Osteopontin is required for mechanical stress-dependent signals to bone marrow cells

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Abstract

Mechanical stress to bone plays a crucial role in the maintenance of bone homeostasis. It causes the deformation of bone matrix and generates strain force, which could initiate the mechanotransduction pathway. The presence of osteopontin (OPN), which is one of the abundant proteins in bone matrix, is required for the effects of mechanical stress on bone, as we have reported that OPN-null (OPN^{−/−}) mice showed resistance to unloading-induced bone loss. However, cellular mechanisms underlying the phenomenon have not been completely elucidated. To obtain further insight into the role of OPN in mediating mechanical stress effect on bone, we examined *in vitro* mineralization and osteoclast-like cell formation in bone marrow cells obtained from hind limb bones of OPN^{−/−} mice after tail suspension. The levels of mineralized nodule formation of bone marrow cells derived from the femora subjected to unloading were decreased compared with that from loaded control in wild-type mice. However, these were not

decreased in cells from OPN^{−/−} mice after tail suspension compared with that from loaded OPN^{−/−} mice. Moreover, while spreading of osteoclast-like cells derived from bone marrow cells of the femora subjected to unloading was enhanced compared with that from loaded control in wild-type mice, this enhancement of spreading of these cells derived from the femora subjected to unloading was not recognized compared with those from loaded control in OPN^{−/−} mice. These data provided cellular bases for the effect of the OPN deficiency on *in vitro* reduced mineralized nodule formation by osteoblasts and on enhancement of osteoclast spreading *in vitro* induced by the absence of mechanical stress. These *in vitro* results correlate well with the resistance to unloading-induced bone loss in OPN^{−/−} mice *in vivo*, suggesting that OPN has an important role in the effects of unloading-induced alterations of differentiation of bone marrow into osteoblasts and osteoclasts. *Journal of Endocrinology* (2007) **193**, 235–243

Introduction

Mechanical stimuli are known to be one of the crucial factors maintaining bone mass (Lian *et al.* 1996, Ducy *et al.* 2000, Manolagas 2000). For instance, while high impact sports and, moreover, very low magnitude, high frequency vibration could increase bone mass, prolonged bed rest and extended spaceflight leads to bone loss (Morey & Baylink 1978, Bikle & Halloran 1999, Rubin *et al.* 2001b). Mechanical stimuli loaded to bone have been thought to cause the deformation of bone matrix and generate strain force, which initiates the mechanotransduction pathway (Burger & Klein-Nulend 1999, Nomura & Takano-Yamamoto 2000). Each cell is attached to its surrounding matrix through membrane receptors called integrin for extracellular matrix protein (Rodan 1991, Ingber 1999). These transmembrane receptors could provide a direct link between the extracellular matrix and cytoskeleton and could mediate

changes in matrix strain (Wang *et al.* 1993, Shyy & Chien 1997, Wozniak *et al.* 2000). However, molecular mechanisms involved in these phenomena are not yet fully understood.

Osteopontin (OPN) is a phosphorylated acidic glycoprotein present in extracellular matrix of mineralized tissues and is one of the abundant non-collagenous proteins in bone matrix produced by osteoblasts, osteoclasts, and probably osteocytes (Rodan 1991, Butler *et al.* 1996, Denhardt & Noda 1998). This protein contains an arginine–glycine–aspartate sequence that is a major integrin-binding site and supports adhesion of bone cells to the mineralized matrix (Giachelli & Steitz 2000, Denhardt *et al.* 2001b). It has been reported that OPN is required for stress-induced bone remodeling, such as ovariectomy or ectopic bone disc implantation (Rittling & Denhardt 1999, Yoshitake *et al.* 1999, Asou *et al.* 2001, Ihara *et al.* 2001, Nemoto *et al.* 2001, Kitahara *et al.* 2003).

