with ice-cold PBS and then resuspended in a lysis buffer (10 mM HEPES-NaOH (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl, 200 mM sucrose, 1% Triton X-100, 0.5 mM DTT, 0.5 mM PMSF, 10 mg/ml leupeptin) (36). Cell lysates (20 μg of protein) were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; Amersham Pharmacia Biotech, Little Chalfont, U.K.). The membranes were treated with 5% skim milk in PBS containing 0.5% Tween 20 (PBS-T), incubated with anti-I-κBα Abs (1/1000 dilution), and then incubated with HRP-conjugated anti-rabbit IgG. ECL reagents (Amersham Pharmacia Biotech) were used for detecting the immunoreactive bands (36).

Results

Osteoclasts express functional receptors for LPS

We first examined whether osteoclasts express LPS receptors using the RT-PCR technique. Expression of TLR2, which appears to be involved in recognition of Gram-positive bacterial cell walls such as peptidoglycan and lipoproteins, was also examined. Bone marrow macrophages were prepared as osteoclast precursors. Both osteoclasts and bone marrow macrophages expressed TLR2, TLR4, and CD14 mRNAs (Fig. 1*A*). Some reports have indicated that in contrast to macrophages, osteoclasts do not express CD14 protein in the cell membrane (46, 47). To examine the expression of CD14 protein in osteoclasts, immunostaining with anti-CD14 Abs was performed on purified osteoclasts formed in vitro and also on bone marrow macrophages as the positive control (Fig. 1*B*). Osteoclasts weakly but detectably expressed CD14 protein. These results suggest that osteoclasts as well as bone marrow macrophages express TLR2, TLR4, and CD14. We previously reported that IL-1 rapidly and transiently induced activation of NF-κB in osteoclasts, concomitantly with the degradation of I-κBα (36). Treatment of osteoclasts and bone marrow macrophages with LPS for 30 min resulted in almost complete disappearance of I-κB in both types of cell lysate (Fig. 1*C*). I-κB reappeared in osteoclasts and bone marrow macrophages after the cells were treated with LPS for 60 min (Fig. 1*C*).

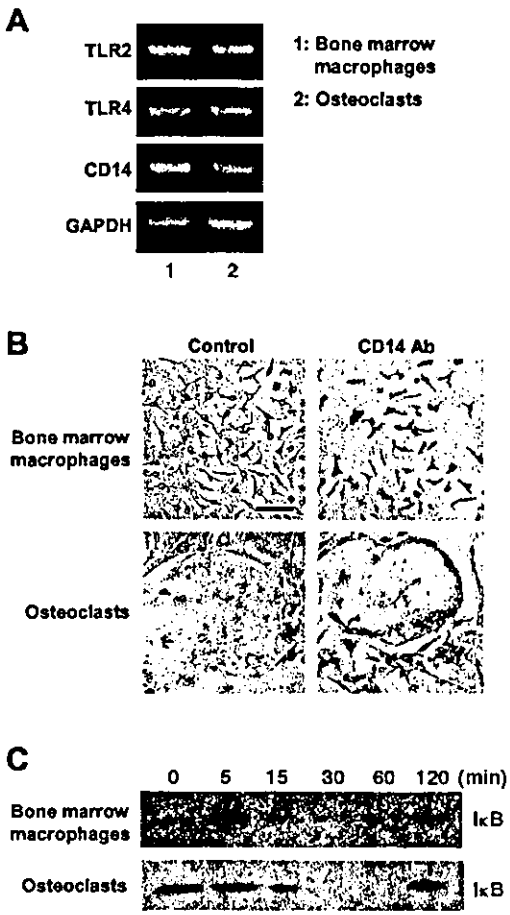

FIGURE 1. Expression of LPS receptors in osteoclasts and bone marrow macrophages. *A*, Expression of TLR2, TLR4, and CD14 mRNAs in bone marrow macrophages and osteoclasts. Total RNA was extracted from bone marrow macrophages and purified osteoclasts, and amplified by PCR for mouse TLR2 (27 cycles), TLR4 (27 cycles), CD14 (27 cycles), or GAPDH (25 cycles) using the respective primer pairs. *B*, Expression of CD14 protein in bone marrow macrophages and osteoclasts. Purified bone marrow macrophages and purified osteoclasts were fixed and incubated with or without anti-mouse CD14 Abs, followed by incubation with the second Abs. Note that osteoclasts as well as bone marrow macrophages were positive for CD14. Bar, 50 μm. *C*, Degradation of I-κBα in bone marrow macrophages and osteoclasts in response to LPS. Bone marrow macrophages and purified osteoclasts were treated with 1 μg/ml LPS for the indicated periods, and the amounts of I-κBα in the cell lysates were determined by immunoblotting.

LPS directly induces the survival of osteoclasts

We previously reported that purified osteoclasts spontaneously died due to apoptosis, and cytokines such as RANKL, IL-1α, and TNF-α promoted the survival of purified osteoclasts (28, 37, 38). The number of purified osteoclasts was decreased in a time-dependent manner, and almost all the osteoclasts disappeared within 36 h (Fig. 2*A*). LPS as well as RANKL significantly reduced the spontaneous apoptosis of osteoclasts (Fig. 2, *A* and *B*). Dose-response experiments showed that LPS at 100 ng/ml significantly stimulated the survival of osteoclasts (data not shown). LPS at 1 μg/ml also stimulated the survival of bone marrow macrophages. When bone marrow macrophages were further cultured in the absence of M-CSF, only 16.7 $\pm$ 1.0% (the mean $\pm$ SEM of four cultures) of bone marrow macrophages survived after culture for 48 h. LPS at 1 μg/ml increased the survival rate of bone marrow macrophages up to 46.3 $\pm$ 1.2%.

We next examined whether LPS stimulates the survival of osteoclasts through TLR4. Purified osteoclasts were prepared in co-cultures of osteoblasts and bone marrow cells obtained from C3H/HeJ mice or from normal C3H/HeN mice (Fig. 3). IL-1α similarly supported the survival of osteoclasts derived from mice of both strains. However, LPS prolonged the survival of osteoclasts derived from C3H/HeN mice, but not from C3H/HeJ mice.

LPS stimulates the target cells to produce proinflammatory cytokines such as IL-1 and TNF-α, which have been shown to support the survival of osteoclasts (28, 37, 38). We therefore examined the possibility that the effect of LPS on the survival of osteoclasts is mediated by such cytokines (Fig. 4). In this experiment, we used IL-1β instead of IL-1α, because bone marrow macrophages produce large amounts of IL-1β in response to LPS (see Fig. 5). IL-1β as well as IL-1α markedly stimulated the survival of osteoclasts (Fig. 4*A*). The survival of osteoclasts supported by LPS was not inhibited by adding mouse IL-1Ra, which strongly inhibited IL-1β-induced survival of osteoclasts (Fig. 4*A*). OPG, a decoy receptor of RANKL, completely suppressed the survival of osteoclasts supported by RANKL, but had no inhibitory effect on LPS-prolonged survival of osteoclasts (Fig. 4*B*). TNF-α failed to promote the survival of osteoclasts derived from TNFR1 knockout mice, but LPS and RANKL stimulated the survival of those osteoclasts to a similar extent (Fig. 4*C*). These results suggest that LPS supports the survival of osteoclasts via TLR4, and that none of the cytokines examined (RANKL, IL-1, and TNF-α) was involved in LPS-enhanced survival of osteoclasts.

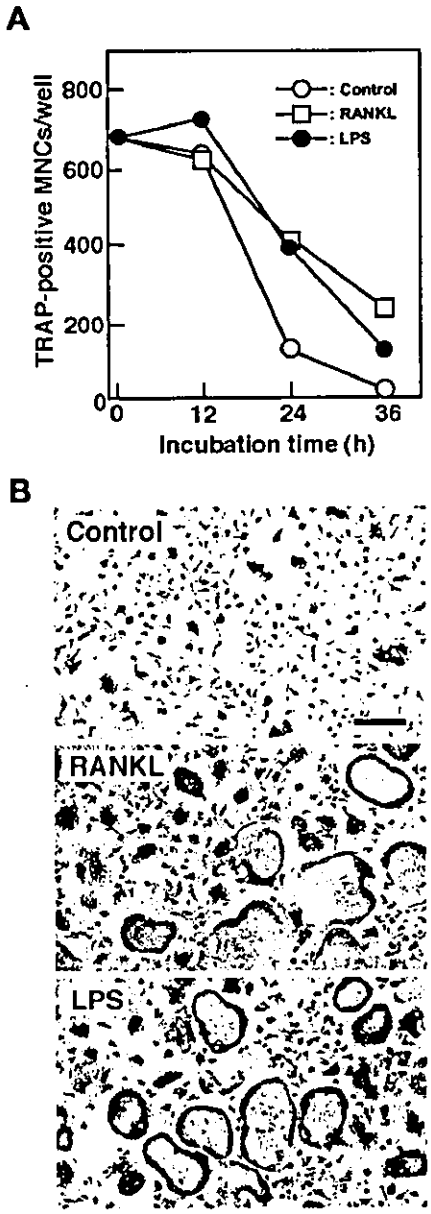

FIGURE 2. Effects of LPS and RANKL on the survival of osteoclasts. *A*, Effects of LPS and RANKL on the survival of purified osteoclasts. Purified osteoclasts were cultured for the indicated periods in the absence (○) or presence of LPS (1 μg/ml) (●) or RANKL (100 ng/ml) (□). Cells were then fixed and stained for TRAP. TRAP-positive MNCs containing more than three nuclei were counted as viable osteoclasts. Values are expressed as the means of triplicate cultures. *B*, TRAP staining of purified osteoclasts cultured for 36 h with vehicle (control), RANKL (100 ng/ml), and LPS (1 μg/ml). TRAP-positive cells appeared as red cells. Bar, 100 μm.

Osteoclasts do not produce proinflammatory cytokines in response to LPS

We next examined whether osteoclasts also produce proinflammatory cytokines in response to LPS, as do macrophages. Purified osteoclasts and bone marrow macrophages were treated with increasing concentrations of LPS, and the concentration of IL-1β in the conditioned medium was measured by ELISA (Fig. 5*A*). Bone marrow macrophages released a large amount of IL-1β in the conditioned medium in response to LPS in dose- and time-dependent manners. In contrast, osteoclasts failed to produce a large amount of IL-1β in the presence or absence of LPS. Immunocytochemical

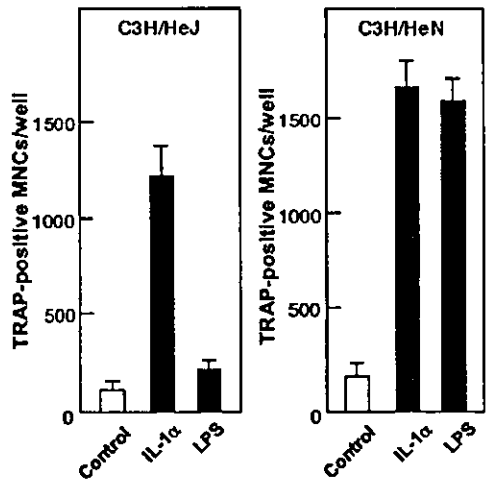

FIGURE 3. Effects of LPS and IL-1α on the survival of osteoclasts derived from C3H/HeJ and C3H/HeN mice. Purified osteoclasts derived from C3H/HeJ and C3H/HeN mice were treated with LPS (1 μg/ml) or IL-1α (10 ng/ml) for 24 h. Cells were fixed and stained for TRAP, and TRAP-positive MNCs containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SEM from quadruplicate cultures.

studies confirmed that bone marrow macrophages produced IL-1β in response to LPS (Fig. 5*B*). These results indicate that bone marrow macrophages lose the ability to produce IL-1β in response to LPS during their differentiation into osteoclasts. Expression of calcitonin receptors is believed to be the most reliable marker of osteoclast differentiation (18, 43). Using the technique of double detection of calcitonin receptors and IL-1β, we determined the stage of differentiation at which the cells stop producing IL-1β (Fig. 5*C*). LPS-treated multinucleated osteoclasts that expressed calcitonin receptors were completely negative for IL-1β staining (Fig. 5*C*). Even small mononuclear cells expressing calcitonin receptors were also negative for IL-1β production in response to LPS (Fig. 5*C*, *inset*).

