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Heparin Inhibits Osteoclastic Differentiation and Function

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Abstract We investigated the effects of Glycosaminoglycans (GAGs) on mouse monocytic cell line in regard to their differentiation, proliferation, and function in vitro. RAW 264.7 cells were cultured with receptor activator of NF-κB ligand (RANKL) and various GAGs. Osteoclastic cells were visualized by staining for tartrate-resistant acid phosphatase (TRAP) and detected using a phenyl-phosphate substrate method. RAW 264.7 cells were also cultured with stimulants contained in BD BioCoat OSTEOLOGIC™ kit, and bone resorption activity was assessed by counting the numbers of resorption pits. We also examined the effect of heparin on cell growth using MTT assay, while the expression level of c-Src protein was determined by immunoblot analysis. Heparin suppressed TRAP-positive multinucleated cell formation and TRAP activity induced by RANKL, whereas the other GAGs showed no effects on osteoclast differentiation. Heparin also inhibited the formation of resorption pits, while the others did not. In the MTT assay, none of the tested GAGs had an influence on RAW 264.7 cell proliferation. However, heparin reduced the level of c-Src protein in RAW 264.7 cells stimulated with RANKL. To determine the affinity of heparin and RANKL, they were subjected by HiTrap heparin column chromatography and each fraction was collected. Western blotting analysis revealed the expression of RANKL in the fraction bound to heparin. The binding of RANKL and heparin was confirmed by quartz-crystal microbalance. These results indicate that the inhibitory effect of heparin toward osteoclastogenesis induced by RANKL is due to the binding of heparin to RANKL. J. Cell. Biochem. 103: 1707–1717, 2008.

Key words: osteoclastogenesis; glycosaminoglycan; heparin; receptor activator of NF-κB ligand; binding

Glycosaminoglycans (GAGs) are acidic polysaccharide complexes involved in a variety of physiological conditions. Recent advances in biological methods have allowed the elucidation of the potential roles of GAGs in key biological processes [Lindahl, 2000; Sasisekharan and Venkataraman, 2000; Casu and Lindahl, 2001; Shriver et al., 2002], including thrombosis [Petitou et al., 1999], angiogenesis [Sasisekharan et al., 1997], viral invasion [Chen et al., 1997; Fry et al., 1999; Shunkla et al., 1999], tumor growth [Hulett et al., 1999; Vlodavsky et al., 1999; Liu et al., 2002], and bone metabolism [Walton et al., 2002]. GAGs are physiologically reactive, highly acidic, negatively charged, and structurally and functionally similar to polysaccharides. They are long chain compounds composed of repeating disaccharide units with a carboxyl group and one or more sulfates, in which one sugar is N-acetylgalactosamine or N-acetylglucosamine. Endogenous GAGs are heparin, heparan sulfate, keratan sulfate, dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, and hyaluronic acid, among which, heparin is the most sulfated and acidic.

Long-term administration with heparin is connected with the risk of development of

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osteoporosis [Jones and Sambrook, 1994; Wolinski-Friedland, 1995], though heparin-induced osteoporosis is a rare adverse effect and its actual rate of incidence remains unclear [Walenga and Bick, 1998]. However, up to one-third of all patients receiving long-term heparin treatment experience a subclinical reduction in bone density [Walton et al., 2002]. The gathering of unequivocal data concerning the real risk of symptomatic heparin-induced osteoporosis is difficult, because heparin therapy is generally not given over a long term [Ginsberg et al., 1990]. Pikul et al. reported that the heparin tended to increase the formation of osteoclasts at lower concentrations, whereas at the highest concentrations it tended to decrease the numbers of osteoclast in rat bone marrow cell culture [Folwarczna et al., 2005]. However, the exact mechanisms by which it causes profound effects on osteoclasts remain unknown.

In the present study, we examined the effects of different GAGs on osteoclastogenesis in vitro, and clarified the precise mechanism by which the heparin regulated osteoclast formation and function induced by receptor activator NF-κB ligand (RANKL).

MATERIALS AND METHODS

Reagents and Antibodies

The GAGs used in this study, (chondroitin, chondroitin sulfate A, B, D, E, and heparin) were kindly supplied by Seikagaku Kogyo Corporation (Tokyo, Japan). Recombinant human-soluble RANKL was purchased from Pepro Tech EC Ltd (London, UK). Human M-CSF (Leukoprol) was purchased from Yoshitomi Pharmaceutical (Osaka, Japan). Anti-c-Src (sc-8056), anti-RANKL (sc-7628), and horseradish peroxidase-conjugated anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MTT and tartrate-resistant acid phosphatase (TRAP) staining kits were purchased from Sigma (St. Louis, MO).

Cell Culture

Mouse monocytic RAW 264.7 cells were maintained in alpha-minimum essential medium (α-MEM; GIBCO, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS; GIBCO), penicillin G (100 U/ml), and streptomycin (100 μg/ml) in collagen-coated dishes (Asahi Techno Glass, Chiba, Japan).

Cell Viability

RAW 264.7 cells were plated in a 96-well plate at a concentration of 5×10^2 cells per well 1 day before the experiment, then the cells were stimulated with RANKL (40 ng/ml) and each GAG (10 or 100 μg/ml). The stimulated cells were cultured for 6 days, after which a stock MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, 2.5 mg/ml; Sigma Chemical Co., St Louis, MO at 20 μl/well) was added to the wells and the plates were incubated for 4 h. Next, acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added and mixed thoroughly, and the plates were read using a Multskan Bichromatic microplate reader (Labsystems, Helsinki, Finland), with a test wavelength of 540 nm and a reference wave length of 620 nm [Okahashi et al., 1997].

