate cells (three or more nuclei) was determined under a microscope. More than 95% of the cells were multinuclear and positive for TRAP activity.

Pit-formation assay

The pit-formation assay was performed by the method of Takada et al. [23] with a slight modification. The prepared cells were inoculated on to dentine slices in 96-well dishes and cultured for 2 h. The cells were then incubated at 37 °C in a 10% CO₂/90% air atmosphere in 5% FBS/ α -MEM containing the indicated samples. At the end of the culture period, all the cells on the dentine slices were brushed off, and the pits formed on the slices were stained with acid haematoxylin. Using a microscope with a mesh glass installed in the ocular lens, the number of mesh squares covering the pits was counted. Differences in mean values were analysed statistically by Student's *t* test.

For the determination of the cytotoxicity of samples, cells on dentine slices were washed with PBS(–) and treated with 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) for 2 h prior to being brushed off the slices. MTT-formazan formed in viable cells was extracted with DMSO, and its absorbance was measured at 540 nm.

Preparation of osteocyte-rich calvarial fragments and isolation of osteocytes

Osteocyte-rich calvarial fragments were prepared from 16-day chick embryo calvariae as described below. The periosteum of the calvariae was removed, and both the non-calcified and the marrow-invaded parts were cut off. The parietal bones were minced into small fragments (1 mm × 1 mm). The fragments were digested with 260 units/ml bacterial collagenase in the isolation medium (25 mM Hepes, 10 mM NaCl, 3 mM K₂HPO₄, 1 mM CaCl₂, 30 mM KCl, 1 mg/ml BSA, 5 mg/ml glucose, 7.5 μ M α -tosyl-L-lysyl chloromethane) at 37 °C for 30 min. The fragments were washed 10 times by shaking with PBS(–). In the process, most of the blood cells and surface bone cells were

removed, such that only osteocytes were contained in the fragment. For the preparation of sections of the collagenase-digested fragments, the fragments were fixed in 2.5% glutaraldehyde, dehydrated in ethanol and embedded in Spurr's resin. Sections of 1 μ m were stained with Toluidine Blue. The osteocyte-rich calvarial fragments were used as a source for the isolation of osteocytes and the purification of osteocyte-derived protein.

Osteocytes were further prepared from the fragments by the method of Tanaka et al. [24]. Briefly, the fragments were decalcified with 5 mM EDTA in PBS(–) for 30 min. After having been washed three times with isolation medium, the fragments were again treated with collagenase under the same conditions. The released cells were collected and washed with 2% FBS/ α -MEM, and filtered through a fine mesh (8 μ m). The filtered cells were then inoculated into 35-mm culture dishes and, after incubation at 37 °C for 30 min, non-adherent cells were removed. More than 95% of the adhesive cells were stellate-shaped, and possessed a large number of cytoplasmic processes. These cells were negative for alkaline phosphatase activity, and were not proliferative.

Preparation of homogenate and conditioned medium of osteocytes

Osteocyte homogenate was prepared as follows. Freshly isolated osteocytes (1 × 10⁶ cells) prepared as described above were homogenized in 500 μ l of PBS(–) containing 1% octyl glucoside. The homogenate was centrifuged at 10000 *g* for 10 min. The supernatant was removed and dialysed against octyl glucoside-free PBS(–) at 4 °C for 18 h.

For preparation of conditioned medium, isolated osteocytes (6 × 10⁴ cells) were cultured in 2% FBS/ α -MEM (1 ml) for 16 h. Then the medium was changed to α -MEM containing 0.1% BSA instead of 2% FBS. After a 2-day culture period, the medium was collected and dialysed against PBS(–) at 4 °C for 18 h.