We finally examined effects of LPS on the production of TNF-α and IL-6 in macrophages and osteoclasts (Fig. 6). In this experiment, freshly isolated peritoneal macrophages as well as bone marrow macrophages were examined, because bone marrow macrophages had been treated with M-CSF in culture. Production of TNF-α and IL-6 in bone marrow macrophages and in peritoneal macrophages was markedly increased in response to LPS. Peritoneal macrophages also produced IL-1β in response to LPS (control, 3.5 ± 2.0 ng/ml; LPS, 79 ± 25 ng/ml, the mean ± SEM of four cultures). In contrast, the concentrations of TNF-α were very low in the conditioned medium of cultures treated with or without LPS after culture for 48 h (Fig. 6). Interestingly, osteoclasts spontaneously produced a large amount of IL-6 even in the absence of LPS. Treatment of osteoclasts with LPS again failed to stimulate IL-6 production. Thus, the responsiveness of macrophages to LPS was quite different from that of osteoclasts: macrophages produced proinflammatory cytokines in response to LPS, but osteoclasts did not.

Discussion

We have shown in this study that osteoclasts as well as bone marrow macrophages, precursors of osteoclasts, express TLR4 and CD14. This receptor system for LPS appeared to be involved in LPS-induced signaling in osteoclasts. Degradation of I-κB was induced in osteoclasts as well as bone marrow macrophages in

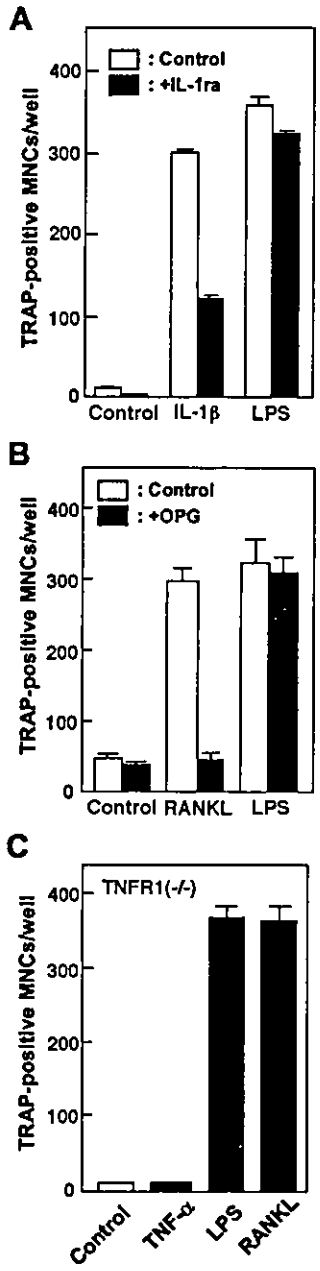

FIGURE 4. Roles of IL-1β, RANKL, and TNF-α in the survival of osteoclasts supported by LPS. *A*, Effect of mouse IL-1Ra on the survival of osteoclasts supported by IL-1β and LPS. Purified osteoclasts were incubated with LPS (1 μg/ml) or IL-1β (1 ng/ml) in the presence or absence of IL-1Ra (1 μg/ml) for 24 h. Cells were then fixed and stained for TRAP, and TRAP-positive MNCs containing more than three nuclei were counted as osteoclasts. *B*, Effects of OPG on the survival of osteoclasts supported by RANKL and LPS. Purified osteoclasts were incubated with LPS (1 μg/ml) or RANKL (100 ng/ml) in the presence or absence of OPG (1 μg/ml) for 24 h. TRAP-positive MNCs containing more than three nuclei were counted as osteoclasts. *C*, Effects of TNF-α and LPS on the survival of osteoclasts derived from TNFR1-deficient mice (TNFR1$^{-/-}$). Purified osteoclasts derived from TNFR1-deficient mice were incubated with mouse TNF-α (10 ng/ml), LPS (1 μg/ml), or RANKL (100 ng/ml) for 24 h. TRAP-positive MNCs containing more than three nuclei were counted as osteoclasts. Values are expressed as the means ± SEM from quadruplicate cultures.

response to LPS within 30 min. The time course of changes in I-κB degradation in osteoclasts after LPS stimulation was very similar to that induced by IL-1. LPS stimulated the survival of osteoclasts derived from normal mice, but not that of osteoclasts from C3H/HeJ mice, which possess a missense mutation in the TLR4 gene. These results suggest that TLR4-mediated signals are essentially involved in osteoclast function supported by LPS.

We previously reported that cytokines such as IL-1, TNF-α, and RANKL enhanced the survival of osteoclasts (28, 37, 38). The survival of osteoclasts supported by LPS was not mediated by any of those cytokines. Moreover, LPS-supported survival of osteoclasts was not inhibited by adding either OPG, a decoy receptor of RANKL, or IL-1Ra. The survival of osteoclasts derived from TNFR1-deficient mice was also supported by the addition of LPS. M-CSF, which is mainly produced by osteoblasts/stromal cells, strongly stimulated the survival of osteoclasts (28, 48). Anti-c-Fms (M-CSF receptor) Abs did not inhibit the LPS-induced survival of osteoclasts (data not shown). These results suggest that LPS directly supports the survival of osteoclasts through a mechanism independent of the production of proinflammatory cytokines.

The signaling cascade of TLR4 is quite similar to that of IL-1Rs (6–9). Both receptors have been shown to use TRAF6 and MyD88 as common signaling molecules (9). We previously reported that IL-1 induced activation of NF-κB and promoted the survival of osteoclasts (36). When osteoclasts were pretreated with antisense oligodeoxynucleotides to p65 and p55 of NF-κB, the expression of the respective mRNAs by osteoclasts was suppressed, and the IL-1-enhanced survival of osteoclasts was inhibited concomitantly (36). In addition, IL-1β as well as RANKL stimulated pit-forming activity of purified osteoclasts cultured on dentine slices. Suda et al. (49) recently reported that LPS induced the degradation of I-κB in prefusion mononuclear osteoclasts, and stimulated their fusion and pit-forming activity. It was also shown that mice deficient in TRAF6 developed osteopetrosis, and that TRAF6 deficiency resulted in defects in the signaling of not only IL-1, but also LPS (14, 15). These results suggest that, like IL-1, LPS directly stimulates osteoclast function through signals mediated by TRAF6 and NF-κB activation. Additional experiments using MyD88-defcient mice will provide more conclusive evidence for the direct action of LPS on macrophages and osteoclasts.

Mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3 (PI-3) kinase have been implicated in the survival and function of cells. Miyazaki et al. (50) first reported that extracellular signal-regulated kinase (ERK) activity relegates the osteoclast apoptosis. Therefore, we examined effects of PD98059, a specific inhibitor of ERK activation, on the survival of osteoclasts in the presence and absence of LPS (1 μg/ml). PD98059 (10^{-6} M) failed to inhibit the survival of osteoclasts supported by LPS (data not shown). SB203580, a specific inhibitor of p38 MAPK, had no inhibitory effects on LPS-enhanced survival of osteoclasts (51). These results suggest that neither ERK-mediated signals nor p38 MAPK-mediated signals are involved in the LPS-induced survival of osteoclasts. In contrast, wortmannin (10^{-8} M), a specific inhibitor of PI-3 kinase, inhibited the survival of osteoclasts induced by LPS (data not shown). This suggests that the PI-3 kinase/Akt (protein kinase B) pathway regulates the survival of osteoclasts.

Wang et al. (52) reported that alendronate induces apoptosis of osteoclasts through up-regulation of Fas expression. RT-PCR analysis revealed that osteoclasts formed in mouse cocultures expressed low levels of Fas ligand and Fas mRNAs, and LPS added to the osteoclast cultures did not significantly influence the expression of Fas ligand and Fas expression (data not shown). These results suggest that LPS-induced survival of osteoclasts is not mediated by suppression of Fas or Fas ligand expression in osteoclasts. Mitochondria play key roles in apoptosis, a central step being the release of cytochrome *c* into the cytoplasm. The Bcl-2 family members are shown to regulate the release of cytochrome *c*

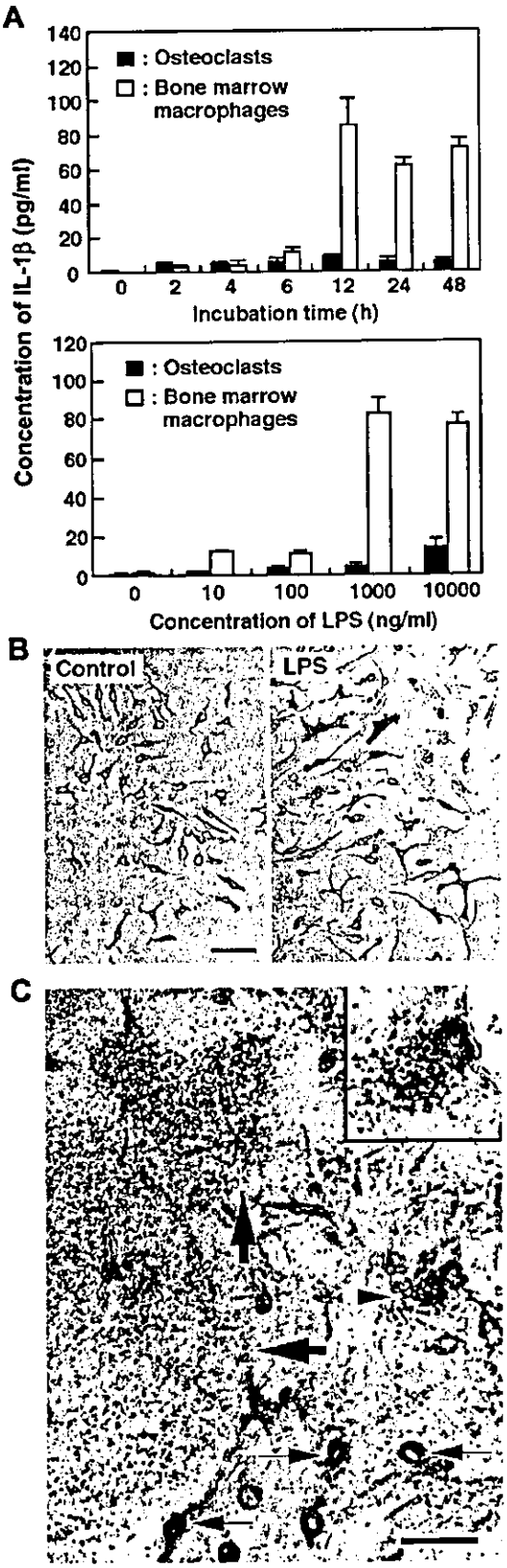

FIGURE 5. Effects of LPS on IL-1β production in bone marrow macrophages and osteoclasts. *A*, Effects of LPS on the production of IL-1β in bone marrow macrophages and osteoclasts. Mouse bone marrow macrophages and purified osteoclasts were incubated with or without increasing concentrations of LPS for indicated periods. The conditioned medium of each culture was collected, and the concentration of IL-1β in the conditioned medium was measured using an IL-1β ELISA kit. Values are expressed as the means ± SEM from quadruplicate cultures. *B*, Expression of IL-1β in bone marrow macrophages in response to LPS. Bone marrow macrophages were treated with or without LPS (1 μg/ml) for 12 h. The cells were then fixed and subjected to immunostaining using anti-IL-1β Abs. Cells producing IL-1β were stained dark brown. Note that bone marrow macrophages produced IL-1β in response to LPS. Bar, 50 μm. *C*, Autoradiography of ^{125}I-labeled calcitonin binding and immunostaining for IL-1β in bone marrow macrophages and osteoclasts. Osteoclast preparations (not purified) were placed on coverslips and treated with LPS (1 μg/ml) for 12 h. The cells were then incubated with ^{125}I-labeled human calcitonin and fixed in 0.1 M sodium cacodylate buffer containing 1% formaldehyde and 1% glutaraldehyde. The specimens were then subjected to immunocytochemistry for IL-1β, followed by autoradiography. Expression of calcitonin receptors was detected as the dense accumulation of grains due to ^{125}I-labeled calcitonin binding. Note that calcitonin receptor-positive multinucleated cells (arrows) as well as small mononuclear cells (arrowheads) were totally negative for IL-1β staining. Macrophages (small arrows) contaminating the osteoclast preparation produce IL-1β in the presence of LPS. *Inset*, Shows an enlargement of the portion indicated in *C*. Bar, 50 μm.