Differentiation of Osteoclasts

Osteoclasts were detected using tartrate-resistant acid (TRAP) staining (Sigma Chemicals) [Kotake et al., 1999]. In brief, bone marrows cells were prepared by removing femurs from 6-week-old female ddY mice and flushing the bone marrow cavity with α-MEM containing 10% FBS. Cells were cultured in 48-well plates (1.5×10^5 cells/well) in the presence of M-CSF (100 ng/ml). After 3 days, non-adherent cells were completely removed from the cultures by pipetting, and adherent cells were used as M-CSF-dependent bone marrow macrophages (M-BMMs) [Kobayashi et al., 2000; Okahashi et al., 2001]. M-BMMs were further cultured for 4 days with RANKL (40 ng/ml) in the presence or absence of GAGs. After the culture, the adherent cells were fixed and stained to detect TRAP-positive cells TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclast-like cells (OCLs) and counted under a microscope.

RAW 264.7 cells were cultured in 96-well plates (5.0×10^2 cells/well) in the presence of RANKL (40 ng/ml) and each GAG. After being cultured for various times, OCLs were visualized by staining for TRAP described above.

TRAP Activity

For the TRAP activity assay, RAW 264.7 cells were cultured in 96-well plates (5.0×10^2 cells/well) in the presence of RANKL (40 ng/ml) or each GAG (100 μg/ml) for 6 days. The treated RAW 264.7 cells were suspended in 25 μl of

phosphate-buffered saline (PBS, pH 7.2), and then frozen and thawed three times. TRAP activities in the supernatants were analyzed using a phenyl-phosphate substrate kit (Sanseiphospha KII-Test-Wako, Wako, Osaka, Japan) according to the manufacturer's instructions [Kind and King, 1954].

Bone Resorption Assay

To estimate bone resorption activity, differentiated RAW 264.7 cells stimulated with RANKL (40 ng/ml) were cultured for 2 days with RANKL (40 ng/ml) and each GAG (100 μg/ml) on BD BioCoat™ Osteologic™ multi-test slides, which consisted of sub-micron synthetic calcium phosphate thin films coated onto various culture vessels (Becton Dickinson and Company, Bedford, MA). The cells were removed using 6% NaOCl and 5.2% NaCl, and the number of the resorption pits formed in each well were counted under a microscope. We also cultured RAW 264.7 cell on dentin slices that had been placed in 96-well plates [Suda et al., 1997]. After preincubation for 1 h, dentine slices were transferred to 48-well plates (1 dentine slices per well) containing 300 μl αMEM containing 10% FBS, and they were further cultured with or without GAG (100 μg/ml) for 2 days. Dentine slices incubated with calcitonin (10^{-8} M) for the same period were regarded as a control. Resorption pits on dentine slices were visualized by staining with Mayer's hematoxylin solution (Sigma Chemicals) as described by Suda et al. [1997]. The number of resorption pits on each slice was counted.

Western Blot Analysis

RAW 264.7 cells (1×10^5) were cultured in αMEM containing 10% FBS in the presence of RANKL (40 ng/ml) and heparin (100 μg/ml) on a 6-well plate. Adherent cells were washed twice with PBS and lysed in a cell lysis buffer (75 mM Tris-HCl containing 2% SDS and 10% glycerol, pH 6.8). The protein contents were measured using a DC-protein assay kit (Bio-Rad, Hercules, CA). The samples were electrophoresed using SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to PVDF membranes (Millipore, Bedford, MA). Nonspecific binding sites were blocked by immersing the membranes in 10% skim milk in PBS for 1 h at room temperature, then washed four times with PBS, followed by incubation in diluted primary antibody for 2 h at room temperature, using anti-c-src and horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies. After washing the membranes, chemiluminescence was produced using an enhanced chemiluminescent (ECL) reagent (Amersham Pharacia Biotech) and detected with Hyperfilm-ECL (Amersham Pharmacia Biotech, Uppsala, Sweden). The membranes were then stained with Coomassie Brilliant Blue G-250 to confirm equal loading.

Heparin-Sepharose Chromatography

The affinity of human-soluble RANKL for heparin was determined by FPLC on a HiTrap heparin column (Amersham Pharmacia Biotech). Binding buffer (10 mM sodium phosphate buffer, pH 7.0) containing RANKL was applied to the column. The column was then eluted with 4 ml of elusion buffer (10 mM sodium phosphate, 2 M NaCl, pH 7.0) following washing with four column volumes of binding buffer and the fractions were collected. The expression of RANKL was detected by Western blot analysis, as described above. In this experiment, the secondary antibody was anti-goat IgG-HRP (Santa Cruz Biotechnology, Inc.). Each fraction was dialyzed using Dialysis Product (Slide-A-Lyzer, PIERCE, Rockford, IL) according to the manufacturer's instructions. RAW 264.7 cells were cultured with dialyzed fractions for 6 days to detect OCLs.

