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Chondroitin Sulfate-E Binds to Both Osteoactivin and Integrin αVβ3 and Inhibits Osteoclast Differentiation

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ABSTRACT

Integrins and their ligands have been suggested to be associated with osteoclast-mediated bone resorption. The present study was designed to investigate whether chondroitin sulfate E (CS-E), which is one of the sulfated glycosaminoglycans (GAGs), is involved in osteoactivin (OA) activity, and osteoclast differentiation. The binding affinity of sulfated GAGs to integrin and its ligand was measured using biotin-labeled CS-E, and the osteoclast differentiation was evaluated by tartrate-resistant acid phosphatase staining and a pit formation assay. CS-E as well as CS-B, synthetic chondroitin polysulfate, and heparin inhibited osteoclast differentiation of bone marrow-derived macrophages. Pre-coating of OA to synthetic calcium phosphate-coated plates enhanced the osteoclastic differentiation of RAW264 cells, and addition of a neutralizing antibody to OA inhibited its differentiation. CS-E bound not only to OA, fibronectin, and vitronectin, but also to its receptor integrin αVβ3, and inhibited the direct binding of OA to integrin αVβ3. Furthermore, CS-E blocked the binding of OA to cells and inhibited OA-induced osteoclastic differentiation. On the other hand, heparinase treatment of RAW264 cells inhibited osteoclastic differentiation. Since binding of OA to the cells was inhibited by the presence of heparan sulfate or heparinase treatment of cells, heparan sulfate proteoglycan (HSPG) was also considered to be an OA receptor. Taken together, the present results suggest that CS-E is capable of inhibiting OA-induced osteoclast differentiation by blocking the interaction of OA to integrin αVβ3 and HSPG. J. Cell. Biochem. 116: 2247–2257, 2015.

KEY WORDS: OSTEOCLASTS; CHONDROITIN SULFATE; OSTEOACTIVIN; INTEGRIN

Bone tissue predominantly consists of mineral crystals and organic matter, the composite of which assures stiffness, and compressive strength [Buckwalter and Cooper, 1987]. The prototype of the mineral crystals is hydroxyapatite, which is a type of calcium phosphate (CaP). The main organic matter includes extracellular matrix molecules (ECMs), such as collagen and non-collagenous proteins. These ECMs contribute not only to maintaining the mechanical strength of bone, but also regulate the growth, and differentiation of osteoblasts and osteoclasts [Celic et al., 1998; Rucci et al., 2009]. Osteoclasts are bone-resorbing multinuclear cells formed by the fusion of hematopoietic precursors in the monocyte/macrophage lineage. It is well-known that receptor activator of the NF-κ B ligand (RANKL) in osteoblasts and macrophage colony-stimulating factor (M-CSF) are involved in the formation of mature osteoclasts from mononuclear osteoclast precursor cells [Quinn et al., 1998]. In addition to RANKL and M-CSF, cell adhesion molecules and the extracellular matrix molecules also contribute to the regulation of osteoclast differentiation. The integrin family of receptors consists of transmembrane heterodimer adhesion molecules with α and β subunits that bind to a variety of matrix proteins, including osteopontin, fibronectin (FN), vitronectin (VN), and bone sialoprotein via an Arg-Gly-Asp (RGD) consensus sequence [Oldberg et al., 1988; Butler, 1989]. It has been reported that integrins present on the surface of pre-osteoclasts interact with integrin ligands in bone matrix and play an important role in osteoclast-mediated bone resorption [Helfrich et al., 1992]. In particular, studies have focused on the function of αVβ3 integrin on osteoclast differentiation and cellular activity [Rodan and Rodan, 1997]. It is becoming increasingly clear that osteoactivin (OA), which is one of the ligands for integrin, is

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expressed in osteoblasts, and involved in osteogenesis [Safadi et al., 2002; Abdelmagid et al., 2014]. OA is a type I transmembrane glycoprotein that has high homology to glycoprotein nmb and dendritic cell-associated heparan sulfate proteoglycan dependent-integrin ligand (DC-HIL) [Shikano et al., 2001].