OPN has also been reported to be regulated by mechanical stress both *in vitro* and *in vivo* (Kubota *et al.* 1993, Harter *et al.* 1995, Klein-Nulend *et al.* 1997, Toma *et al.* 1997, Carvalho *et al.* 1998, Meazzini *et al.* 1998, Miles *et al.* 1998, Terai *et al.* 1999, Denhardt *et al.* 2001a, You *et al.* 2001, Morinobu *et al.* 2003). These observations could be related to the reciprocally coordinated regulation of OPN in bone remodeling. Recently, we reported that OPN is necessary for unloading-induced enhancement of bone resorption and suppression of bone formation *in vivo* (Ishijima *et al.* 2001, 2002). These data suggest that OPN plays a key role in conveying the effect of mechanical stress to osteoclasts and osteoblasts. However, molecular mechanisms of OPN function in mechanotransduction pathways are not completely elucidated.

Bone marrow stromal cells are known to play an integral part in bone formation by providing an osteoprogenitor cell source capable of differentiating into mature osteoblasts in response to mechanical stresses (Mauney *et al.* 2004, Koike *et al.* 2005). On the other hand, the osteopenic response to reduced mechanical stress has been known to be well associated with the decrease in the number of osteoprogenitor cells, proliferation and differentiation in bone marrow cells (Bikle *et al.* 1994, Keila *et al.* 1994, Barou *et al.* 1998, Kostenuik *et al.* 1999, Sakata *et al.* 1999, Sakai *et al.* 2002). These data indicate that mechanical stress, whenever it is applied or reduced, could affect the characteristics of not only osteoblasts and osteoclasts but also bone marrow cells. In addition, OPN is considered to be involved in these processes (Ishijima *et al.* 2001, 2002, Mauney *et al.* 2004).

Thus, in this study, we examined characteristics of bone marrow cells in the femora in OPN^{-/-} mice after tail suspension using mineralized nodule formation and osteoclast-like cell formation assay to elucidate the role of OPN in osteoblastic and osteoclastic differentiation of bone marrow cells regulated by mechanical stress.

Materials and Methods

Animals

Female wild-type and OPN^{-/-} mice in a 129/SV × C57BL/6 F2 background (Rittling *et al.* 1998) derived from the original heterozygous crosses were maintained as separate colonies. These mice were kept under controlled conditions at 24 °C with a ratio of 12 h light:12 h darkness cycle with the light cycle starting at 0700 h, fed with standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) and given tap water. Twelve-week-old female OPN^{-/-} and wild-type mice (24 mice in total) were used in the experiments and randomly assigned in equal numbers to loaded control and tail suspension groups. All experiments were conducted according to the institutional guidelines for animal welfare.

Tail suspension model

The mice were subjected to tail suspension as described previously (Ishijima *et al.* 2001). Briefly, a tape was applied to the surface of the tail to set a metal clip. The end of the clip was fixed to the overhead bar and the height of the bar was adjusted to maintain an approximately 30° head-down tilt with the hind limbs elevated above the floor of the cage. Mice were permitted to move within the cage using their forelimbs while their hind limbs were kept free of weight bearing. One-half of the mice in the tail suspension group was subjected to tail suspension for 7 days (*n*=6 per group). Loaded control mice were also housed individually under the same conditions without tail suspension for the same duration (7 days). After tail suspension, mice were anesthetized with pentobarbital and were killed by cervical dislocation. The femora and tibiae were separated from adherent muscles and connective tissues other than periosteum. The tibiae were fixed with 70% ethyl alcohol solution for subsequent analysis of bone and the femora were used for the experiments to evaluate bone marrow cells as described below. All experiments in this study were conducted twice (*n*=3 per group) and all data (*n*=6 per group) were evaluated.

Body weight of the animals

To monitor the effects of tail suspension on the general condition of these animals, body weight of the mice in first experiment (*n*=3 per each group, 12 in total) was measured every day during the experimental periods. Body weights of either loaded or tail suspended mice were not altered during experiments in both genotypes (data not shown). This confirms that stress can be considered minimal in our experiments, as previously concluded (Bikle *et al.* 1985). It is reported that extremely low-level stimulus may provide an effective biomechanical intervention for the bone loss (Rubin *et al.* 2001a). Therefore, when the mice were weighed, the tail-suspended mice were permitted to stand on all four legs for periods of no longer than 10 s. Body weight of the mice in second experiment (*n*=3 per each group, 12 in total) was not measured. As these two independent experiments showed similar results, we considered that the effect of mechanical stimulus by body weighing is minimal.