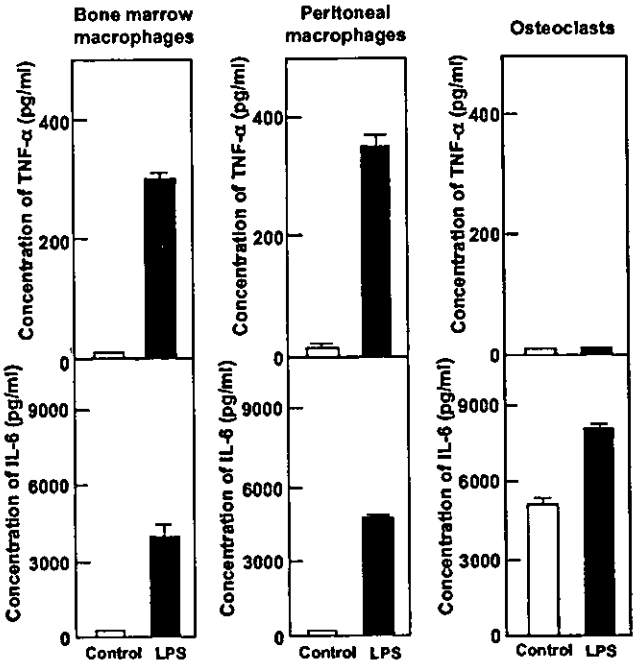

FIGURE 6. Effects of LPS on the production of TNF-α and IL-6 in bone marrow macrophages, peritoneal macrophages, and osteoclasts. Mouse bone marrow macrophages, peritoneal macrophages, or purified osteoclasts were incubated for 48 h in the presence or absence of LPS (1 μg/ml). The conditioned medium was collected, and the concentrations of TNF-α (*upper panel*) and IL-6 (*lower panel*) in the conditioned medium were measured using the respective ELISA kits. Values are expressed as the means ± SEM from quadruplicate cultures.

(53). We previously reported that most osteoclasts died during the first 24 h after purification, but expression levels of Bcl-2 and Bcl-x_L remained unchanged in purified osteoclasts for 18 h (54). Further studies will elucidate the relationship between antiapoptotic signaling pathways and mitochondrial activities in osteoclasts.

Kikkawa et al. (55) reported that osteoclasts transiently produce TNF-α in response to LPS. In agreement with their finding, the concentration of TNF-α was increased in the culture medium of purified osteoclasts after treatment with LPS at 1 μg/ml for 2–12 h (control, 11 ± 1.5 pg/ml; LPS, 74 ± 23 pg/ml at 12 h, the mean ± SEM of four cultures), but returned to the basal level after 24–48 h (control, 10.5 ± 0.87 pg/ml; LPS, 11 ± 0.51 pg/ml at

48 h). The survival of osteoclasts derived from TNFR1-deficient mice was also supported by the addition of LPS. These results suggest that TNF-α production by osteoclasts is essentially different from that by macrophages, and TNF-α transiently released from osteoclasts is not involved in the LPS-supported survival of osteoclasts.

Bone marrow macrophages differentiate into osteoclasts in the presence of RANKL and M-CSF (Fig. 7). LPS stimulates the production of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6 in bone marrow macrophages and in freshly isolated peritoneal macrophages, but not in osteoclasts. Interestingly, osteoclasts constitutively produced a high level of IL-6, which was unaffected by adding LPS (Fig. 7). This finding about the IL-6 production by osteoclasts is consistent with the findings of Roodman et al. (56), who showed that human osteoclasts expressed a relatively high level of IL-6, and osteoclasts in patients with Paget's disease produced increased levels of IL-6 compared with normal subjects. These results suggest that the characteristics of bone marrow macrophages and osteoclasts are quite different from each other in terms of cytokine production in the presence or absence of LPS.

We previously examined chronological changes in the expression of osteoclast- and macrophage-associated phenotypes during the differentiation of osteoclast precursors into multinucleated osteoclasts (44). Calcitonin receptor-positive mononuclear cells of a small size, which appeared before the emergence of multinucleated osteoclasts, expressed macrophage-associated phenotypes such as nonspecific esterase, Mac-1, and Mac-2. Nonspecific esterase and Mac-1 in calcitonin receptor-positive cells disappeared during the differentiation of these cells into multinucleated osteoclasts (44). The present study showed that even small calcitonin receptor-positive mononuclear cells failed to produce IL-1β in response to LPS. These results suggest that osteoclast precursors quit cytokine production (except for IL-6 production) in response to LPS as soon as the differentiation pathway of osteoclast precursors is determined (Fig. 7). Loss of inflammatory responsiveness to LPS in osteoclasts must be requirement for performing essential roles in physiological bone turnover. Further studies will be necessary to elucidate the mechanism of regulation of proinflammatory cytokine production in macrophages and osteoclasts.

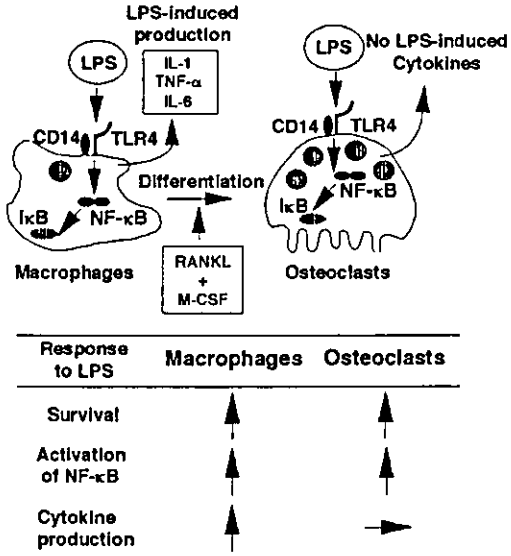

FIGURE 7. Roles of LPS in the function of bone marrow macrophages and osteoclasts. Bone marrow macrophages differentiate into osteoclasts in the presence of RANKL and M-CSF. Bone marrow macrophages and osteoclasts express functional receptors for CD14 and TLR4. LPS induces the degradation of I-κB similarly in bone marrow macrophages and osteoclasts, resulting in the activation of NF-κB. LPS also stimulates the survival of bone marrow macrophages and osteoclasts. Bone marrow macrophages produce proinflammatory cytokines such as IL-1, TNF-α, and IL-6 in response to LPS. In contrast, osteoclasts fail to up-regulate proinflammatory cytokine production in response to LPS. Osteoclasts constitutively produce a large amount of IL-6 even in the absence of LPS. Thus, the characteristics of osteoclasts and macrophages are different from each other with respect to the proinflammatory cytokine production in response to LPS.

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S 12911-2 Inhibits Osteoclastic Bone Resorption In Vitro

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ABSTRACT

The potential anti-osteoporotic activity of the strontium compound, S12911, was tested on osteoclast-like cells and on cultured fetal mouse long bones. From 1 mM Sr^{2+}, S12911 reduced both basal and stimulated bone resorption by decreasing osteoclast activity and ruffled border formation.

The aim of this study was to evaluate the effects of S 12911-2 on osteoclastic bone resorption using in vitro systems. Osteoclast-like cells, produced in vitro by co-culture of mouse bone marrow cells with primary osteoblasts, were allowed to settle on dentine slices, and the area of resorption pits formed after 48 h was measured using an image analysis system. S 12911-2, at a minimal active concentration of 1 mM Sr^{2+}, significantly reduced pit formation by these cells ($p < 0.05$). Pretreatment of slices for 48 h with S 12911-2 (5 mM Sr^{2+}) did not produce appreciable inhibition of resorption. Bone resorption in cultured fetal mouse long bones was assessed by measuring the release of pre-incorporated ^{45}calcium. S 12911-2 inhibited resorption in control cultures (18.9%, $p \leq 0.05$) and in bones cultured with the active form of vitamin D_3 [1,25$(OH)_2D_3$] (44.5%, $p \leq 0.05$). S 12911-2 had no effect on the number of osteoclasts observed histochemically in longitudinal sections prepared from fetal mouse long bones. Electron microscopy of mouse long bones treated with S 12911-2 (3 mM Sr^{2+}) showed osteoclasts with clear zones facing the bone surface, but without well-developed ruffled borders; untreated bones contained osteoclasts with normal ruffled borders. These results indicate that S 12911-2 inhibits osteoclast activity. This effect is directly linked to the presence of strontium, is effective on basal and stimulated resorption, and involves a decrease in ruffled border formation by osteoclasts. (J Bone Miner Res 2003;18:1082–1087)

Key words: S 12911-2, bone resorption, osteoclast

INTRODUCTION

S 12911-2 is a novel agent that is being developed for the prevention and treatment of postmenopausal osteoporosis. It comprises an organic moiety, ranelic acid, and two atoms of stable strontium.

Results of animal and human studies have suggested that S 12911-2 may be useful for the treatment of osteoporosis in humans, without the risk of altering the bone mineral.[1,2] In in vitro experiments with cultured rat calvariae, S 12911-2 has been shown to stimulate bone formation[3] and inhibit bone resorption.[4] S 12911-2 has also been found to inhibit bone resorption in isolated rat osteoclasts.[4] In ovariectomized rats, S 12911-2 has been shown to partially prevent bone loss by reducing histomorphometric indices of bone resorption, whereas indices of bone formation remained elevated.[5] The decrease in bone resorption was associated with a decrease in both the osteoclast surface and the number of osteoclasts per unit area of bone surface. In intact rats, long-term (2 years) administration of S 12911-2 increased bone mass and bone strength, with no adverse effect on bone mineralization.[6]

Osteoclasts are the primary bone-resorbing cells. However, their inaccessibility and their fragility when isolated have hampered detailed analysis of their function and regulation.[7] Mammalian osteoclasts can be obtained directly by disaggregation of bone, but the numbers obtained are usually small. Alternatively, a relatively large number of

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osteoclasts can be produced from mononuclear precursors derived from hemopoietic progenitor cells. This process requires the presence of primary osteoblasts and osteoclast differentiation factor (RANKL), which is normally presented as a membrane-associated cytokine on the surface of osteoblasts.[8,9]

The purpose of this in vitro study was to further investigate the effects of S 12911-2 on the osteoclast resorption step of bone remodeling. Two in vitro culture systems were used. In the first culture system, bone marrow cells from mice were co-cultured with primary osteoblasts to produce multinucleated osteoclast-like cells (OCLs).[10,11] The OCLs produced by this method were then used to investigate the effect of S 12911-2 on the formation of resorption pits by OCLs in dentine slices. In the second culture system (organ culture of mouse fetal long bones), the degree of bone resorption was assessed in cultured bones by the release of pre-incorporated ^{45}calcium (^{45}Ca) under basal or vitamin D–stimulated resorption conditions. The number and morphology of osteoclasts were also estimated using enzyme histochemistry and electron microscopy.

MATERIALS AND METHODS

Experimental procedures were conducted in compliance with the principles approved by the Council of the American Physiological Society for the care and use of animals. This study was also reviewed and approved by the Showa University Animal Care and Use Committee.