Proteoglycans (PGs), such as decorin and biglycan, which are members of the family of small leucine-rich proteoglycans (SLRPs), exist in bone tissue, and regulate bone metabolism [Parisuthiman et al., 2005; Mochida et al., 2009]. PG is a class of glycosylated proteins that has covalently-linked glycosaminoglycan (GAG), such as chondroitin sulfate (CS), dermatan sulfate (DS), heparin (Hep), heparan sulfate (HS), and keratan sulfate. Decorin and biglycan contain CS or DS as a GAG chain [Cheng et al., 1994]. In addition to SLRPs, heparan sulfate proteoglycans (HSPGs), such as syndecan, also exist in bone tissue, and regulate bone metabolism [Bertrand et al., 2013]. It is well-known that bone metabolism is regulated not only by the core protein of PG, but also by the GAG chain through interactions with growth factors and cytokines. For example, Hep binds to a variety of proteins, including RANKL [Ariyoshi et al., 2008], osteoprotegerin [Théoleyre et al., 2006], fibroblast growth factors [Gospodarowicz et al., 1984], and bone morphogenetic protein (BMP) [Paralkar et al., 1991]. Hep also binds to integrin ligands such as FN, VN, and OA via their heparin binding domain [Shikano et al., 2001; Chillakuri et al., 2010; Tang et al., 2010]. In addition to Hep, several CS structure variants bind to growth factors and cytokines, which lead to the regulation of osteoclast differentiation and function. We previously reported that CS-E binds to BMP-4 and enhances osteoblast differentiation [Miyazaki et al., 2008]. We have also demonstrated that CS-E inhibits osteoclast differentiation of the murine macrophage cell line RAW264 [Miyazaki et al., 2010]. Together, these data suggest that CS-E may inhibit osteoclast differentiation by blocking the interaction of integrin with ligands such as FN.

In this report, we investigated the effect of a variety of CS structural isomer variants on osteoclast differentiation of murine bone marrow cells after stimulation with M-CSF and RANKL. This is the first report to describe a mechanism of action for how CS-E inhibits osteoclast differentiation through blocking interactions with adhesion molecules, such as OA, and integrin αVβ3.

MATERIALS AND METHODS

MATERIALS

The murine macrophage cell line RAW264 was obtained from the Riken Cell Bank (Tsukuba, Japan). DMEM/F-12 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MEMα and gentamicin were purchased from Invitrogen Corporation (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from Biological Industries, Ltd. (Kibbutz Beit Haemek, Israel). CS-A (GlcUA1-3GalNAc(4S))$_n$ from whale cartilage, CS-B (the so-called DS) (IdoUA1-3GalNAc(4S))$_n$ from porcine skin, CS-C (GlcUA1-3GalNAc(6S))$_n$ from shark cartilage, CS-D (GlcUA(2S)1-3GalNAc(6S))$_n$ from shark cartilage, CS-E (GlcUA1-3GalNAc(4S,6S))$_n$ from squid cartilage, and HS from pig kidney were obtained from PG Research Co., Ltd. (Kodaira, Japan). A synthetic chondroitin polysulfate (CPS) (GlcUA(2S,3S) 1-3GalNAc(4S,6S))$_n$, was purchased from Novartis Animal Health Inc. (Basel, Switzerland). Heparin from porcine intestine was purchased from Wako Pure Chemical Industries. The fluoresceinamine-labeled CS-B, CS-C, CS-D, CS-E, CPS, Hep, and HS were obtained from PG Research. The molar percentage of bound fluoresceinamine groups to the disaccharide unit was almost the same among these GAGs. Recombinant human RANKL was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Recombinant human M-CSF, human plasma VN, recombinant mouse OA Fc Chimera (OA-Fc), recombinant human OA-Fc, polyclonal neutralizing antibody against mouse OA, recombinant human integrin αVβ3, and anti-mouse IgG were purchased from R&D Systems Inc (Minneapolis, MN). Human plasma FN was purchased from Wako Pure Chemical Industries and heparinase III was obtained from Sigma–Aldrich Corporation (St. Louis, MO).