Micro-X-ray computed tomography (μ-CT) analysis of bone

For measurements of the bone volume/tissue volume (BV/TV), the bones were subjected to μ-CT analysis, using Musashi (Nittetsu-ELEX, Tokyo, Japan). The data were subsequently quantified using a Luzex-F automated image analysis system (NIRECO, Tokyo, Japan). The fractional BV/TV was measured in the area of 0.39 mm² with its closest and furthest edges at 0.34 and 0.62 mm distal to the growth plate of the proximal ends of the tibiae.

Bone marrow cell cultures

After 7 days of tail suspension, bone marrow cells were obtained from the right femora of either loaded or unloaded mice. The bone marrow was flushed from the bones. A single cell suspension was prepared by gentle pipetting and the number of total bone marrow cells was counted using a hemacytometer. The cells were plated into a 9.6 cm² culture well at a concentration of 5.0×10^3 cells/cm² and cultured in 5% CO₂ and 95% air in a humidified atmosphere.

Formation of mineralized nodule

Bone marrow cells were cultured in six-well plates in Minimum Essential Medium alpha modification (Sigma) supplemented with 10% fetal bovine serum (Life Technologies), $1 \times$ antibiotic-antimycotic (Life Technologies), 2.2 mg/ml sodium bicarbonate (WAKO, Osaka, Japan), 50 µg/ml ascorbic acid (Sigma) and 10 mM β -glycerophosphate (Sigma). The medium was changed every 3 days in culture. On the 21st day in culture, the cells were fixed with ethyl alcohol for 10 min, stained with a saturated solution of Alizarin Red S (Sigma) for 15 min, and washed with water. After drying the samples in the air, the dish surface area covered with dark stain representing mineralized nodules was measured using a Luzex-F automated image analysis system (NIRECO).

Osteoclast-like tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cell development

Bone marrow cells were cultured in Minimum Essential Medium alpha modification supplemented with 10% fetal bovine serum, $1 \times$ antibiotic-antimycotic, 2.2 mg/ml sodium bicarbonate, 10 ng/ml macrophage colony-stimulation factor (M-CSF; R&D Systems, Minneapolis, MN, USA), and 100 ng/ml soluble receptor activator of NF- κ B ligand (RANKL; supplied by Snow Brand Milk Products, Tokyo, Japan). The medium was changed on the fourth day in culture. On the ninth day in culture, the cells were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 5 min and fixed in ethyl alcohol with acetone for 1 min and dried in air. The samples were treated with 5 mg naphthol AS-MX phosphate (Sigma) dissolved in 0.5 ml *N,N*-dimethylformamide (WAKO) containing 50 mM sodium tartrate (WAKO) and 30 mg fast red violet LB salt (Sigma) in 50 ml 0.1 M sodium acetate buffer (pH 5.0) for 10 min at room temperature. After rinsing the samples with water, the number of TRAP-positive multinucleated (cells containing more than two nuclei) osteoclast-like cells was counted under a light microscope. Quantitative analysis of the cell spreading was conducted according to the method described previously (Teti *et al.* 1998). After taking the micrographs of the cells, the cell maximal diameter (stretch) was measured by using a Luzex-F automated image analysis system (NIRECO). For each sample, over 80 osteoclast-like cells were evaluated.

Statistical analysis

The results were presented as mean values $\pm$ S.E.M). Statistical analysis was performed by Mann-Whitney's *U*-test. A *P* value of <0.05 was considered to be statistically significant.

Results

OPN^{-/-} mice showed resistance to bone loss induced by tail suspension

We previously reported that OPN^{-/-} mice when they were subjected to tail suspension for 2 and 4 weeks showed resistance to unloading-induced reduction in BV/TV (Ishijima *et al.* 2001). In this study, neither the increase in osteoclast number nor the suppression of osteoblastic activities was observed in OPN^{-/-} mice subjected to unloading. Although OPN is known to be involved in bone resorption (Yoshitake *et al.* 1999), OPN deficiency-induced reduction in suppression of bone formation caused by unloading cannot be explained by the role of OPN in bone resorption because bisphosphonate did not block unloading-induced reduction in bone formation (Kodama *et al.* 1997). Therefore, these data clearly indicate that OPN has a crucial role in the process of signal transduction pathways of mechanical stress in bone. However, one possibility remained obscure that bone loss induced by the absence of mechanical stress by tail suspension could be recovered after 2 and/or 4 weeks of tail suspension in OPN^{-/-} mice, since unloading-induced reduction of bone formation rate was not recognized in OPN^{-/-} mice (Ishijima *et al.* 2001). Therefore, in this study, OPN^{-/-} and wild-type mice were subjected to tail suspension for 1 week to clarify this possibility. As presented in Fig. 1, in wild-type mice, BV/TV in the secondary spongiosa of the tibia after 7 days of tail suspension was significantly reduced compared with