Kinetic Analysis Using Quartz-Crystal Microbalance (QCM)

A 27-MHz QCM (Afinix Q; Initium Inc., Tokyo, Japan) was employed to analyze the affinity of RANKL and heparin. RANKL (2 μl; 10^{-11} M) was immobilized directly on the gold electrode surface of the QCM ceramic sensor chip, after which the sensor chip was soaked in a chamber containing 8 ml of PBS at 25°C until frequency equilibrium was attached. Heparin (100 μg/ml; volume 100 μl) was applied into the equilibrated solution containing the RANKL-immobilized sensor chip. The binding of Heparin to RANKL was determined by monitoring the alterations in frequency resulting from changes in mass on the electrode surface [Shinmyouzu et al., 2007].

Statistics Analysis

The significance of differences was determined using an unpaired Student's *t*-test with

Bonferroni correction for multiple comparisons. All data are expressed as the mean ± SD.

RESULTS

Effects of GAGs on Cell Growth

We examined the effects of RANKL and the different GAGs on the proliferation of RAW 264.7 cells using an MTT assay, and found that neither RANKL nor any of the GAGs had an effect on RAW 264.7 cell proliferation after 6 days (Fig. 1).

Heparin Inhibited Osteoclast Differentiation Induced by RANKL

We examined the effect of GAGs on the differentiation of M-BMMs into osteoclastic cells. As shown in Figure 2A, CoS-B, CoS-E, and heparin remarkably inhibited the TRAP-positive multinucleated cell formation. Heparin inhibited the differentiation of RAW 264.7 cells into OCLs in the presence of RANKL (Fig. 2B,C), whereas the other GAGs did not. The number of TRAP-positive multinucleated cells was determined to be 33 cells/well when being cultured with RANKL (40 ng/ml) and heparin (100 μg/ml). The inhibitory effect of heparin on osteoclastogenesis was in a dose-dependent manner up to 100 μg/ml (Fig. 2D). On the other hand, none of the GAGs had an effect on OCL formation in the absence of RANKL (data not shown).

Heparin Downregulated TRAP Activity Induced by RANKL

We examined the effect of GAGs on the TRAP activity of RAW264.7 cells stimulated with RANKL using a phenyl-phosphate substrate methods. As shown in Figure 3, RANKL enhanced TRAP activity in RAW 264.7 cells. However, when the cells were incubated with both RANKL and heparin, TRAP activity was lower than that in cells treated with RANKL alone. On the other hand, other GAGs had no effect on RANKL-induced TRAP activity in RAW 264.7 cells.

Effects of Heparin on Bone Resorption by Mouse Osteoclasts Bone In Vitro

To determine whether heparin affects osteoclast function, differentiated RAW 264.7 cells were cultured on OsteologicTM multitest slides with RANKL (40 ng/ml) in the presence of each GAGs (100 μg/ml). Heparin (100 μg/ml) inhibited bone resorption formed by RANKL-stimulated RAW 264.7 cells on Osteologic™ plates (Fig. 4A,B). When the cells were cultured with the other GAGs, there were no changes in numbers of pits formed on the plates. When

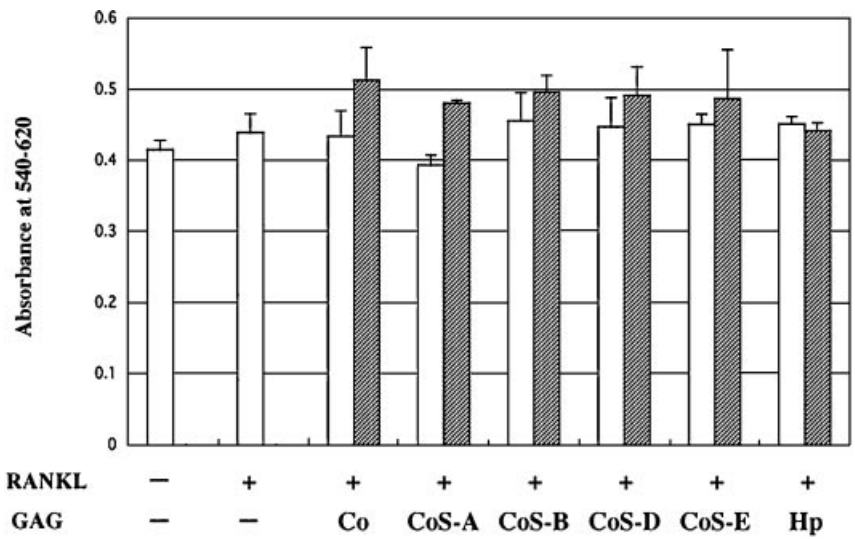

Fig. 1. Effects of GAGs on proliferation of RAW 264.7 cells. RAW 264.7 cells were stimulated with RANKL (40 ng/ml) and various GAGs for 6 days. Cell viability was determined as described in ''Materials and Methods''. Co, chondroitin; CoS-A, chondoitin sulfate A; CoS-B, chondoitin sulfate B; CoS-D, chondoitin sulfate D; CoS-E, chondoitin sulfate E; Hp, heparin. Data are expressed as the mean ± standard deviation of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. Student's *t*-test, $^*P < 0.05$. □, 10 μg/ml; ▨, 100 μg/ml.

the differentiated RAW 264.7 cells on dentin slices, heparin also showed inhibitory effect on the pit-forming activity of osteoclasts (data not shown).