Animals and chemicals

Newborn mice and 6-week-old male *ddy* mice were obtained from the Shizuoka Laboratories Animal Center (Shizuoka, Japan). 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] was purchased from Wako Pure Chemical Co. (Osaka, Japan). Salmon calcitonin was obtained from Peptide Institute Inc. (Osaka, Japan). Bacterial collagenase was obtained from Sigma Chemical Co. (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Cell Culture Laboratories (Cleveland, OH, USA). ^{45}Ca was purchased from New England Nuclear Products (Boston, MA, USA). Collagen gel matrix (cell matrix, type I-A) was obtained from Nitta Gelatin Co. (Osaka, Japan). Dentine was provided by Nishide Inzai Co. (Tokyo, Japan). Test compounds of S 12911-2, S 12911-0 and S 12911-5 were provided by Technologie Servier (Orléans, France). Concentrations of S 12911-2 and S 12911-0 are expressed as added concentrations of cations (Sr^{2+} or Ca^{2+}) in the culture medium that contained a basal calcium concentration of 1 mM. For S 12911-5, concentrations are expressed as matching concentrations of ranelic acid as compared with those obtained with S 12911-2. Other chemicals and reagents were of analytical grade.

Preparation of mouse OCLs

Mouse OCLs were prepared as described by Akatsu et al.[10] Briefly, osteoblasts were isolated from calvariae of newborn *ddy* mice as described previously.[12] Culture dishes (10 cm in diameter; Corning, Acton, MA, USA) were coated with a 0.2% collagen gel matrix. Osteoblasts (5×10^5 cells) were co-cultured with bone marrow cells (6×10^6 cells) obtained from 6- to 9 week-old *ddy* male mice in αMEM containing 10% FBS in the collagen-coated dishes.[10] 1,25$(OH)_2D_3$ (10 nM) was added to the co-culture to induce development of OCLs. After culturing for 6 days, cells were recovered by digesting the collagen gel with 0.2% bacterial collagenase. The purity of OCLs in this preparation was 3–4%. Results are expressed as mean ± SEM, usually for four replicates.

Dentine slice resorption pit assay

The pit-forming activity of OCLs was assayed as previously reported.[13] The OCL preparation obtained from a 10-cm culture dish was suspended in 10 ml of αMEM containing 10% FBS and plated on dentine slices (4 mm in diameter) in 96-well culture plates (0.1 ml/slice/well). After a 90-minute incubation, the slices were transferred to 24-well plates and incubated for another 48 h in 0.5 ml of αMEM containing 10% FBS, with or without test compounds such as S 12911-2, S 12911-0, and S 12911-5. Dentine slices were then ultrasonicated in 1 M NH_4OH to remove adherent cells. Resorption pits on the slice were stained with Mayer's hematoxylin, and the resorbed area was measured using an image analysis system (LA-525; PIAS Co., Tokyo, Japan). The results were expressed as the percentage of resorbed area relative to the whole surface area of the dentine slice.

Long bone resorption assay

Pregnant *ddy* mice were injected subcutaneously with 25 μCi of ^{45}Ca on day 16 of gestation. On gestation day 17, shafts of the radii and ulnas were excised from fetuses and incubated in MEM. After preincubation for 24 h, the bones were transferred to 0.5 ml MEM plus 0.2% bovine serum albumin (BSA) and cultured for 48 h in the presence or absence of test compounds. Bone resorptive activity was expressed as the percentage release of ^{45}Ca from prelabeled bones.[14]

Enzyme histochemistry and electron microscopy

Radii and ulnas were obtained from fetal mice on gestation day 17 and incubated as described above. The long bones were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) and decalcified with 0.5 M EDTA for 4 days. Samples for enzyme histochemistry were dehydrated with a graded ethanol series, embedded in Technovit 8100 (Heraeus Kulzer, Germany), and sectioned longitudinally for the demonstration of TRACP. After treatment with ethanol:acetone (1:1) for 1 minute, sections were incubated at room temperature in acetate buffer (0.1 M sodium acetate, pH 5.0) containing napthol AS-MX phosphate as substrate and red-violet LB salt, in the presence of 50 mM sodium tartrate.[15] $TRACP^+$ cells appeared dark red and were assumed to be osteoclasts.

Samples for electron microscopy were dehydrated with ethanol and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). Ultra-thin sections were prepared, stained with uranyl acetate and lead citrate, and examined using a Hitachi HU-12A electron microscope with an accelerating voltage of 75 kV.

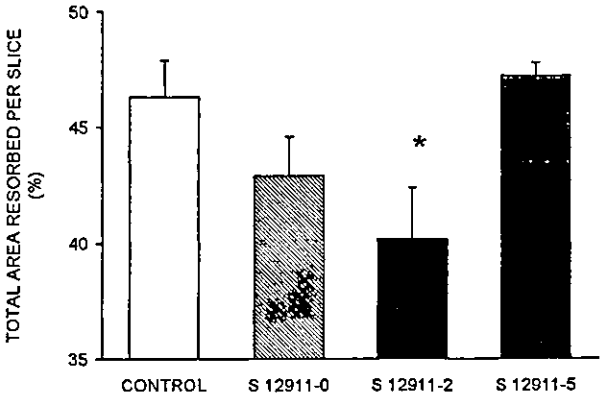

FIG. 1. Effects of incubation with S 12911-2 (1 mM Sr^{2+}), S 12911-0 (1 mM Ca^{2+}), and S 12911-5 (0.5 mM ranelic acid) on resorption pit formation in dentine slices. Error bars show SEM; $n = 4$ cultures; $^*p \leq 0.05$ vs. control.

TABLE 1. EFFECTS OF S 12911-2 AND OTHER TEST COMPOUNDS ON BONE RESORPTION IN ORGAN CULTURES OF MOUSE LONG BONES

	*Bone resorption (%)**	
	Control	*1,25(OH)$_2$D$_3$ (at 10 nM)*
Control	26.8 ± 2.4	69.2 ± 5.4
S 12911-2 (3 mM Sr^{2+})	18.9 ± 3.3†	44.5 ± 6.7‡
Salmon calcitonin (1 nM)	11.6 ± 1.0†	28.5 ± 4.0‡
S 12911-0 (3 mM Ca^{2+})	25.5 ± 2.5	50.7 ± 4.8
S 12911-5 (1.5 mM ranelic acid)§	21.9 ± 6.1	64.1 ± 5.3

* Mean ± SEM of four cultures. Bone resorption was measured by release of ^{45}Ca from prelabeled bones.
† $p \leq 0.05$ vs. control without 1,25(OH)$_2$D$_3$.
‡ $p \leq 0.05$ vs. control with 1,25(OH)$_2$D$_3$.
§ S 12911-5 was added to obtain a concentration of 1.5 mM of ranelic acid to match that obtained by addition of S 12911-2 or S 12911-0.

Statistical analysis

The significance of any differences between groups was determined using the Student's *t*-test.

RESULTS

Effect of S 12911-2 on pit-forming activity of osteoclasts

Under control conditions, the proportion of the area of dentine slices occupied by pits formed by OCLs was 46.3 ± 1.6% ($n = 4$). Addition of S 12911-2 (1 mM Sr^{2+} in the culture medium) during the incubation period significantly reduced the pit area (40.2 ± 2.2%; $p < 0.05$). The addition of S 12911–0 (1 mM Ca^{2+}) did not reduce the pit-occupied area significantly (42.9 ± 1.7%) nor did the addition of S 12911–5 (0.5 mM ranelic acid; 47.2 ± 0.6%). These results indicate that the reduction in pit area was strontium dependent and that ranelic acid had no effect in this model (Fig. 1). No significant difference was observed between S 12911-2 and S 12911-0.

The effect of pretreating the dentine slices with S 12911-2 for 48 h before incubation with OCLs was also investigated. Pretreatment with S 12911-2 (1 or 5 mM Sr^{2+}) did not produce significant inhibition of pit formation. Although the addition of S 12911-2 during incubation with OCLs reduced pit formation significantly, pretreatment did not enhance this inhibitory effect. The effects of incubation with S 12911-2 at a concentration of 1 or 5 mM Sr^{2+} were similar.

Effect of S 12911-2 on bone resorption in organ culture

The effect of S 12911-2 on osteoclast activity was investigated in organ culture of fetal mouse long bones, using the release of ^{45}Ca from prelabeled bone as a measure of bone resorption. As would be expected for a valid method of measuring bone resorption, treatment of cultured long bones with 1,25(OH)$_2$D$_3$ (10 nM) markedly stimulated bone resorption (Table 1). S 12911-2 (3 mM Sr^{2+}) significantly inhibited both basal (18.9 ± 3.3%, $p \leq 0.05$) and 1,25(OH)$_2$D$_3$-stimulated (44.5 ± 6.7%, $p \leq 0.05$) bone resorption. Salmon calcitonin (1 nM) also inhibited bone resorption, in both the presence and absence of 1,25(OH)$_2$D$_3$. S 12911–0 (3 mM Ca^{2+}) and S 12911–5 (1.5 mM ranelic acid), however, had no significant effect on either basal or stimulated bone resorption, indicating that the inhibition was strontium dependent (Table 1). No significant difference was observed between S 12911-2 and S 12911-0.

Effect of S 12911-2 on osteoclast number

Osteoclasts in longitudinal sections of long bone organ cultures were visualized by TRACP enzyme histochemistry and counted. The numbers of $TRACP^+$ osteoclasts were similar whether bones were cultured in control medium, in 10 nM 1,25(OH)$_2$D$_3$, or in 3 mM S 12911-2 (3 mM Sr^{2+}). The mean values (± SEM of four cultures) were 27.3 ± 0.9 for the control medium, 28.3 ± 1.1 for 1,25(OH)$_2$D$_3$, and 27.0 ± 0.7 for S 12911-2.

Effect of S 12911-2 on osteoclast ultrastructure

Osteoclasts on the bone-resorbing surface in cultured fetal long bones treated with 1,25(OH)$_2$D$_3$ showed well-developed ruffled borders and clear zones (Fig. 2A). On the other hand, osteoclasts in bone treated (in vitamin D-free medium) with S 12911-2 (3 mM Sr^{2+}; Fig. 2B) had only clear zones facing the bone surface and failed to form ruffled borders.