Figure 1 Trabecular bone volume of the tibiae of loaded and unloaded mice. Fractional trabecular bone volume (BV/TV) was quantified based on the image of microcomputed tomography (μ CT) pictures of the tibiae after 1 week of either tail suspension (Susp) or loading (Load) in wild-type (wild-type) or OPN^{-/-} mice (OPN^{-/-}). Data are expressed as means and standard errors for wild-type and OPN^{-/-} mice. **P* < 0.05

that in loaded control. In contrast, this reduction of BV/TV was not observed in OPN^{-/-} mice even after 7 days of tail suspension (Fig. 1). These data denied the possibility that OPN^{-/-} mice showed early recovery from unloading-induced bone loss as mentioned above. Therefore, these results, combined with our previous studies (Ishijima *et al.* 2001, 2002), clearly indicate that OPN is necessary for unloading-induced bone loss *in vivo*.

Formation of mineralized nodules of bone marrow cells obtained from femora subjected to unloading was reduced compared with that in of cells from loaded control, and OPN is required for this process

To examine the cellular activities underlying the phenomenon in OPN^{-/-} mice, we next evaluated the characteristics of bone marrow cells after tail suspension. The numbers of bone marrow cells in the unloaded right femora were not altered compared with loaded control in either genotype after 7 days of tail suspension (Fig. 2).

In our previous report, while bone formation rate and mineral apposition rate were significantly reduced after tail suspension in wild-type mice, such reduction was not recognized in OPN^{-/-} mice (Ishijima *et al.* 2001, 2002). In this study, we first examined the cells involved in bone formation by estimating the efficiency in mineralized nodule formation. We used bone marrow cells prepared from mice after unloading in both genotypes. Mineralized nodules were observed in the bone marrow cells from both loaded and tail-suspended wild-type and OPN^{-/-} animals cultured in the presence of ascorbic acid and β -glycerophosphate in the cells (Fig. 3A). However, the formation of mineralized nodules in the cells obtained from tail-suspended wild-type animals was less compared with the cells obtained from loaded wild-type animals (Fig. 3A, wild-type Load versus Susp). On the other

Figure 2 Number of bone marrow cells of loaded and unloaded mice. Bone marrow cells were obtained from the right femora after tail suspension (Susp) or loading (Load) in wild-type mice (wild-type) or OPN^{-/-} mice (OPN^{-/-}). The number of the cells was counted as described in Materials and Methods. Data are expressed as means and standard errors for wild-type and OPN^{-/-} mice.

hand, in OPN^{-/-} mice, this reduction in the formation of mineralized nodules in the cells obtained from tail suspended animals was not observed compared with the cells obtained from loaded animals (Fig. 3A, OPN^{-/-} Load versus Susp).

Quantitative analysis of the mineralized nodule formation showed that *in vitro* formation of mineralized nodules in the cells obtained from the femora in loaded OPN^{-/-} mice was similar compared with that of the cells from loaded wild-type mice (Fig. 3B, OPN^{-/-} Load versus wild-type Load). Formation of mineralized nodules in the cells from tail-suspended wild-type mice was significantly reduced compared with that of the cells from loaded wild-type mice (Fig. 3B, 84% reduction, $P < 0.05$). In contrast, in the cells obtained from OPN^{-/-} mice, the reduction of formation of mineralized nodules in cells obtained from tail suspended mice was not recognized when it was compared with that of the cells obtained from loaded mice (Fig. 3B, 21% reduction, $P = 0.28$). These results showed that unloading decreased the ability to form mineralized nodules in bone marrow cells and OPN has a crucial role in transmitting the signals of mechanical stress to the bone marrow cells.