Heparin Inhibited RANKL-Induced Expression of c-Src Protein in RAW 264.7 Cells

Next, we investigated the effects of GAGs on the expression of c-Src in RAW 264.7 cells stimulated with RANKL by Western blotting analysis. As shown in Figure 5, the expression level of c-Src protein was increased in cells following stimulation with RANKL, while it was downregulated following the addition of heparin for up to 72 h.

Affinity of RANKL for Heparin

To clarify whether RANKL binds to heparin, we examined the affinity of RANKL for heparin using an FPLC on heparin column. RANKL (1 μg) was subjected on HiTrap heparin column chromatography, and unbound and bound fractions were collected separately. Western blotting analysis revealed the expression of RANKL in the fraction that bound to heparin (Fig. 6A). In addition, we cultured RAW 264.7 cells with unbound and bound fractions that were dialyzed with dialysis products, and with RANKL (550 ng) obtained after FPLC and dialysis. As shown in Figure 6B, RAW 264.7 cells were differentiated into TRAP-positive multinucleated

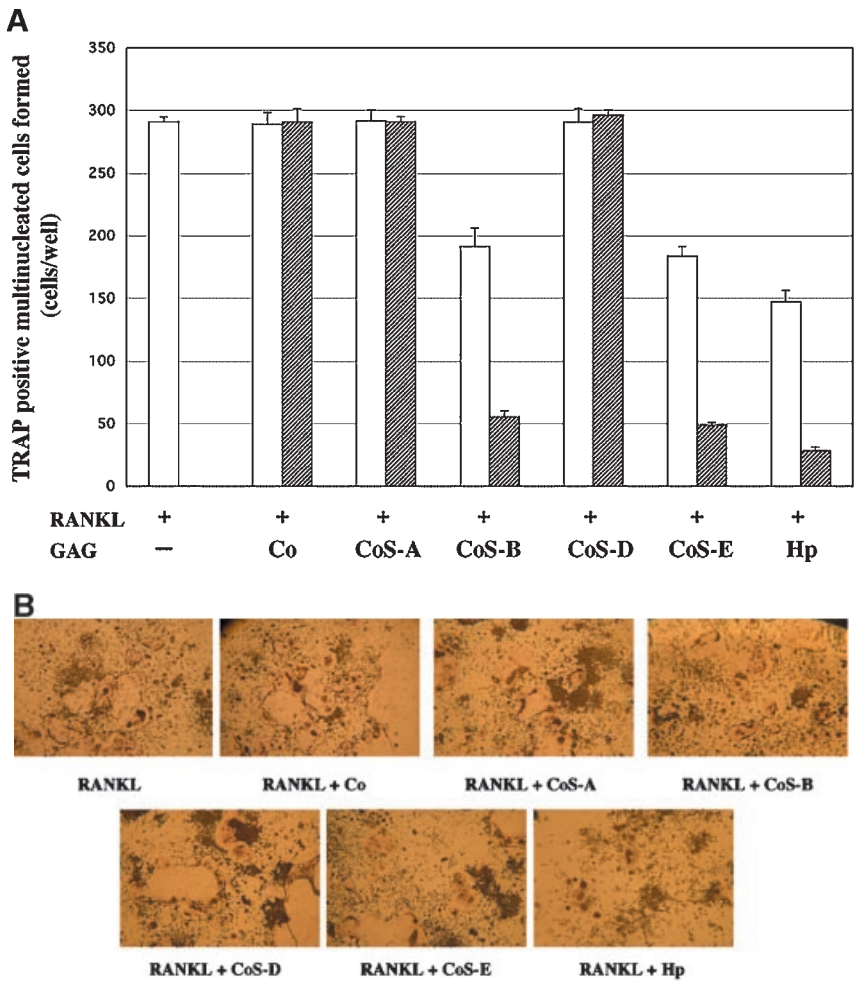

Fig. 2. Effects of GAGs on osteoclast differentiation induced by RANKL. M-BMMs were stimulated with RANKL (40 ng/ml) and various GAGs for 4 days. **A**: The number of OCLs was counted after TRAP staining. RAW 264.7 cells were stimulated with RANKL (40 ng/ml) and various GAGs for 6 days. **B**: Images showing OCL formation. **C**: The number of OCLs was counted after TRAP staining. **D**: Data shown are the results from a dose-dependent experiment. Co, chondroitin; CoS-A, chondoitin sulfate A; CoS-B, chondoitin sulfate B; CoS-D, chondoitin sulfate D; CoS-E, chondoitin sulfate E; Hp, heparin. Data are expressed as the mean ± standard deviation of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. Student's *t*-test, $^*P < 0.05$. □, 10 μg/ml; ▨, 100 μg/ml.