DISCUSSION

Osteoclast preparations obtained directly by mechanical disaggregation of newborn rat long bones have been used widely for pit resorption assays. However, this method produces only approximately 1000 osteoclasts from each newborn rat, making the design of rigorous experiments difficult. The procedure used here, involving the co-culture of primary osteoblastic cells with bone marrow cells, can yield up to 40,000 OCLs per dish.[10] It has been shown that OCLs produced in this way possess essentially all the characteristics of directly isolated osteoclasts (e.g., morpholog-

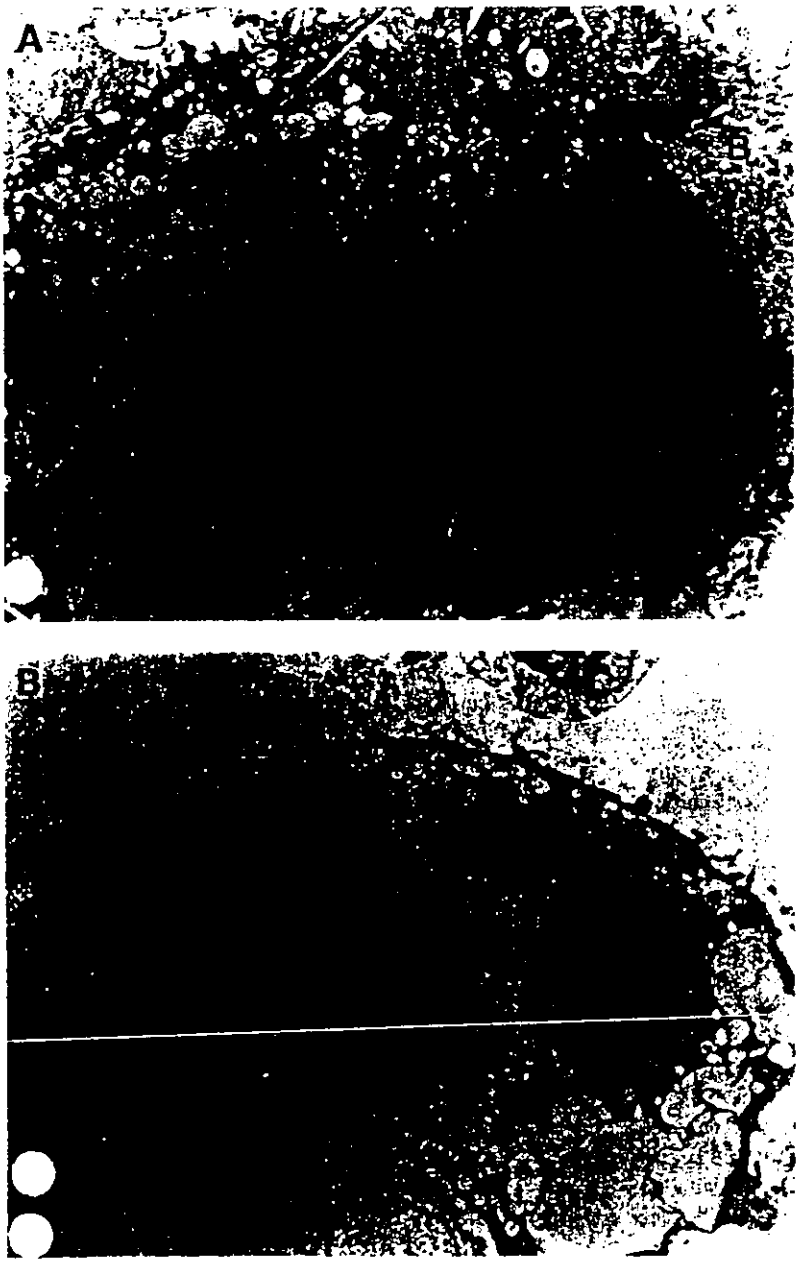

FIG. 2. Electron micrographs of osteoclasts in cultured mouse fetal long bones treated with (A) $1,25(OH)_2D_3$ or (B) S 12911-2 (3 mM Sr^{2+}). B, bone; CZ, clear zone; RB, ruffled border. (A and B) Magnification, ×5000.

ically similar, $TRACP^{+}$[10]), suggesting that they are suitable for use in studies of mammalian osteoclast function. The relatively large number of OCLs obtainable allows reliable experimental protocols to be performed.

In this study, we used dentine rather than bone slices to observe and quantify the formation of resorption pits. Dentine has a homogeneous structure and is free of vascular canals and osteocyte lacunae, which can make the identification of pits in bone slices difficult.[13]

Several parameters can be used to quantify pit formation by osteoclasts, including the number, depth, volume, and surface area of resorption pits. In the present study, with relatively large numbers of OCLs available, it was possible to generate a large numbers of pits, although the large number of pits generated made it difficult to distinguish individual pits. However, the resorbed surface area has been shown to be closely correlated with the volume of resorption pits,[16] with the surface area being relatively easy to quantify using an image analyzer. Furthermore, it has been shown that light microscope images of resorption pits stained with Mayer's hematoxylin correspond almost exactly to pits visualized by scanning electron microscopy,[13] but the procedure is far simpler. The relatively large resorbed areas obtained in the present study (approximately 20–40% of the total slice area) confer greater accuracy and reproducibility compared with assays using directly isolated authentic osteoclasts, when the resorbed area may be only 1% of the slice surface.[13]

Using in vitro assay systems, it has been shown that S 12911-2, at a concentration of 1 mM Sr^{2+} in the culture medium, inhibits the formation of resorption pits by OCLs in dentine slices. S 12911-2, at a concentration of 3 mM Sr^{2+}, was also found to reduce bone resorption significantly in fetal mouse long bone cultures, as measured by the release of pre-incorporated ^{45}Ca. Incubation of the slices with S 12911-0 (3 mM Ca^{2+}) or S 12911-5 (1.5 mM ranelic acid) had no inhibitory effects on bone resorption. Thus, the observed effects are directly linked to the presence of strontium, while ranelic acid has no relevant effect in this model. To relate these findings to the in vivo setting, studies in rats have shown that a dose of S 12911-2 (167.8 mg/kg/day) is equivalent to a serum strontium concentration of 0.13 mM.[1] Furthermore, in clinical trials, a daily dose of S 12911-2 of 2 g leads to a minimum steady-state plasma strontium concentration of approximately 10.6 μg/ml, or 0.12 mM (H Breuel, unpublished data, 2002). It has been demonstrated that postmenopausal women treated daily with 2 g of S 12911-2 and with similar plasma strontium concentrations had a 41% reduction in relative risk of experiencing a first new vertebral fracture.[17] However, this corresponds to concentrations in the circulating compartment and not the effect compartment. As strontium is distributed preferentially into bone,[18] it could be speculated that, as demonstrated with calcium,[19] strontium concentrations in the bone microenvironment could reach higher levels within the millimolar range, although the concentration varies with the bone structure.

Osteoclastic bone resorption involves two major processes: the activation of pre-existing mature osteoclasts and the recruitment of new osteoclasts. The present study suggests that the effects of S 12911-2 may be caused by inhibition of osteoclast function. Electron microscopy revealed that osteoclasts treated with S 12911-2 (3 mM Sr^{2+}) failed to form well-developed ruffled borders, whereas osteoclasts from bones cultured in the presence of $1,25(OH)_2D_3$, which stimulates bone resorption, were found to have well-developed ruffled borders. The ruffled border represents the resorption specialization phase of the osteoclast. It can be regarded as an extensive area of cell surface where the secretion of enzymes, as well as the uptake of matrix components, occurs during the resorption process. For example, bone resorption involves the secretion of protons through vacuolar H^+-ATPases, recruited from intracellular membrane compartments to the ruffled border of the cell.[20–22] Furthermore, osteoclasts in osteopetrotic (*ia/ia*) rats, which are incapable of bone resorption, show extended clear zones and defective ruffled border formation.[23] Thus, the failure of the S 12911-2-treated long bone osteoclasts to form ruffled borders suggests that S 12911-2 has a direct inhibitory effect on the bone-resorbing activity of pre-existing osteoclasts. This is consistent with the finding that S 12911-2 inhibited the pit-forming activity of mature OCLs placed on dentine slices. Furthermore, the structure of clear zones was not significantly affected by treating cells with S 12911-2. This suggests that the attachment of osteoclasts to bone is not affected by S 12911-2.

Other studies of S 12911-2 in animal models of bone resorption indicate that S 12911-2 inhibits osteoclast function. In mice calvaria cultured for 2 days in the presence of S 12911-2, Baron and Tsouderos[4] showed that S 12911-2 inhibits bone resorption in a dose-dependent manner. They also performed a rat osteoclast assay, involving the culture of rat osteoclasts on bovine cortical bone slices. Although incubation of the osteoclasts in the presence of S 12911-2 had only small inhibitory effects, preincubation of the bone slices dose-dependently ($R = 0.74$, $p < 0.0001$) inhibited subsequent bone resorption by up to 65% after preincubation with 1 mM S 12911-2, an inhibition equivalent to the effect of salmon calcitonin at 200 pg/ml in the assay used. The inhibitory effect was even higher (73 ± 16%, $p < 0.0001$) when S 12911-2 was also present while the rat osteoclasts were being cultured.

As in the present study, preincubation of the slices or incubation of the cells with S 12911-0 or S 12911-5 had no inhibitory effects at the concentrations tested (up to 1 mM Ca^{2+} and up to 0.5 mM ranelic acid). Similarly, preincubation and/or incubation with S 12911-2 did not affect the number of osteoclasts present on the bone slices at the end of the culture period.

The results from Baron and Tsouderos[4] showed that S 12911-2 inhibits bone resorption in both organ and isolated osteoclast cultures. The fact that preincubation of bone slices significantly affects subsequent bone resorption by isolated osteoclasts, without affecting their attachment or viability, suggests that this effect may be, at least partly, caused by a direct and/or matrix-mediated inhibition of bone-resorbing activity of the osteoclast by S 12911-2. These results also show that S 12911-2 reduces the expression of the osteoclast markers, carbonic anhydrase II (CAII) and vitronectin receptor (VNR), in chicken bone marrow culture in a dose-dependent manner.[4] The expression of both CAII and the α_v subunit of the VNR was reduced by 40–50% ($p < 0.001$) with S 12911-2 (1 mM Sr^{2+}), with the first significant effects being detected at 0.1 mM Sr^{2+} (10–30%, $p < 0.01$). It was concluded that the effects of S 12911-2 may be caused by its inhibition of the differentiation of osteoclasts as well as their function.

It has been suggested that S 12911-2 acts by decreasing bone resorption and increasing bone formation.[2,5] A number of in vivo and in vitro studies support this suggestion. S 12911-2 has been shown in vivo to partially prevent bone loss by reducing bone resorption, whereas bone formation remained elevated.[5] Previous in vitro experiments have shown that S 12911-2 increases the replication of preosteoblastic cells in cultured rat calvariae (by approximately 60% after 96-h treatment) and subsequently increases bone formation.[3]

The findings of the present in vitro study provide support for the effect on bone resorption proposed by the above hypothesis. This effect is directly linked with the presence of strontium, is effective on basal and stimulated resorption, and is partially explained by the decrease in ruffled border formation. These findings also provide further support for S 12911-2 as a potential therapeutic option in the treatment of osteoporosis.

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SRCL/CL-P1 Recognizes GalNAc and a Carcinoma-Associated Antigen, Tn Antigen

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SRCL /CL-P1 was recently identified as a scavenger receptor with a C-type lectin domain, which was expressed in vascular endothelial cells and could bind to Gram-positive and Gram-negative bacteria, yeast and oxidized LDL. We found that SRCL was expressed in some but not all nurse-like cells examined. Furthermore, to characterize the C-type lectin domain of SRCL, the secreted form of the C-type lectin domain (LEC-AP) of SRCL, which was fused to the signal sequence of IgG and alkaline phosphatase, was expressed in 293/EBNA-1 cells and the culture medium was used for the *in vitro* binding assay. LEC-AP specifically bound to GalNAc-conjugated gel in a Ca^{2+}-dependent manner, and this binding was inhibited by free GalNAc, L-, D-fucose, D-galactose, lactose, and especially T antigen and Tn antigen. Furthermore, we examined whether or not SRCL could take up saccharide-conjugated particles. 293/EBNA-1 cells stably expressing SRCL were found to take up GalNAc but not mannose-conjugated particles on confocal microscopy. The binding of GalNAc-conjugated particles to these cells was quantitatively measured by comparing the x-means of individual cell populations. An approximately 2.1-fold increase in immunofluorescence intensity was observed for the SRCL transfectants compared to control vector transfectants. Our results provide a basis for understanding the scavenger function of SRCL as to carbohydrate-containing ligands.

Key words: C-type lectin, GalNAc, nurse-like cells, SRCL, Tn-antigen

Abbreviations: BSA, bovine serum albumin; BP, biotinylated-polyacrylamide probe; CS-A, chondroitin sulfate A; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; IC_{50}, 50% inhibition concentration; PCR, polymerase chain reaction; 293E, 293/EBNA-1 cells; SRCL, scavenger receptor with C-type lectin.

Nurse cells were first recognized in the thymus, and to express MHC class II, to interact with T cells and to stimulate the proliferation of T cells (*1–3*). We previously identified and characterized nurse-like cells (NLC) from various human tissue sites from their ability to make T cells crawl beneath them (pseudoemperipolesis) and to express the MHC class II molecule (*4*, *5*). NLC are thought to be responsible for initiating T cell-mediated immune responses to antigens in extrathymic organs (*4*).