Unloading enhances spreading of osteoclast-like cells and this enhancement of spreading of osteoclast-like cells was impaired due to the absence of OPN in vitro

We next examined the cells involved in bone resorption by *in vitro* osteoclastogenesis induced by the treatment with RANKL and M-CSF in the bone marrow cells. We utilized these two cytokines, RANKL and M-CSF, as described elsewhere (Ross & Teitelbaum 2005). The numbers of TRAP-positive multinucleated osteoclast-like cells developed in the cultures of bone marrow cells obtained from loaded OPN^{-/-} mice were similar to those in loaded wild-type mice (Table 1). Unloading of both OPN^{-/-} and wild-type mice did not affect the numbers of osteoclast-like cells developed in the bone marrow cells obtained from these mice (Table 1).

However, as shown in the pictures in Fig. 4A, unloading enhanced spreading of osteoclast-like cells in bone marrow cell cultures compared with that of bone marrow cell cultures obtained from loaded wild-type mice. Quantitative analyses according to the method described previously (Teti *et al.* 1998) indicated that the maximal diameters of the osteoclast-like cells developed in the marrow cells from tail suspended wild-type mice were significantly greater than those from loaded mice in wild-type (Fig. 4B, 66.6% increase, $P < 0.05$).

Spreading of the TRAP-positive cells derived from bone marrow cells obtained from loaded OPN^{-/-} mice was similar in comparison with that of the loaded wild-type mice (Fig. 4A). However, as shown in the pictures in Fig. 4A, spreading of the TRAP-positive cells derived from bone marrow cells obtained from unloaded OPN^{-/-} mice was similar compared with that of cells from loaded OPN^{-/-} mice. Quantification of the spreading by measuring maximal diameters of the TRAP-positive multinucleated cells indicated that unloading-induced enhancement of spreading of

Figure 3 Mineralized nodule formation in the cultures of bone marrow cells obtained from loaded and unloaded mice. (A) Bone marrow cells from right femora after tail suspension (Susp) or loading (Load) in wild-type mice (wild-type) or OPN^{-/-} mice (OPN^{-/-}) were cultured in the presence of 50 µg/ml ascorbic acid and 10 mM β-glycerolphosphate. On the 21st day in culture, the cells were stained with a solution of Alizarin Red as described in Materials and Methods. Two representative samples of each group are presented. (B) Quantification of the number of mineralized nodules formed in the cultures of bone marrow cells obtained from loaded and unloaded (Susp) mice. The percentage of surface area covered with mineralized nodules was measured as described in Materials and Methods. Data are expressed as means and standard errors for wild-type and OPN^{-/-} mice. Asterisk (*) indicates statistically significant difference from respective control ($P < 0.05$).

these cells was not recognized in the bone marrow cells obtained from OPN-deficient mice (Fig. 4B, OPN^{-/-} Susp versus OPN^{-/-} Load).

Discussion

This study showed that OPN is a prerequisite for bone loss induced by the absence of mechanical stress and, moreover, affects osteoblastic and osteoclastic differentiation of bone marrow cells when they are subjected to loss of mechanical stress.

Osteocytes and osteoblasts are thought to regulate bone structure and bone mass by responding to mechanical stress (Burger & Klein-Nulend 1999). In addition to these cells, bone marrow cells may also be regulated by mechanical stress as well as growth factors, cytokines, and hormones (Sakai *et al.* 1998, 2002, Sakata *et al.* 1999). As osteoblasts and osteoclasts differentiate from mesenchymal and hematopoietic stem cells respectively, the changes of local bone turnover are well associated with osteoblastic and osteoclastic differentiation of bone marrow cells. Thus, we observed the characteristics of bone marrow cells in the femora in OPN^{-/-} mice after tail suspension to elucidate the role of OPN in osteoblastic and osteoclastic differentiation of bone marrow cells regulated by

Table 1 Quantification of the number of tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like cells formed in the cultures of bone marrow cells obtained from loaded or unloaded mice. Data are expressed as means and standard errors for wild-type and OPN^{−/−} mice