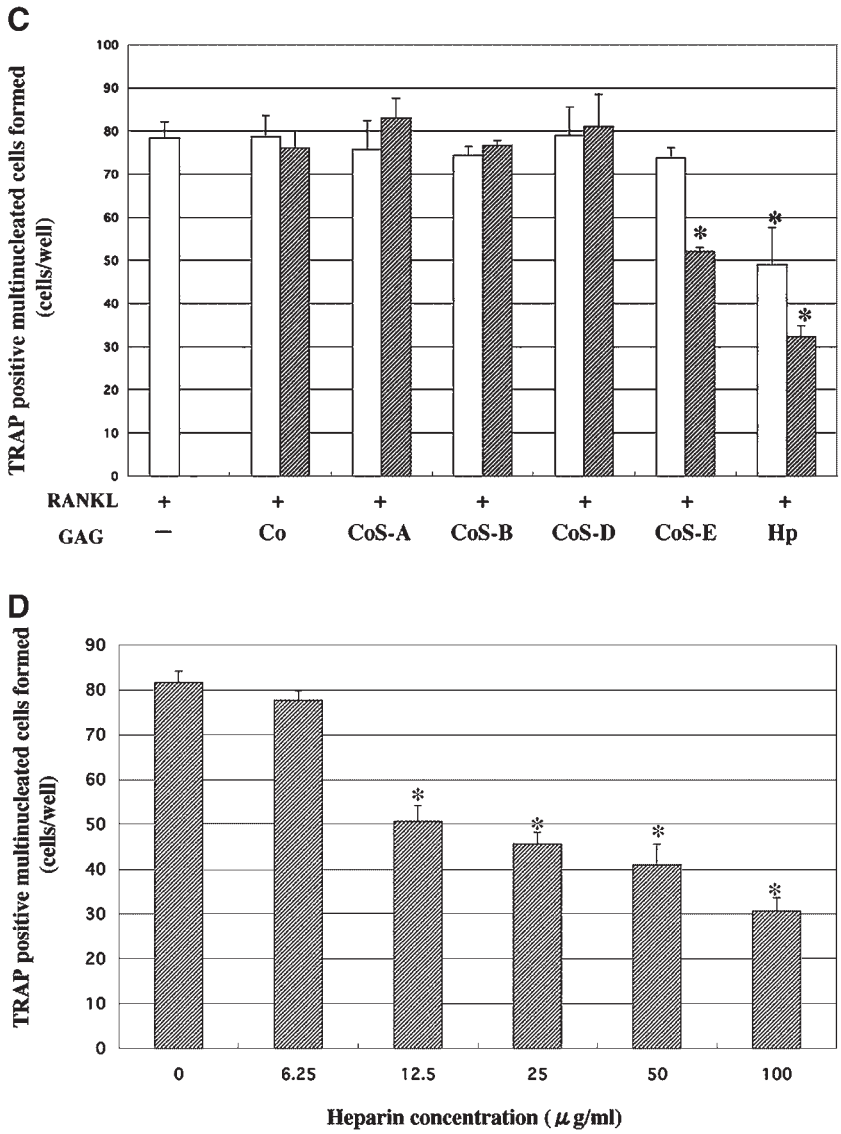

Fig. 2. (*Continued*)

cells after being cultured with the bound fraction (×10 dilution). To determine the competitive ability of heparin with RANK through binding to RANKL, we examined the affinity between heparin and RANKL using a QCM technique. Following the binding of heparin to RANKL, RANK was unable to bind to RANKL (Fig. 7A). On the other hands, after RANK was bound to RANKL, heparin could bind to RANKL (Fig. 7B).

DISCUSSION

In the present study, we used a homogenous clonal population of murine monocytic RAW 264.7 cells to elucidate the direct effects of RANKL and heparin on osteoclast differentiation and function. This cell line is known to express RANK and differentiate into TRAP-positive cells when cultured with bone slices and RANKL [Hsu et al., 1999]. The main advantage of this system is that it does not contain any osteoblastic/bone marrow stromal cells, which may also be targets of RANKL and heparin actions. We found that heparin inhibited OCL formation induced by RANKL in M-BMM culture system (Fig. 2A). Although CoS-B, CoS-E, and heparin had inhibitory effect on the OCL formation derived from M-BMM (Fig. 2A), CoS-B had no inhibitory effect on the OCL formation derived from RAW 264.7 cells

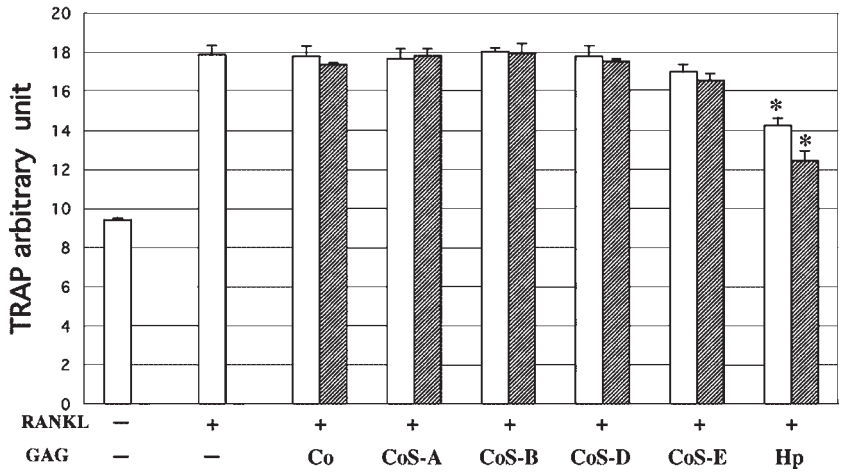

Fig. 3. Effects of GAGs on TRAP activity induced by RANKL. RAW 264.7 cells were stimulated with RANKL (40 ng/ml) and various GAGs (100 μg/ml) for 6 days. TRAP activity was determined as described in ''Materials and Methods''. Co, chondroitin; CoS-A, chondoitin sulfate A; CoS-B, chondoitin sulfate B; CoS-D, chondoitin sulfate D; CoS-E, chondoitin sulfate E; Hp, Heparin. Data are expressed as the mean ± standard deviation of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. Student's *t*-test, $*P < 0.05$. □, 10 μg/ml; ▨, 100 μg/ml.