CELL CULTURE

Bone marrow cells were isolated from seven week-old male ddY mice as described by Takeshita et al. [2000]. All procedures were approved by in house Animal Research Committee at PG Research. Tibia and femora were aseptically removed and the marrow was forced out into a petri dish containing MEMα, 10% heat inactivated FBS, and 10 μg/mL gentamicin. The marrow suspension was filtered with a Cell Strainer (Becton Dickinson, Franklin Lakes, NJ) to remove bone particles and aggregated cells. The bone marrow cells were carefully agitated and harvested by centrifugation. After red blood cells were lysed using 0.75% ammonium chloride and washed, the bone marrow cells were cultured in the same medium with M-CSF (100 ng/mL) using a 10-cm suspension culture dish (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) at 37°C in a humidified atmosphere of 5% CO_2. On day 3, after removing nonadherent cells, the adherent cells were harvested by TrypLESelect (Invitrogen). These M-CSF–dependent bone marrow macrophages (MDBMs) were used for the subsequent assays.

RAW264 cells were maintained in DMEM/F-12 containing 10% FBS and 10 μg/mL gentamicin (growth medium). The cells were detached with TrypLESelect and used for assays.

COATING OF CELL ADHESION MOLECULES FOR CELL CULTURE

Cell adhesion molecules, such as recombinant mouse OA-Fc, FN, VN, and integrin αVβ3 (10 μg/mL in PBS), were added to the 48-well CaP plate (PG research) and incubated at 37°C for 1 h. After washing the plate with PBS and growth medium, the plates were used for assays.

TARTRATE-RESISTANT ACID PHOSPHATASE (TRAP) STAINING ASSAY

The MDBMs were inoculated into 48-well plates at a density of 5×10^3 cells/well and cultured in a MEMα containing 10% heat inactivated FBS, 50 ng/mL M-CSF, 50 ng/mL RANKL, and 10 μg/mL gentamicin. The sulfated GAGs and other reagents were added at the same time. After five days, cells were fixed with 4% paraformaldehyde and then stained with a tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma) following the manufacturer's protocol. After washing the wells with pure water, the number of TRAP-positive multinuclear cells in each well was determined and

photographed. The area of TRAP-positive cells was measured with an image analyzing software, Image J software (NIH, Bethesda, MD).

PIT ASSAY

The MDBMs were inoculated into the CaP plates at a density of 5×10^3 cells/well and cultured in MEMα containing 10% heat inactivated FBS, 100 ng/mL M-CSF, 100 ng/mL RANKL, and 10 μg/mL gentamicin. RAW264 cells were inoculated into the CaP plates at a density of 5×10^3 cells/well and cultured in a growth medium containing 100 ng/mL RANKL. The sulfated GAGs or other regents were added at the same time. After six days, the plates were treated with 5% sodium hypochlorite for 5 min to remove the cells. After washing the plates with pure water and then allowing them to dry, the pit area in each well was photographed, and measured with an image analyzing software, Scion image software (Frederick, MD) or Image J software.

PREPARATION OF BIOTIN-LABELED CS-E (BIOTIN-CS-E)

Labeling of biotin to CS-E was performed according to the methods described by Ogamo et al. [1982]. The uronic acid residues of CS-E were reacted with 6-(Biotinylamino) hexanoylhydrazine and the biotin labeled product was prepared.

BINDING ASSAY OF GAGS TO INTEGRIN AND INTEGRIN LIGANDS

The binding affinity of biotin-CS-E to OA-Fc was investigated according to the following method. A 96-well plate (MaxiSorp, Nunc, Roskilde, Denmark) was first coated with anti-mouse IgG (10 μg/mL in PBS) at room temperature. The plate was then washed with PBS containing 0.05% Tween 20 (T-PBS) and blocked with Block Ace (DS Pharma Biomedical Co., Ltd, Suita, Japan). Recombinant mouse OA-Fc (10 μg/mL) was added and bonded to the plate. After washing the plate, various concentrations of biotin-CS-E were added to each well and incubated.