	Tail suspension	Number of osteoclast-like cells (per well)
Genotype		
Wild-type	−	398.0 ± 161.9
Wild-type	+	408.3 ± 226.7
OPN ^{−/−}	−	417.0 ± 199.6
OPN ^{−/−}	+	401.3 ± 197.1

Bone marrow cells obtained from tail suspended (susp) or loaded (load) wild-type mice (wild-type) or OPN^{−/−} mice (OPN^{−/−}) were cultured in the presence of M-CSF and RANKL. The TRAP-positive multinucleated osteoclast-like cells were counted as described in Materials and Methods.

mechanical stress. It is known that unloading decreases bone formation in unloaded bones and cultured osteoprogenitor cells retain a memory of their prior loading history (Kostenuik *et al.* 1997). In our previous study, bone formation did not decrease in OPN^{−/−} mice when they were subjected to tail suspension, while it was reduced in wild-type mice (Ishijima *et al.* 2001). The results of *in vitro* mineralized nodule formation of bone marrow cells from unloaded bone in this study could be one of the mechanisms of resistance to unloading-induced suppression of bone formation in OPN^{−/−} mice in our *in vivo* previous study described above. Therefore, lack of reduction in bone formation by the loss of mechanical stress in the absent of OPN would be resulted in part in the loss of signals for differentiation of osteoblastic precursor cells in bone marrow cells.

Recent data revealed that unloading induces resistance to insulin-like growth factor-I (IGF-I) by inhibiting activation of the IGF-I signaling pathways through down-regulation of $\alpha_v\beta_3$ integrin in bone marrow cells (Sakata *et al.* 2004). IGF-binding protein-5 (IGFBP-5), which is one of the six forms of IGFBPs, binds to OPN, and these interactions are considered to modulate the cooperative actions between the IGF-I receptor and integrin receptor signaling pathways (Nam *et al.* 2000). Although the precise role of OPN in the loss of signals for differentiation of osteoblastic precursor cells in bone marrow cells in response to reduction in mechanical stress is unclear, one of the possibilities could be that OPN may be involved in these interactions between IGF-I, IGF receptor (IGF-R), IGFBP-5, and $\alpha_v\beta_3$ integrin in bone marrow cells. In addition, OPN may have a role in alterations of blood flow in hind limbs of unloaded animals, because OPN is reported to be involved in vasculogenesis (Asou *et al.* 2001, Yumoto *et al.* 2002, Pritzker *et al.* 2004, Cheriya & Hussein, 2005). Blood perfusion is reduced in the bones of the hind limbs of tail-suspended animals (Colleran *et al.* 2000). The loss of alkaline phosphatase activity after unloading is closely associated with the suppression of platelet endothelial cell adhesion molecule-1 (CD31) signaling on the bone marrow cell surface (Sakuma-Zenke *et al.* 2005). Although further

study is necessary to elucidate this possibility, OPN may be involved in this process.

In our previous study, unloading-induced enhancement in osteoclastic bone resorption was not observed in OPN^{−/−} mice (Ishijima *et al.* 2001). Therefore, we have gone on to ask whether the failure of the increase in osteoclast number following unloading is intrinsic to osteoclastogenesis in the bone marrow cells in OPN^{−/−} mice. The efficiencies of the formation of TRAP-positive multinucleated cells in the cultures of bone marrow cells obtained from the femora of unloaded and loaded mice in the presence of soluble RANKL and M-CSF were similar regardless of unloading or loading in either wild-type or OPN^{−/−} mice. These results suggest that unloading does not affect the proportions of bone marrow cells, which are able to differentiate into osteoclasts *in vitro* at least under these conditions. Thus, the mechanism underlying the failure in unloading-induced increase in the number of osteoclasts in OPN^{−/−} mice is not intrinsic.

Previous study reported that the number of TRAP-positive osteoclast-like cells induced by bone marrow cells of the tibiae subjected to tail suspension was significantly increased compared with cells from loaded control (Sakata *et al.* 1999). These data are not consistent with our data in which the number of TRAP-positive osteoclast-like cells induced by bone marrow cells of the femora was similar regardless of unloading or loading in either wild-type or OPN^{−/−} mice (Table 1). However, for instance, while osteoclast-like cells were induced from bone marrow cells in the presence of soluble RANKL and M-CSF in our study, these were induced from bone marrow cells in the presence of human parathyroid hormone in the previous study (Sakata *et al.* 1999). Therefore, the differences in the results of osteoclast-like cell formation assay between the previous study by Sakata *et al.* and our study may be due to the differences in experimental procedures.