Preparation of homogenate of osteocyte-rich calvarial fragments and purification of osteocyte-derived protein that inhibits bone resorption

The homogenate of osteocyte-rich calvarial fragments was prepared as follows. Osteocyte-rich calvarial fragments prepared as described above were homogenized in 20 mM Tris/HCl (pH 8.0) containing 2 M NaCl and 5 mM EDTA with a Bio-mixer (Nissei) on ice for 15 min. The homogenate was centrifuged at 10000 *g* for 15 min, and the supernatant was removed and dialysed against 20 mM Tris/HCl (pH 8.0) at 4 °C for 18 h.

The dialysed homogenate was adsorbed to a column of Q-Sepharose fast flow equilibrated with 20 mM Tris/HCl (pH 8.0) and eluted with a linear gradient of 0–1 M NaCl in the same buffer. The active fractions eluted at 0.18–0.28 M NaCl were collected, lyophilized and reconstituted with distilled water. This material was dialysed against 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl and applied to a column of Sephadex G-50 equilibrated with the same buffer. The active fractions were diluted with distilled water and applied to a column of Mono Q equilibrated with 20 mM phosphate buffer (pH 7.0). The flow-through fraction was applied to a column of Superose 12 equilibrated with 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl. The fractions from Superose 12 were analysed on SDS/PAGE. The bone-resorption-inhibitory activity of each fraction was determined by the pit-formation assay. The active fraction, which contained a single band of 18.5 kDa, was subjected to preparative native PAGE [25]. The protein was cut from the native PAGE gel, extracted with 10 mM potassium

phosphate buffer (pH 7.0) and dialysed against the same buffer. The purity of the protein was analysed on SDS/PAGE with silver staining. The extracted protein was examined for its effect on bone resorption.

Microinjection

Rabbit osteoclasts were isolated by the method described above. The cells were inoculated on to slide glasses and incubated for 12–16 h. FITC-labelled α -actinin was microinjected into the cytoplasm of osteoclasts using glass capillaries. The osteoclasts with labelled podosomes were selected 30 min after injection, and re-injected with 10 mM potassium phosphate buffer (pH 7.0) or the 18.5 kDa protein. After an additional 30 min incubation, the podosomes in the cells were examined under a fluorescence microscope.

Analysis of N-terminal amino acid sequence

For analysis of the N-terminal amino acid sequence of the bone-resorption-inhibitory protein, the protein on the SDS/PAGE gel was transferred to poly(vinylidene difluoride) membranes at 0.8 mA/cm² for 2 h and stained with Coomassie Brilliant Blue. The sequence of the active protein was determined using an ABI Model 473A Protein Sequencer. Sequence data were analysed and compared with the databases of GenBank and FASTA.

RESULTS

Effects of crude preparations of osteocytes on osteoclastic bone resorption

Homogenates of freshly isolated chick calvarial osteocytes were examined for their effects on bone resorption in the pit-formation assay using unfractionated bone cells from mice (Table 1). The homogenate was used in the assay at a concentration of 25 or 50 μ g of protein/ml (equivalent to 1.25×10^4 or 2.5×10^4 osteocytes). At both concentrations, pit formation was significantly inhibited by the osteocyte homogenate (Table 1). In contrast, the homogenates of osteoblastic cell lines (MC3T3-E1 and ROS17/2.8), prepared by the same method as described for the osteocyte homogenate, did not inhibit pit formation at 50 μ g of protein/ml. The number of viable cells on the dentine slices was not affected by the addition of any of the homogenates (Table 1), indicating that the inhibitory activity of the osteocyte homogenate was not a result of cytotoxicity.

The conditioned medium of purified osteocytes was also

Table 2 Effect of conditioned medium of osteocytes on pit formation

Unfractionated bone cells from mice were inoculated on to dentine slices (6×10^5 /well) and incubated for 3 days in the absence or presence of salmon calcitonin or osteocyte conditioned medium (CM) diluted 1:5 or 2:5 (v/v). Values are means for two wells.