SRCL was recently identified as a novel scavenger-like receptor with a C-type lectin domain (*6*). SRCL is composed of five domains, an extracellular C-type lectin domain at the C-terminus, a collagen-like domain, a coiled-coil domain, a transmembrane domain and a short N-terminal cytoplasmic domain. SRCL can bind to Gram-positive and Gram-negative bacteria when expressed in Chinese hamster ovary cells (*6*). Recently, the same receptor (CL-P1) was cloned by another group, and found to be expressed in umbilical vein and arterial endothelial cells (*7*). Moreover, when expressed in cells, SRCL/CL-P1 bound to and endocytosed yeasts and heavily oxidized LDL. These ligands are thought to bind to the collagen domain of SRCL due to the sequence similarity to other members of the scavenger receptor family. Therefore, it is possible that SRCL has a function in the process of atherogenesis as well as in protection against bacterial and yeast pathogens.

The C-type lectin domain of SRCL, another putative ligand binding domain, remained to be characterized. The Ca^{2+}-dependent (C-type) animal lectins comprise a family of proteins that contain at least one carbohydrate-recognition domain (CRD). The majority of C-type lectins bind to either D-mannose, D-glucose, and related sugars (Man-type ligands), or D-galactose, GalNAc and its derivatives (Gal-type ligands), and comprise a large family of recognition molecules especially in the immune system (*8–14*). For example, macrophages could recognize malignant tumor cells through Tn antigens, the universal carcinoma markers and autoantigens, through macrophage lectin (*9*). Moreover, a recently identified C-type lectin, DC-SIGN, is thought to participate in the interaction between dendritic cells and T cells for antigen presentation (*14*). SRCL has one C-type lectin domain that contains the carbohydrate recognition motif of QPD (Gal-type) (*6*) and exhibits sequence homology to several Gal-type C-type lectins, especially human macrophage lectin (*9*) and hepatic asialoglycoprotein (*15*). Thus, it is important to study the C-type lectin expressed in immunocom-

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petent cells including nurse-like cells and vascular endothelial cells for understanding the immune system.

In the present study, we found that SRCL was expressed in some nurse-like cells, and that its C-type lectin domain could bind specifically to several carbohydrates including GalNAc, T and Tn antigen in a Ca2+-dependent manner. Furthermore, 293/EBNA-1 cells overexpressing SRCL were shown to take up GalNAc-conjugated particles.

MATERIALS AND METHODS

Reagents—The following were obtained commercially from the sources shown: Carbohydrate-conjugated Sepharoses (GalNAc-, mannose-, galactose-, and fucose-sepharose) from E-Y Laboratories, mannose- and GalNAc-BP from Seikagaku Kogyo, and GalNAc, D-galactose, D -glucose, D -fucose, L-fucose, D-fructose, D -ribose, D-xylose, L -xylose, D-arabinose, L -arabinose, lactose, melibiose, sucrose, raffinose, mannan, fucoidan, heparin, dextran, and LPS from SIGMA. T antigen (Thomsen-Friedenreich antigen, Galβ1–3GalNAcα-benzene), Tn antigen (*N*-acetylgalactosamine α1-*O*-serine), and chondroitin sulfate A were purchased from CALBIOCHEM. Galactan (gum Arabic) and arabinogalactan were from ALDRICH. methyl-β-D-galactopyranoside were from Lancaster.

Cell Culture and Transfection—Nurse-like cell lines, HS729T (ATCC.HTB153), FHs173WE (ATCC.HTB158), HSN27E (*4*), SK-LMS-1 (ATCC.HTB88), RA189SM (*16*), and Hs67 (ATCC.HTB163), and non-nurse cell lines, Hs913T (ATCC.HTB152), SK-HEP-1 (ATCC.HTB52), A549 (ATCC.CCL185), SIHA (ATCC.HTB35), Hs683 (ATCC.HTB138), MC/CAR (ATCC.CRL8083), Jurkat (ATCC.TIB152), HL60 (ATCC.CCL240), and IMR-32 (ATCC.CCL127), and 293/EBNA-1 (Invitrogen, San Diego, USA) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Stably transfected 293/EBNA-1 cells expressing full-length SRCL, secreted alkaline phosphatase (SEAP) and LEC-AP chimeras were generated by transfection in 6-cm dishes with ~1 μg of plasmid DNA using Lipofectamine-plus reagent (Gibco BRL, Rockville, MD, USA). Cells expressing transfected receptors were selected in 200 μg/ml Hygromycin (Sigma) for 1 week and maintained in 100 μg/ml Hygromycin.

Expression Vector Construction—The SRCL expression vector was constructed in pDREF (*17*). The full-length cDNA of SRCL was cloned into the pCR2.1 vector by PCR from SRCL-cDNA using primers, 414F 5′-GTCGACACG-GTCACCATGAAAGAC-3′ and 414R 5′-GCGGCCGCCC-CATCACAGTCCGTTAT-3′.

After digestion with restriction enzymes, *Sal*I and *Not*I, the resulting SRCL insert was ligated into the *Sal*I–*Not*I site of pDREF, generating the plasmid "pDREF-SRCL".

For construction of the LEC-AP expression vector, the C-type lectin domain of SRCL (corresponding to 584 to 742 aa) was amplified by PCR using primers, F10Bam 5′-GGATCCGGCCCATCAGGAGCGGTG-3′ and R10Xba 5′-TCTAGATAATGCAGATGACAGTAC-3′.

After digestion of the resultant PCR product with *Bam*HI and *Xba*I, the cDNA was subcloned into the pDisplay vector (Invitrogen) for fusion to the signal sequence of IgG. Subsequently, chimeric constructs containing the signal sequence were subcloned into pDREF-AP, which was the same vector as pDREF-SEAP used in Ref. *18* and has no signal sequence, generating plasmid "pDREF-LEC-AP." Construction of the secreted-type AP expression vector (pDREF-SEAP) was carried out as shown below. Briefly, SEAP was cloned from the pSEAP2-Enhancer vector (Clontech) into pCR2.1 (Stratagene) by PCR using primers, Xba-AP primer, (5′-CGCTCT-AGAAGCTCCGGAATCATCCCAGTTGAGGAGGAGAAC-3′) and AP(HIS)6-NOT primer (5′-CGCGCGGCCGCTCA-GTGATGGTGATGGTGATGACCCGGGTGCGCGGCGT-CGGT-3′), to generate a HIS-tag and a 3′-*Not*I restriction enzyme site. This SEAP contains no signal sequence. The PCR product was digested with *Bam*HI and *Not*I, and then inserted into the *Bgl*II–*Not*I sites of the pDisplay vector to generate the chimeric SEAP fused to IgG signal sequence. Subsequently, the chimeric SEAP containing the IgG signal sequence was subcloned into pDREF to generate expression vector "pDREF-SEAP."

Northern Blotting Analysis—Total RNA (15 μg/lane) was separated on 1.0% agarose gels containing formaldehyde. After transfer to a Hybond-N$^+$ membrane (Amersham Pharmacia Biotech), hybridization was carried out as previously described by Hieshima *et al.* (*18*). A probe was prepared by PCR amplification using primers F1 (5′-TGGAAGAACTTCACAGAC-3′) and R1 (5′-ATGGCCAT-GACCCCAGTT-3′).

Saccharide-BP Uptake Study—HEK293/EBNA-1 cells (293E cells) stably expressing SRCL or ML (human macrophage lectin) grown for 2 days on Lab Tek Chamber Slides (Nunc) precoated with 1 μg/ml Fibronectin overnight, were incubated for 1 h at 37°C in buffer (D-MEM,10% FCS containing 2 mM $CaCl_2$) with biotinylated saccharide-polyacrylamide polymer (GalNAc-BP and Man-BP) at the concentration of 50 μg/ml. After three washes in the same buffer, the cells were fixed for 10 min at room temperature in 4% formaldehyde in PBS. The cells were permeabilized in PBS containing 1% BSA and 0.1% Triton X-100. After three washes with the same buffer, the cells were incubated with FITC-conjugated avidin (Amersham) for 1 h at room temperature. After three washes with PBS 1% BSA, the cells on slides were mounted with a mounting solution (Slow Fade, Molecular Probes) and then examined under a confocal microscope. For flow cytometric analysis, cells were detached from the culture dish with ice-cold PBS containing 2 mM EDTA before fixation. After fixation, the cells were permeabilized with 0.1% Triton X-100 in PBS, incubated with FITC-conjugated avidin for 1 h before a wash in PBS/1% BSA, and then analyzed with FACScan.

Production of the LEC-AP Fusion Protein—293/EBNA-1 cells were transfected with expression vector pDREF-LEC-AP. This vector, PDREF, could be episomally maintained in the cytoplasm of 293/EBNA-1 cells, and it is very easy to isolate stable transfectants of a mixed population. The transfected cells were maintained for a few weeks in D-MEM with 10% FCS containing Hygromycin B (100 μg/ml), and the culture supernatant was collected. Alkaline phosphatase activity in the supernatant was measured by means of a chemiluminescent assay using a Great EscAPe detection kit (Clontech).

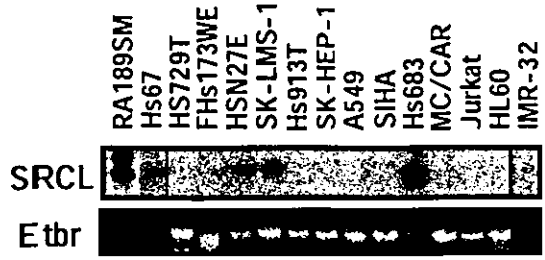

Fig. 1. **Northern blotting analysis of human SRCL expression in human cell lines.** Aliquots of total RNA, 15 μg, from human cells were electrophoresed through 1% agarose gels, transferred to Hybond-N$^+$ membranes, and then blotted with ^{32}P-labeled probes. Ethidium bromide staining of the gels demonstrated equal loading of the samples. Total RNA was purified from nurse-like cell lines HS729T, FHs173WE, HSN27E, SK-LMS-1, RA189SM, and Hs67, and non-nurse cell lines Hs913T, SK-HEP-1, A549, SIHA, Hs683, MC/CAR, Jurkat, HL60, and IMR32.

Binding Assay for the C-Type Lectin Domain of SRCL—For the binding and competition experiment on the C-type lectin domain of SRCL, 20 μl (bed volume) of either GalNAc-, fucose, Gal-, or mannose-conjugated gel was incubated and rotated for 1 h at room temperature with culture medium containing expressed LEC-AP or SEAP in the absence or presence of EDTA, monosaccharide or oligosaccharide in 400 μl of D-MEM containing 10% FCS, with 40 mM HEPES (pH7.4) and 2 mM Ca^{2+}. For binding inhibition by EDTA, Ca^{2+}-depleted buffer was used. After incubation, the gel was spun down in a centrifuge, and washed with the same buffer three times and then incubated for 30 min at 65°C to inactivate native AP. AP activity bound to the gel was measured using a Great EscAPe detection kit and a chemiluminescence detector (Lumat). Data were expressed in relative light units and AP activity in the absence of inhibitor was expressed as 100%.

RESULTS

Identification of the SRCL cDNA in Nurse-Like Cells—We used the subtraction cloning method to isolate cDNA preferentially expressed in a human nurse-like cell. The isolated cDNA was identical to the recently reported human SRCL type I/CL-P1 (*6*, *7*). We have identified several cell lines as nurse-like cells from their pseudoemperipolesis activity (Suzuki, R., unpublished observation). SRCL expression was examined in NLCs and non-nurse cell lines by Northern blotting. NLC lines HS729T, FHs173WE, HSN27E, SK-LMS-1, RA189SM, and Hs67, and non-nurse cell lines Hs913T, SK-HEP-1, A549, SIHA, Hs683, MC/CAR, Jurkat, HL60, and IMR-32 were examined (Fig. 1). RA189SM cells are the primary cultured cells isolated from synovial tissues of patients with rheumatoid arthritis. These cells could present antigens to T cells and stimulate T cell proliferation (*16*). SRCL was expressed in FHs173WE (faint but detectable), HSN27E, Hs683, SK-LMS-1, RA189SM, and Hs67. It is possible that SRCL is expressed in nurse-like cells.