(Fig. 2C). We have no ready expression for this phenomenon, but we think it is caused by the contamination of a small amount of stromal cells in bone marrow cells. *Another possibility is that RAW 264.7 cells is a transformed cell line, while RAW 264.7 cells exhibit many similarities with authentic osteoclast precursors*. Thus, we focused on pre-osteoclastic cells in the subsequent experiments to examine the effects of RANKL and heparin on differentiation and function. Our results showed that the inhibitory effect of heparin on OCL formation is involved in the RANKL-mediated signaling pathway in mouse bone marrow cells as well as in RAW 264.7 cells.

Bone resorption is a multi-step process initiated by the proliferation of immature osteoclast precursors, which is followed by the commitment of those cells to the osteoclast phenotype, and degradation of the organic and

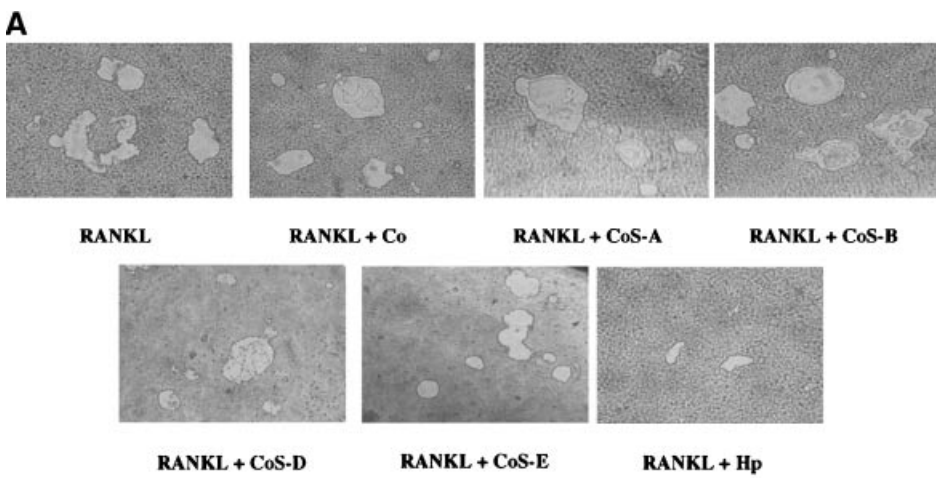

Fig. 4. Effects of GAGs on bone resorption induced by RANKL. RAW 264.7 cells were cultured with RANKL (40 ng/ml) and various GAGs (100 μg/ml) on Osteologic™ plates for 14 days. **A**: Images showing pit formation. **B**: The number of resorption pits was counted. Co, chondroitin; CoS-A, chondoitin sulfate A; CoS-B, chondoitin sulfate B; CoS-D, chondoitin sulfate D; CoS-E, chondoitin sulfate E; Hp, heparin. Data are expressed as the mean ± standard deviation of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. Student's *t*-test, $*P < 0.05$.

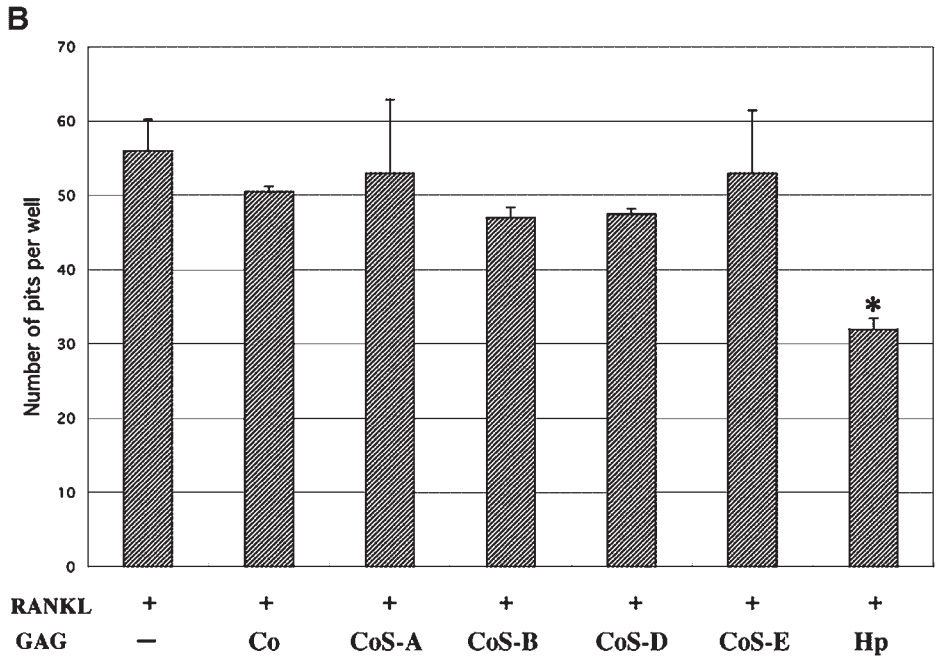

Fig. 4. (*Continued*)

inorganic phases of bone by mature resorptive cells. Like their in vivo counter parts, in vitro-generated osteoclasts are capable of bone resorption. When being cultured with bone or dentin, osteoclasts excavate resorptive lacunae, which are similar to the structures formed when the cells degrade bone in vivo, and the number and size of resorption lacunae formed can be used for quantitative measurements of osteoclast activity [Fuller et al., 1994]. In the present study, we used Osteologic™ slides coated with calcium phosphate substrate and found that heparin downregulated the pit-forming activity of OCLs stimulated with RANKL. These results suggest that heparin inhibits both the differentiation and function of RAW 264.7 cells induced by RANKL.