Addition	Concn./dilution	Pit formation (% of control)
None		100.0
Calcitonin	0.1 μ M	26.3
Osteocyte CM	1:5 (v/v)	117.8
	2:5 (v/v)	105.9

Figure 1 Photomicrograph of a section from a chick embryonic calvarial fragment digested by collagenase

Table 3 Inhibition of pit formation by a homogenate of osteocyte-rich calvarial fragments and stability of the inhibitory activity

Unfractionated bone cells from mice were inoculated on to dentine slices (6×10^5 /well) and incubated for 3 days in the absence or presence of the calvarial homogenate (73 μ g of protein/ml). Values are means $\pm$ S.D., $n = 3$. Significance is indicated in comparison with untreated control value (* $P < 0.05$, ** $P < 0.01$).

Addition	Pit formation (% of control)
None	100.0 $\pm$ 10.2
Calvarial homogenate	
No treatment	31.8 $\pm$ 14.1**
37 °C, 30 min	43.7 $\pm$ 10.3**
60 °C, 30 min	66.4 $\pm$ 6.4*
100 °C, 30 min	91.4 $\pm$ 21.2
Trypsin (10 μ g/ml, 1 h)	87.3 $\pm$ 9.0

examined in the pit-formation assay (Table 2). The conditioned medium was added to the assay at a dilution of 1:5 or 2:5 (v/v) (equivalent to 1.2×10^4 or 2.4×10^4 osteocytes). At both concentrations the conditioned medium had no effect on pit formation, suggesting that the osteocyte-derived inhibitor(s) was not secreted into the extracellular environment under normal culture conditions.

The homogenates of osteocyte-rich calvarial fragments were also examined for their effects on bone resorption. A photomicrograph of a section from a fragment is shown in Figure 1. The fragment showed no marrow invasion, and there were few cells on the surface. More than 98 % of the cells were present in the osteocytic lacunae within the bone matrix, suggesting that the fragment contained only osteocytes. The homogenate of the fragments showed inhibitory activity in the pit-formation assay (Table 3). In order to examine the nature of the inhibitor(s), we denatured the homogenate by heat treatment or hydrolysed it enzymically with trypsin. Untreated homogenate at 73 μ g of protein/ml inhibited pit formation by 68.2 % (Table 3). The inhibitory activity was decreased to 33.6 % by mild heat treatment (60 °C, 30 min), and was abolished by severe heat treatment

Table 1 Effects of osteocyte homogenate on pit formation and viability of unfractionated bone cells from mice

Unfractionated bone cells from mice were inoculated on to dentine slices (6×10^5 /well) and incubated for 3 days in the absence or presence of salmon calcitonin or homogenates of osteocytes, MC3T3-E1 cells or ROS17/2.8 cells. Values are means $\pm$ S.D., $n = 3$. Significance is indicated in comparison with the untreated control value (** $P < 0.01$).

Addition	Concn.	Viable cells (% of control)	Pit formation (% of control)
None	—	100.0 $\pm$ 3.8	100.0 $\pm$ 18.1
Calcitonin	0.1 μ M	107.9 $\pm$ 3.1	29.5 $\pm$ 6.2**
Osteocyte homogenate	25 μ g of protein/ml	102.2 $\pm$ 1.9	43.0 $\pm$ 3.9**
	50 μ g of protein/ml	99.0 $\pm$ 4.2	20.7 $\pm$ 11.1**
MC3T3 homogenate	50 μ g of protein/ml	92.8 $\pm$ 12.7	108.2 $\pm$ 13.7
ROS homogenate	50 μ g of protein/ml	104.6 $\pm$ 6.5	109.1 $\pm$ 8.2

Figure 2 Superose 12 elution profile (A) and SDS/PAGE (B) of a bone-resorption-inhibitory protein

An aliquot of each fraction from Superose 12 was analysed by a pit-formation assay and by SDS/PAGE (15% polyacrylamide gel) with silver staining.