Construction of a C-Type Lectin Domain-AP Fusion Protein—The alkaline phosphatase (AP) activity allowed quantitative determination of specific binding. We constructed secreted-type SRCL-AP fusion proteins as described under "MATERIALS AND METHODS." The C-type lectin domain of SRCL (584–742) was fused to the signal sequence of IgG and AP (18–506) in frame in its N- and C- terminus (LEC-AP) (Fig. 2). The chimeric protein was expressed in 293/EBNA-1 cells and the culture medum was used for the *in vitro* binding assay of saccharide-conjugated gels. The SEAP was produced in the same manner as LEC-AP (Fig. 2). For the binding of SEAP to the gel, the same amount of alkaline-phosphatase (AP) activity as that of LEC-AP was used. LEC-AP bound to Gal-, Fuc-, and GalNAc-conjugated gel with 4.0-, 17.8-, 43.7-, and 239.6-fold increases compared to the SEAP control, respectively (Fig. 3A, LEC-AP, black bar). As shown by the results, SRCL could most strongly bind to GalNAc-gel, so we used the GalNAc-gel in subsequent experiments.

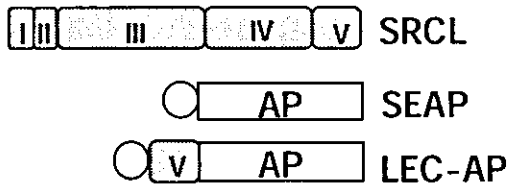

Fig. 2. **Schematic representation of the domain structures of SRCL, AP, and LEC-AP chimera proteins.** I: cytoplasmic. II: transmembrane. III: coiled-coil. IV: collagen. V: C-type lectin: AP: alkaline phosphatase. The circles represent the signal sequence of IgG. Secreted- alkaline phosphatase corresponding to the amino acid sequence (18–506) was fused to either the lectin domain of SRCL which corresponds to the amino acid sequence (584–742) for construction of LEC-AP, or IgG for SEAP

The binding of LEC-AP to GalNAc-gel was inhibited in the presence of 5 mM EDTA, indicating that the interaction was Ca^{2+}-dependent (Fig. 3B). The carbohydrate-binding specificity of LEC-AP was examined as to competitive inhibition using various monosaccharides at the concentration of 100 mM (Fig. 3B). Among the free monosaccharides tested, GalNAc, D-galactose, methyl-D-Gal, D- and L-fucose were found to be the most efficient competitors. Then, increasing amounts of competitors including GalNAc, L-fucose, and D-galactose were added to the binding assay mixture, and the IC_{50} values of GalNAc, L-fucose, and D-galactose were determined to be 6, 12, and 16 mM, respectively (Fig. 3C). Then, several disaccharides and trisaccharides, such as β-lactose, melibiose, sucrose and raffinose, were found to inhibit the binding. In particular, β-lactose effectively competed for the binding of LEC-AP to GalNAc-gel. The IC_{50} value of β-lactose was about 0.5 mM (Fig. 4A).

We next examined the effects of various oligosaccharides on the binding of LEC-AP to GalNAc-gel. Galactan and also arabinogalactan effectively competed for the binding at the concentration of 1 mg/ml (Fig. 4B).

LEC-AP Recognizes T and Tn Antigens—We next examined the effects of T and Tn antigens, carcinoma-associated antigens, on the binding of LEC-AP to GalNAc-gel. Both T and Tn antigens specifically inhibited the binding of LEC-AP to GalNAc-gel. The IC_{50} values of T-antigens and Tn-antigens were <3 mM and <1 mM, respectively (Fig. 5).

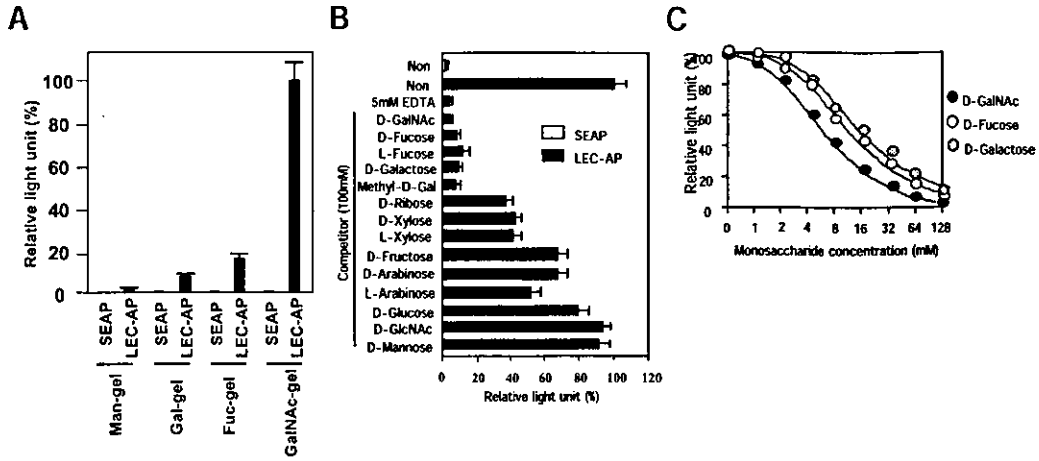

Fig. 3. **In vitro binding and binding competition assaying of LEC-AP chimeras on GalNAc-conjugated gel.** (A) Culture medum containing LEC-AP proteins was applied to 20 μl (bed volume) of mannose-, galactose-, fucose-, or GalNAc-conjugated Sepharose gel at room temperature for 1h. Culture medium containing expressed SEAP was used as a negative control. (B) Culture medum containing LEC-AP proteins was applied to GalNAc-gel in the absence or presence of the indicated concentrations of competitors as described under "MATERIALS AND METHODS." As a negative control, the same level of AP activity as that of LEC-AP was used for in vitro binding. In (A) and (B), data are means ± SD for triplicate determinations and are representative of three experiments. (C) Inhibition of binding of LEC-AP to GalNAc-conjugated gel by free GalNAc, D-fucose, or D-galactose. Briefly, 20 μl (bed volume) of GalNAc-gel was incubated with LEC-AP in the presence of increasing concentrations of free GalNAc, L-fucose, or D-galactose. Data representative of three experiments was shown.

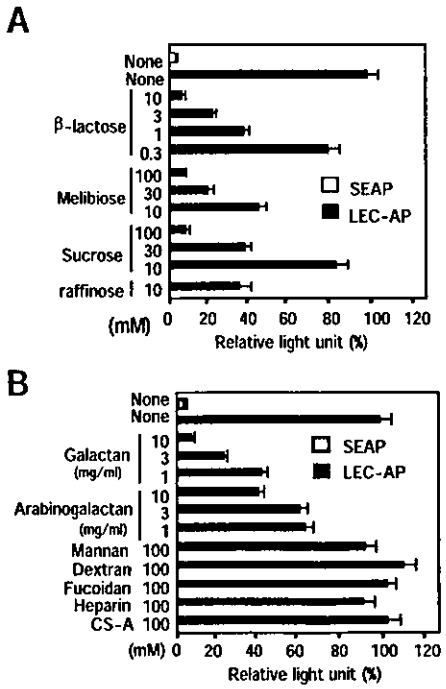

Fig. 4. ***In vitro* binding competition assay of LEC-AP to GalNAc-gel by di- and oligosaccharides.** Culture medium containing LEC-AP proteins was applied to GalNAc-gel in the absence or presence of the indicated concentration of di- and trisaccharides (A), or oligosaccharides (B). In (B), the concentrations of galactan and arabinogalactan are expressed in mg/ml, and those of others were expressed in mM. Data are means ± SD for triplicate determination and are representative of three experiments.

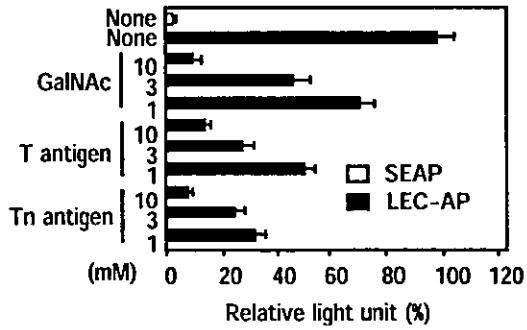

Fig. 5. ***In vitro* binding competition assaying of LEC-AP on GalNAc-gel with T and Tn antigens.** Briefly, 20 μl (bed volume) of GalNAc-gel was incubated with LEC-AP in the presence of the indicated concentrations (mM) of free T antigen and Tn antigen, and free GalNAc as a positive control. AP activity bound to the GalNAc-gel was measured. Data are means ± SD for triplicate determinations and are representative of three experiments.

Carbohydrate-Binding Activity of SRCL—To determine whether or not SRCL bound to carbohydrate chains in transfected cells, the full-length SRCL cDNA was cloned into the pDREF vector (pDREF-SRCL) and then the expression vector was transfected into 293/EBNA-1 cells. Stable transfectants were assayed for the binding of biotinylated carbohydrate ligands at 37°C. As a positive control, pDREF-ML, a human macrophage lectin (*9*), was used. In SRCL-transfected cells, specific binding and uptake were observed on confocal microscopy only when *N*-acetyl-D-galactosamine-BP (GalNAc-BP) was used as the ligand (Fig. 6A). Mannose-conjugated BP did not show any binding (Fig. 6A). To confirm ligand uptake by SRCL, we observed the cells detached from the bottom of a slide and rounded cells because we could easily detecting the cell interior signals by observing such rounded cells. The GalNAc-BP uptake by SRCL was evident because a series of optical sections revealed dot-like signals in both the cell interior (Fig. 6B, white arrow) and on the cell surface (Fig. 6B, black arrow).

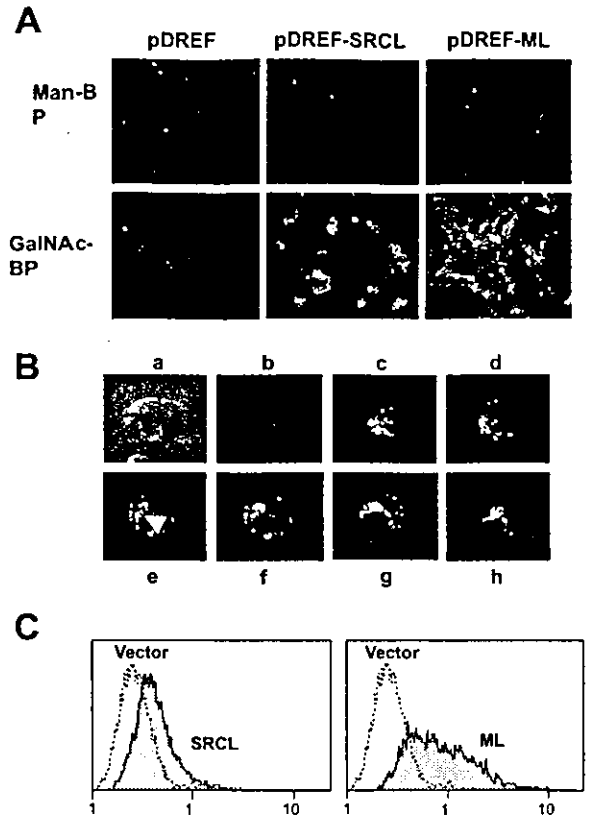

Fig. 6. **The uptake of GalNAc-conjugated polyacrylamide particles by SRCL-transfected cells.** (A) The uptake of GalNAc-BP by 293/EBNA-1 cells expressing SRCL. Cells stably transfected with pDREF-SRCL or control vector pDREF were incubated with Man- or GalNAc-BP (50 μg/ml) for 1 h at 37°C. As a positive control, human macrophage lectin (pDREF-ML) expressed in 293/EBNA-1 cells was used. After washing, the cells were fixed and stained with FITC-avidin and visualized by fluorescence microscopy. (A) Serial section photographs (b–h) showing the uptake of dot-like signals in the cell interior. Cells stably transfected with pDREF-SRCL were incubated with GalNAc-BP (50 μg/ml) for 1 h at 37°C. After washing, the cells were fixed and stained with FITC-avidin, and the rounded cells were visualized by fluorescence microscopy. (a) Phase contrast microscopy. (A) Quantitative measurement of the binding of the GalNAc-BP to SRCL-expressing cells by FACS analysis. Representative data for three experiments are shown. The *x*-means of individual cell populations were calculated and expressed as fold increases compared to in control vector transfectants.