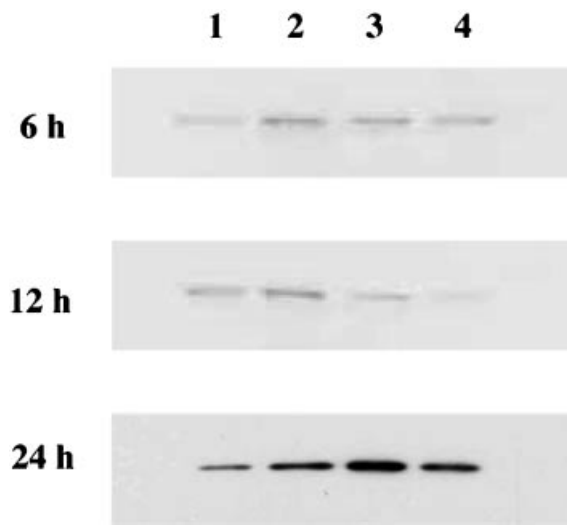

Fig. 5. Expression of c-Src in RAW 264.7 cells. RAW 264.7 cells were stimulated with RANKL (40 ng/ml) in the presence or absence of heparin (100 μg/ml) for the indicated times and whole lysates were subjected to Western blot analysis. **Lane 1**: control; **Lane 2**: heparin; **Lane 3**: RANKL; **Lane 4**: RANKL and heparin.

RANKL increases the levels of c-Src protein, another marker molecule of osteoclast differentiation [Teitelbaum, 2000], which is a widely expressed non-receptor tyrosine kinase that is particularly abundant in platelets, neural tissues [David-Pfeuty and Nouvian-Dooghe, 1990; Clark and Brugge, 1993], and osteoclasts [Horne et al., 1992; Tanaka et al., 1992]. c-Src plays an essential role in osteoclast function, as mice in which the src gene has been disrupted show normal osteoclast development, however, fail to resorb bone, resulting in osteoporosis [Soriano et al., 1991]. We found that the expression of c-Src induced by RANKL was inhibited by heparin, which suggests that the inhibitory effects of heparin on osteoclastic formation and function are responsive to the regulation of c-Src.

Many studies have reported that the binding of heparin to peptides [Chevanne et al., 1999] and chemokines [Witt and Lander, 1994] may be critical for their activities that are dependent

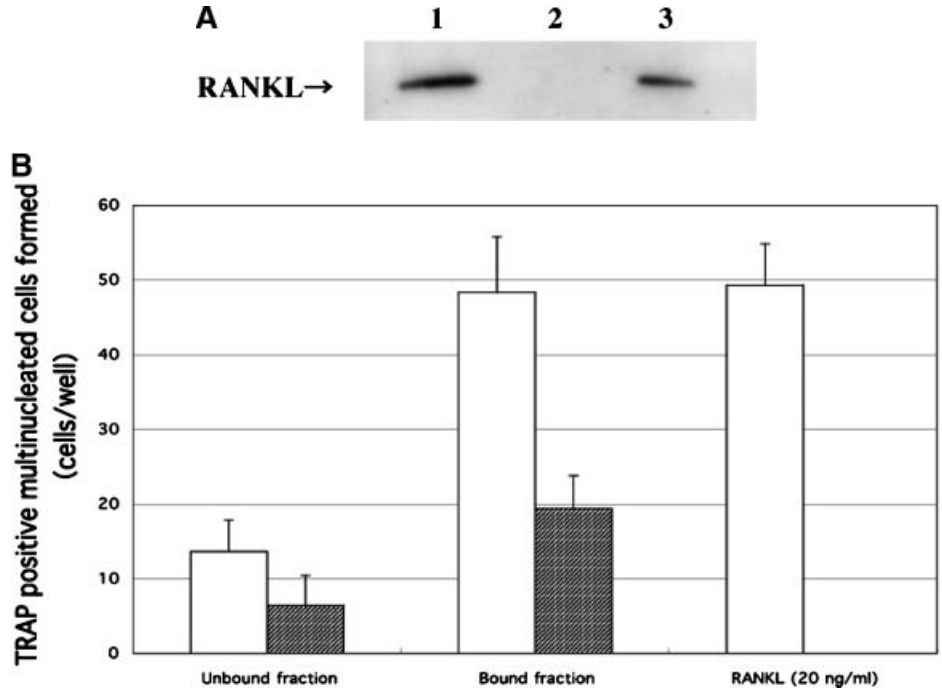

Fig. 6. Binding ability of heparin to RANKL. RANKL (1 μg) was applied to heparin column chromatography and each fraction was collected. **A**: RANKL protein in each fraction was detected by Western blot analysis. **Lane 1**, RANKL as a positive control; **Lane 2**, unbound fraction (90 ng of protein); **Lane 3**, bound fraction (460 ng of protein). **B**: RAW 264.7 cells were cultured with each fraction for 6 days and the number of OCLs was counted. Data are expressed as the mean ± standard deviation of triplicate cultures. The experiment was performed three times, with similar results obtained in each experiment. Student's *t*-test, $^*P < 0.05$. □ ×5 dilution; ▨ ×10 dilution.