(100 °C, 30 min) or trypsin treatment (10 µg/ml, 37 °C, 1 h), indicating that the inhibitor(s) contained a protein moiety.

Analysis of a bone-resorption-inhibitory protein derived from osteocytes

The osteocyte-derived protein that inhibited osteoclastic bone resorption was purified from osteocyte-rich calvarial fragments as described in the Materials and methods section. A homogenate of the fragments was fractionated by the column systems of Q-Sepharose fast flow, Sephadex G-50, Mono Q and Superose 12. The Superose 12 elution profile (Figure 2A) and SDS/PAGE of each fraction (Figure 2B) are shown. Fractions 16 and 17, which markedly inhibited pit formation, contained a band of apparent molecular mass 18.5 kDa (Figure 2). This protein was isolated by preparative native PAGE. The protein extracted from the gel showed a single form, of 18.5 kDa, on SDS/PAGE with silver staining (Figure 3). The purified protein was then subjected to activity studies.

The 18.5 kDa protein on the SDS/PAGE gel was transferred to a poly(vinylidene difluoride) membrane, and the N-terminal amino acid sequence was analysed. The N-terminal 25-amino-acid sequence of the protein was established, and revealed to show 68 % identity with a part of chick and bovine Rho-GDI (where GDI is GTP-dissociation inhibitor), a cytoplasmic protein (Figure 4).

Figure 3 SDS/PAGE of the purified inhibitory protein

Purified protein extracted from the native PAGE gel was analysed by SDS/PAGE (12% polyacrylamide gel) and stained with silver. Lane 1, molecular mass markers; lane 2, purified protein.

Figure 4 N-terminal amino acid sequence of the 18.5 kDa protein compared with those of chick and bovine Rho-GDI

Vertical lines between two amino acids indicate identity.

Figure 5 Effect of the 18.5 kDa protein on pit formation by unfractionated bone cells from mice and rabbits, and by human giant tumour cells

Unfractionated bone cells from mice (6×10^5 /well; ○) or rabbits (3×10^5 /well; □), or human giant tumour cells (8×10^4 /well; ▲), were inoculated on to dentine slices and incubated for 3 days in the absence or presence of the 18.5 kDa protein at the indicated concentrations. Values are expressed as a percentage of the appropriate control value.

Table 4 Effect of the 18.5 kDa protein on pit formation by purified osteoclasts

Purified osteoclasts (200/well) or unfractionated bone cells (4×10^5 /well) from rabbits were inoculated on to dentine slices and incubated for 24 h in the absence or presence of the 18.5 kDa protein (1.65 μ g/ml of medium). Values are means $\pm$ S.D., $n = 3$. Significance is indicated in comparison with the corresponding untreated control value (* $P < 0.05$).

Addition	Pit formation (% of control)
Unfractionated bone cells	
None	100.0 $\pm$ 17.0
18.5 kDa protein	47.4 $\pm$ 11.5*
Purified osteoclasts	
None	100.0 $\pm$ 13.3
18.5 kDa protein	47.9 $\pm$ 25.6*

Figure 6 Microinjection of the 18.5 kDa protein into rabbit osteoclasts

Rabbit osteoclasts were microinjected with buffer (A) or the 18.5 kDa protein (22 μ g/ml) (B) 30 min after preinjection of FITC-labelled α -actinin, and then incubated for a further 30 min. The two photographs were taken at the same magnification ($\times 220$).

Effect of the 18.5 kDa protein on osteoclastic bone resorption

The bone-resorption-inhibitory activity of the purified 18.5 kDa protein was determined in pit-formation assays using unfractionated bone cells from mice or rabbits, or human giant tumour cells (Figure 5). Pit formation by all three cell types was inhibited by the 18.5 kDa protein with a similar dose range. IC_{50} values were 24–56 nM.