In this *in vitro* study, we found that spreading of TRAP-positive osteoclast-like cells induced by bone marrow cells of femora subjected to tail suspension was enhanced compared with cells of loaded femora in wild-type mice. Adherence of osteoclasts to the substrate and polarizing reorganization of their morphology are critical to the resorbing function (Teitelbaum 2000). Osteoclasts are target cells for several cytokines and growth factors, among which M-CSF is essential for both osteoclast development and function. Mature osteoclasts also contain the M-CSF receptor, c-fms, which is required for the survival, spreading, and migration of these cells. It has been revealed that M-CSF induces spreading of osteoclasts (Nakamura *et al.* 1998, Teti *et al.* 1998, Grey *et al.* 2000). This effect of M-CSF requires the integrity of the $\alpha_v\beta_3$ integrin and signals through the c-fms, non-receptor type tyrosine kinase, c-src, phosphatidylinositol 3-kinase, and phospholipase C γ (Duong *et al.* 1998, Ducy *et al.* 2000, Nakamura *et al.* 2001, Sanjaya *et al.* 2001). Although, no major histological alterations in osteoclasts in OPN^{−/−} mice have been observed in our previous study *in vivo* (Rittling *et al.* 1998, Yoshitake *et al.* 1999, Ishijima *et al.* 2001), our present

Figure 4 TRAP-positive osteoclast-like cell formation in the cultures of bone marrow cells obtained from loaded and unloaded mice. (A) Bone marrow cells from tail suspended (Susp) or loaded (Load) wild-type mice (wild-type) or OPN^{-/-} mice (OPN^{-/-}) were cultured in the presence of 10 ng/ml M-CSF and 100 ng/ml RANKL. TRAP staining was conducted as described in Materials and Methods. Two representative independent plates out of six plates in each group are presented. (B) Spreading of TRAP-positive osteoclast like cells formed in the cultures of bone marrow cells obtained from tail suspended (Susp) or loaded (Load) wild-type mice (wild-type) or OPN^{-/-} mice (OPN^{-/-}). The maximal cell diameter was measured in at least 80 osteoclast-like cells as described in Materials and Methods. Data are expressed as relative values against loaded wild-type (WT) cells (100%). Means and standard errors are indicated for wild-type and OPN^{-/-} mice. Asterisk (*) indicates statistically significant difference from respective control ($P < 0.05$).

in vitro data suggest that response of bone marrow cells to these cytokines may be increased due to unloading and OPN may be involved in this process. Our observations on the bone marrow cells obtained from mice after tail suspension are consistent with those of our previous data, in which unloading enhanced the excretion of deoxypyridinoline in urine as a systemic biochemical marker of bone resorption in wild-type mice and this enhancement of the excretion of deoxypyridinoline in urine was not recognized in OPN^{-/-} mice (Ishijima *et al.* 2001). Although which cells are involved and how they sense mechanical stress in bone is still unclear (Rodan, 1991), some reports have shown that osteoclasts respond to mechanical stress and, as a result, alter their bone-resorbing activity (Rubin *et al.* 1999, Kurata *et al.* 2001). These results suggest that OPN has an important role in

spreading of osteoclasts in response to mechanical stress, which may modulate the function of these cells.

In conclusion, these data suggest that OPN may be involved in the regulation not only of osteoclastic spreading but also of osteoblastic bone nodule formation *in vitro* regulated by the alterations of mechanical stress. Based on this and our previous studies (Ishijima *et al.* 2001, 2002), because further study is required to elucidate the precise role of OPN on signal transduction pathways of mechanical stress in bone, we are planning to conduct microarray analysis using mRNA extracted from bone marrow cells in hind limb bones of OPN^{-/-} mice subjected to unloading. Overall, our data support the notion that OPN plays a role in the signal transduction pathways of mechanical stress to bone marrow cells as well as osteoblasts and osteoclasts.

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