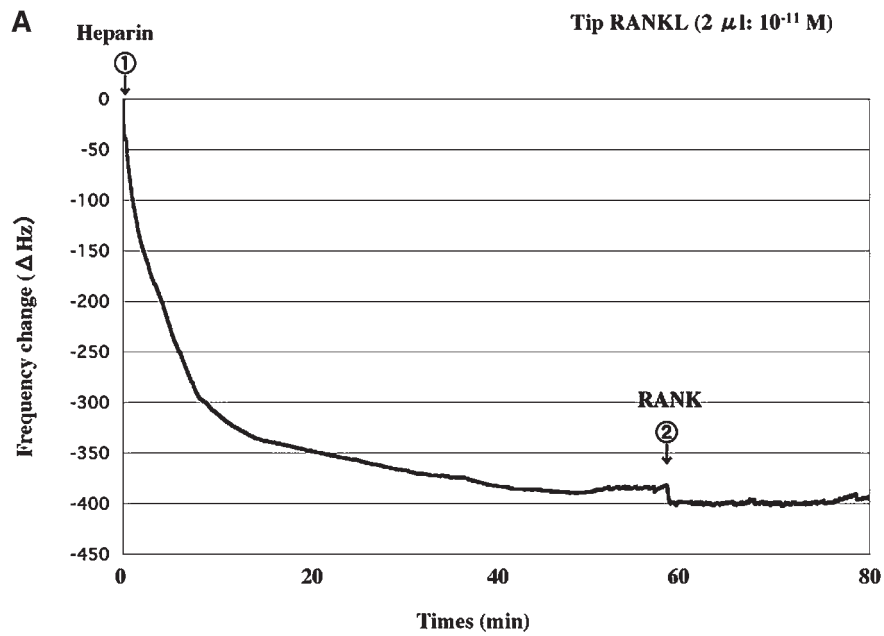

Fig. 7. Competitive ability of heparin with RANK through binding to RANKL. To examine the competitive ability of heparin against RANKL–RANK binding, RANKL (2 μl; 10^{-11} M) was immobilized on a QCM ceramic sensor chip soaked in a PBS solution at 25°C as described in ''Materials and Methods'', after which heparin (100 μg/ml; volume 100 μl) or RANK (100 μg/ml; volume 30 μl) was injected into the solution. **A**: Heparin was injected into the solution (①), and then RANK was injected after the solution was equilibrated (②). **B**: RANK was injected into the solution (①), and then heparin was injected after the solution was equilibrated (②).

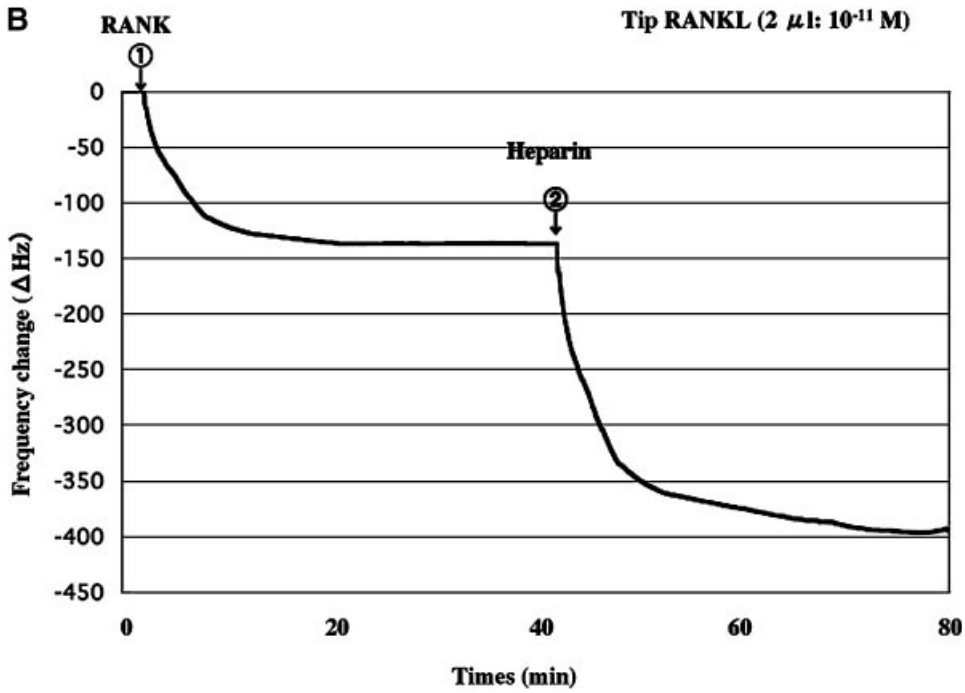

Fig. 7. (*Continued*)

on the specification of immobilization. However, there are no known reports that indicate heparin inhibits osteoclastogenesis through binding to RANKL. In the present study, we found that heparin remarkably bound to RANKL. To our knowledge, this is the report of first the ability of heparin to bind to RANKL. On the basis of these findings, we concluded that such binding ability is involved in the inhibition of osteoclast formation and function. Further, the fraction that was bound to heparin expressed RANKL protein and induced the formation of osteoclasts. Additional studies are needed to examine the correlations of heparin and RANKL regarding bone metabolism including bone formation in physiological conditions.

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