In order to investigate whether this inhibitory protein acts directly on osteoclasts or through other effector cells, the protein was examined in the pit-formation assay using purified rabbit osteoclasts. The 18.5 kDa protein showed inhibitory effects on pit formation by purified osteoclasts (Table 4). The inhibitory activity towards purified osteoclasts corresponded with that towards unfractionated bone cells, suggesting that the 18.5 kDa protein acts directly on osteoclasts and not via other effector cells.

To examine whether the 18.5 kDa protein can act on osteoclasts within the cells, the protein was microinjected into rabbit osteoclasts and the podosomes in the cells were examined. FITC-labelled α -actinin pre-microinjected into osteoclasts was incorporated into podosomes which lined the periphery of the cells.

Although the podosomes were still observed after the injection of buffer (Figure 6A), microinjection of the 18.5 kDa protein caused disruption of the podosomes within 30 min of the injection (Figure 6B) [percentage of osteoclasts without podosomes at 30 min after injection: buffer, 8.7% ($n = 25$); 18.5 kDa protein, 87.5% ($n = 32$)].

DISCUSSION

In the process of bone resorption, osteoclasts encounter a large number of osteocytes excavated from the bone tissue. For instance, osteocytes released from the collagen matrix have been shown to be engulfed into osteoclasts by phagocytosis [2,17–20]. Therefore it is possible that osteocytes may transmit some signals to osteoclasts after the osteoclast–osteocyte encounter or after incorporation of osteocytes into osteoclasts. In the present study, marked inhibitory activity towards osteoclastic bone resorption was found for a chick osteocyte homogenate and a collagenase-digested chick calvarial homogenate. The inhibitory activity was not detected in the conditioned medium from chick osteocytes. These results suggest that some bone-resorption-inhibitory protein(s) is present in osteocytes, and that this inhibitory protein(s) is not secreted into extracellular environment under normal conditions. Our data also suggest that the inhibitory activity emerges after osteoblasts have differentiated into osteocytes, as the homogenates of osteoblastic cell lines such as MC3T3-E1 and ROS17/2.8 had no effect on osteoclastic bone resorption.

An osteocyte-derived inhibitory protein was prepared from chick calvarial fragments from which non-osteocytic cells on the bone surface had been removed by collagenase digestion. The purified protein, of molecular mass 18.5 kDa, significantly inhibited pit formation by unfractionated bone cells from mice and rabbits, as well as that by human giant tumour cells. These data indicate that this protein forms a part of the regulatory system of osteoclastic bone resorption in several species. In addition, this protein was shown to act directly on osteoclasts to inhibit their bone-resorbing activity, because the inhibitory activity was observed in a pit-formation assay using purified osteoclasts.

The N-terminal amino acid sequence of the 18.5 kDa protein was 68% identical to a partial sequence of chick and bovine Rho-GDI. The Rho family of proteins belongs to the Ras-related small G-protein superfamily [26–28]. It is known that Rho is concerned with cytoskeletal control via the actomyosin system [29–31]. It has been shown that microinjection of exoenzyme C3 of *Clostridium botulinum*, by which Rho was ADP-ribosylated and inactivated, caused disappearance of the F-actin-containing podosomes of osteoclasts, resulting in their functional inactivation [32]. Rho-GDI is an GDP/GTP-exchange-inhibitory protein for the Rho family members Rho, Rac and G25K [33–36]. The microinjection of Rho-GDI into Swiss 3T3 cells was shown to make the cells become round by causing the disappearance of stress fibres [36]. Microinjection of the 18.5 kDa protein also led to the disappearance of the podosomes from osteoclasts, suggesting that the protein may be taken up by the cells and modulate the function of Rho by a mechanism similar to that of Rho-GDI.

In conclusion, we found that an osteoclastic bone-resorption-inhibitory protein is present in chick calvarial osteocytes. This inhibitory protein appears to affect osteoclasts directly, resulting in inhibition of their function. Our findings suggest that osteocytes may participate in the regulation of osteoclastic bone resorption.

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