Moreover, we performed quantitative FACS analysis of the binding of GalNAc-BP (Fig. 6C) to SRCL-expressing cells. In this experiment, we detached 293 cells bound to GalNAc-BP by treating the cells with 2 mM EDTA. With this treatment, the GalMAc-BPs bound to the cell surface was thought to be dissociated from the cells. However, all of the bound-BP was not completely dissociated. Thus, it seems likely that the GalNAc-BPs bound to SRCL is internalized into the cells. The binding of GalNAc-BP to cells was quantitatively measured by comparing the x-means of individual cell populations. Approximately 2.1- and 5.4-fold increases in immunofluorescence intensity compared to in control vector transfectants were observed in the SRCL and ML transfectants, respectively. The binding of SRCL to GalNAc-BP ligands was weak than that of ML. We thought that these binding activities were dependent on the gene expression levels, because the expression of SRCL was lower than that of ML. Thus, this type of quantitative analysis was not so important but confirmed that SRCL expressed in 293/EBNA-1 cells specifically binds to and takes up GalNAc-conjugated polyacrylamide particles.

DISCUSSION

In this study we showed that SRCL is expressed in several nurse-like cells, the C-type lectin domain of SRCL specifically interacts with Gal-type ligands, and T- and Tn antigens, and SRCL-transfected cells specifically internalize GalNAc-conjugated particles. The human SRCL was originally identified as a bacteria binding receptor (*6*). Subsequently, the same receptor (CL-P1) was independently identified by other groups as a receptor of oxidized-LDL (ox-LDL) on vascular endothelial cells (*7*). SRCL/CL-P1 has both scavenger receptor-like and collectin-like structures. The alternative splicing form of SRCL, SRCL type II, which has no C-type lectin domain, was found to be able to bind to bacteria (*6*), so that C-type lectin domain thought to be dispensable for the binding to bacteria. We examined whether or not LEC-AP bound to such microorganisms including *S. aureus*, *Escherichia coli*, and zymosan. However, no specific binding was observed (data not shown). However, LEC-AP could bind to galactan and arabinogalactan with moderate specificity. Although the structures of these materials were complex and not uniform, the similar structural components of these materials were thought to be present in some bacterial cell walls (*19*, *20*), especially in Gram-positive bacteria. Thus, the C-type lectin of SRCL may recognize some bacteria.

The C-type lectin domain of SRCL has been shown to contain the QPD motif, which was expected to bind to D-galactose and GalNAc (*6*). In the present study, SRCL expressed in 293E cells was actually shown to possess binding activity as to GalNAc-conjugated BP. Moreover, in the *in vitro* binding assay, the C-type lectin domain-AP chimera expressed in 293E cells was shown to possess affinity to Gal-type ligands, D-galactose (D-Gal), GalNAc, L-D-fucose, T-antigen, and Tn-antigen. The binding affinity of LEC-AP as expressed as IC_{50}, as to several carbohydrates examined in this study was equivalent to that observed for other C-type lectins (*21*, *22*). The binding specificity was very similar to that of human macrophage C-type lectin (HML), which recognizes GalNAc, D-Gal and Tn antigen (*9*, *21*, *23*). Therefore SRCL may play a role as a scavenger receptor for asialoglycoprotein in nurse-like cells as well as vascular endothelial cells.

Collectins comprise a family of proteins that contain at least two characteristic structures, a collagen-like region and a carbohydrate recognition domain (CRD). There are four groups in this family: mannan-binding protein, surfactant protein A, surfactant protein B and membrane-bound type collectin,SRCL/CL-P1. SRCL could recognizes GalNAc-type carbohydrate, so it is a novel type of collectin.

GalNAc is an about threefold more potent inhibitor than D-Gal, indicating that the *N*-acetamide group at C-2 in the pyranose ring participates in the interaction. L-Arabinose, which has the same configuration as D-Gal or

L-Fuc but lacks the CH_2OH or CH_3 of C6, respectively, was inactive at levels up to 10-fold higher than the molar amount of D-Gal used, indicating that the CH_2OH or CH_3 of C6 is necessary for the binding. Among the oligosaccharides examined, β-lactose (Galβ1–4Glc) was the best inhibitor, the activity being about 4-, 10-, and 10- fold greater than those of D-Gal, raffinose (Galα1–6Glcβ1–2Dfruf), and melibiose (Galα1–6Glc), respectively. Tn antigen (GalNAcα1-*O*-Ser), which has the α-anomer linkage of GalNAc, also showed strong inhibitory activity. These results indicated that SRCL recognizes both the α- and β-anomers of D-Gal.

It is interesting to note that a lectin with Galβ1–4GlcNAc binding activity on microvascular endothelial cells can contribute to retention and secondary tumor formation of blood-borne tumor cells (*24*). Thus, SRCL may be identical to these lectin-like substances observed in the endothelium and may participate in the retention of tumor cells.

Another point of interest is our finding that the C-type lectin domain of SRCL could bind to T and Tn antigens. T (Galβ1–3GalNAcα-Ser/Thr) and Tn (*N*-acetylgalactosamineα1-*O*-serine), found on mucin-type glycoproteins, has been well documented to be expressed on a variety of human carcinoma cells, and thought to be autoantigens (*25, 26*) and to participate in metastasis (*27, 28*). A direct link has been demonstrated between vessel invasion by malignant cells and the cell-surface density of Tn antigens (*29*). These epitopes might contribute to the recognition of malignant cells by immune cells and are thought to stimulate anti-tumor immunity (*25, 30*). Several proteins have been described that specifically recognize the Tn antigen, including monoclonal antibodies (*31, 32*), plant lectins (*33*), human macrophage lectin (*9*), and glycosyltransferase (*34*). Among them, human macrophage lectin (ML), which has a C-type lectin domain and is expressed in macrophages, specifically binds to the Tn antigen for the recognition of malignant cells (*9, 21*). Therefore, it is possible that SRCL has a similar function to that of ML.

The presence of SRCL in immunocompetent cells such as nurse-like cells and vascular endothelial cells suggests its role as a scavenger receptor for antigen presentation. Cytokine-activated human vascular endothelial and skin nurse-like cells play roles in initiating immune responses by interacting with immunocompetent cells *via* their class II MHC molecules (*4, 35–37*). The RA189SM cells shown in Fig 1, which were primary cultured nurse-like cells isolated from synovial tissues of patients with rheumatoid arthritis, and can maintain and activate T cells *in vitro*, are thought to be involved in the development of rheumatoid arthritis (*16*). As SRCL was expressed in these cells and was suggested to have endocytic activity based on the results in Fig. 6, SRCL may function in the uptake of soluble glycoconjugates released from pathogens and endogenous glycoproteins bearing oligosaccharides having terminal galactose residues including T- and Tn antigens for antigen presentation to T cells by MHC class II molecules, thereby initiate immune responses like DEC-205 (*13*). It is important to examine whether or not SRCL in nurse-like cells and endothelial cells actually functions as a molecular scavenger as to Gal-type ligands. The isolation of antibodies specific to SRCL now in progress will help us to answer these questions.

Our finding that SRCL is expressed in several nurse-like cells, and recognizes T- and Tn antigens reveals a novel function for SRCL. Additional studies on the function of SRCL may provide more insight into the roles of nurse-like cells and endothelial cells.

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Comparison of the activities of multinucleated bone-resorbing giant cells derived from CD14-positive cells in the synovial fluids of rheumatoid arthritis and osteoarthritis patients

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Objective. To investigate the morphology and function of multinucleated bone-resorbing giant cells derived from CD14-positive cells in the synovial fluids (SF) of patients with rheumatoid arthritis (RA) or osteoarthritis (OA).

Methods. CD14-positive cells were obtained by magnetic-activated cell sorting of primary cultures of mononuclear cells from the SF. Multinucleated bone-resorbing giant cells were induced from the CD14-positive cells in the presence or absence of cytokines. We examined various characteristics, including osteoclast markers, fusion index and bone-resorption activities of the multinucleated giant cells.

Results. Multinucleated giant cells were induced from the CD14-positive cells in the SF of the RA and OA patients by the addition of interleukin (IL)-3, IL-5 and IL-7, or granulocyte–macrophage colony-stimulating factor (GM-CSF), respectively. These multinucleated giant cells were positive for tartrate-resistant acid phosphatase (TRAP), carbonic anhydrase II, actin, vitronectin receptor and the calcitonin receptor. However, the average values for the number of nuclei, fusion index and bone-resorption functions of the SF cells from the RA patients were significantly higher than those derived from the OA patients.

Conclusion. These results suggest that the induction and activities of multinucleated bone-resorbing giant cells may play a pivotal role in bone destruction, and that these processes may be enhanced significantly in RA patients.

KEY WORDS: Rheumatoid arthritis, Synovial fluid, CD14-positive cell, Osteoclast.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by invasive synovial hyperplasia, which leads to progressive destruction of the joint. Although the precise mechanism of joint destruction has not yet been elucidated, osteoclasts appear to play a pivotal role in the joint destruction seen in RA patients. Osteoclasts are multinucleated bone-resorbing cells that are derived from CD34-positive haematopoietic stem cells [1–3]. Osteoclasts in the RA joint actively resorb bone at the site at which the proliferating synovial membrane invades the adjacent bone [4]. The osteoclast progenitors are members of the monocyte/macrophage lineage [1], and they differentiate into the mononuclear precursors of osteoclasts (preosteoclasts) [1, 5, 6]. The mononuclear preosteoclasts express tartrate-resistant acid phosphatase (TRAP), which is not produced by peripheral blood monocytes [7]. Mature osteoclasts are generated by the fusion of these mononuclear preosteoclasts, while they are in close contact with stromal cells in the bone marrow [1, 8–10]. Rheumatoid synovial fibroblasts participate in bone destruction by inducing osteoclastogenesis [11–13]. Bone-resorptive cytokines, such as tumour necrosis factor-α (TNFα), interleukin 1 (IL-1), IL-6 and soluble IL-6 receptor (sIL-6R) in the synovial fluid or in the serum are reportedly involved in the immune responses and activation of inflammation seen in RA patients [14–20]. High levels of IL-6 and sIL-6R, IL-17 and fibroblast growth factor (FGF)-2 in the synovial fluids of patients with RA appear to enhance osteoclastogenesis and promote joint destruction [21–23].

Nurse cells were first described in 1980 [24, 25] and are believed to play an important role in the differentiation, maturation and apoptosis of murine thymocytes [26–28]. Thymocytes initially adhere to thymic nurse cells and then crawl underneath them in a process that is referred to as pseudoemperipolesis. We reported previously on the presence of nurse-like cells in the synovial tissues and bone marrow of patients with RA, and suggested an important role for these cells in the pathogenesis of RA [29–31].

Recently, we reported that multinucleated bone-resorbing osteoclast-like cells were generated from peripheral monocytes that differentiated into TRAP-positive mononuclear cells when induced by RA nurse-like cells (RA-NLCs) [32]. In addition, certain cytokines in the synovial fluids (SF) of RA patients are responsible for osteoclast-like cell formation. We detected TRAP-positive mononuclear cells, which differentiated into multinucleated bone-resorbing giant cells, in the SF of patients with RA [32]. Although the presence of the bone-resorbing cells in RA joints is well known, the characteristics and functions of multinucleated bone-resorbing giant cells remain unknown. In the present study, we evaluated differences between RA and OA patients in the

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