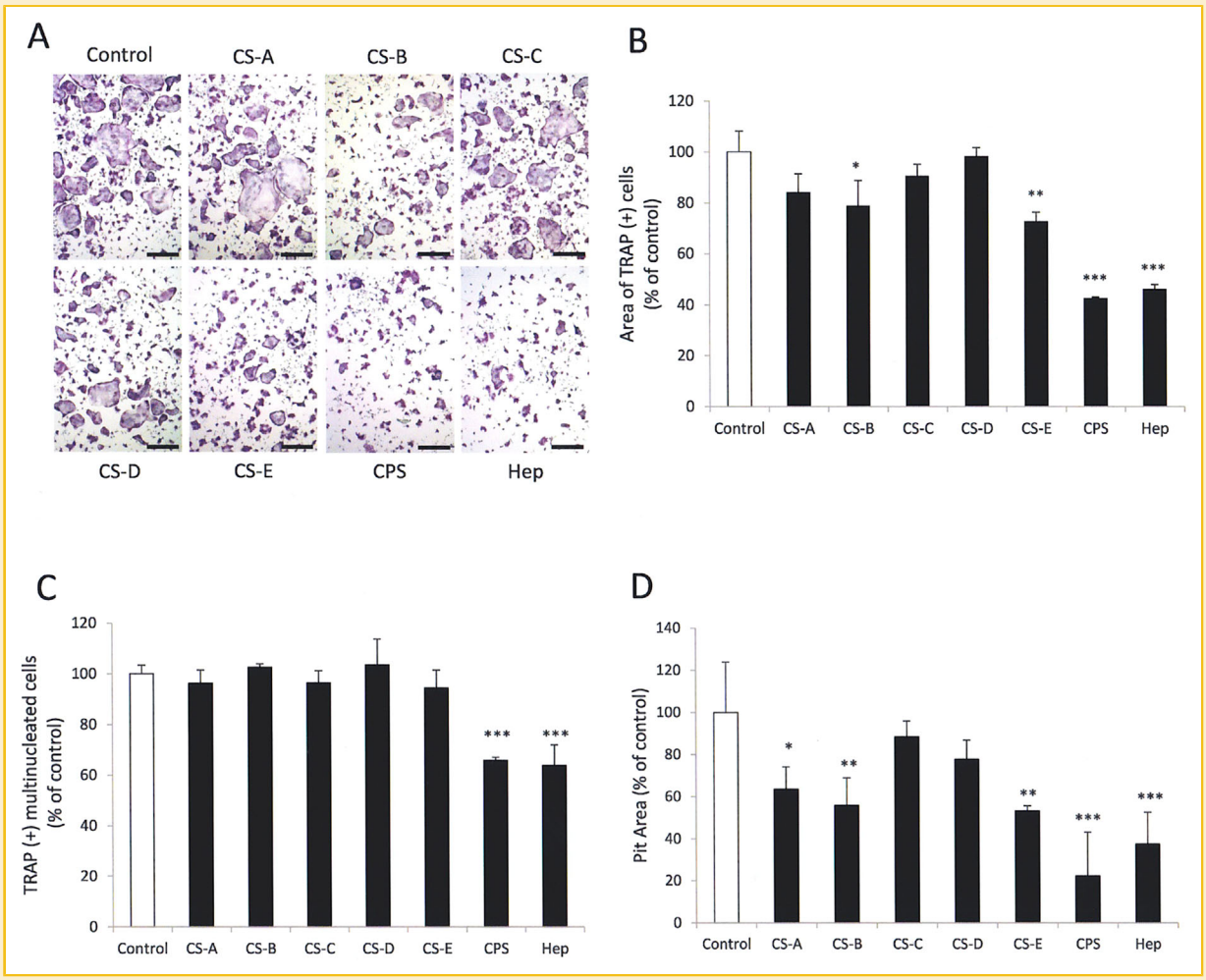

Fig. 1. Effect of sulfated GAGs on the osteoclastic differentiation of MDBMs. A: Photographs using a microscope after TRAP staining of MDBMs. Bars = 200 μm. B: The area of TRAP-positive cells measured by image analyzing software. C: The number of TRAP-positive multinucleated (more than three nuclei) cells. MDBMs were cultured on a 48-well plate at a density of 5×10^3 cells/well in the presence or absence of sulfated GAGs for 5 days while incubated with M-CSF and RANKL. D: Evaluation of pit formation using a CaP plate. MDBMs were cultured on the CaP plate at a density of 5×10^3 cells/well in the presence or absence of sulfated GAGs for 6 days while incubated with M-CSF and RANKL and the pit area was subsequently measured. The concentration of each sulfated GAG was 100 μg/mL. Data are shown as the percentage of the RANKL-containing control (Mean ± SD, $n = 3$). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

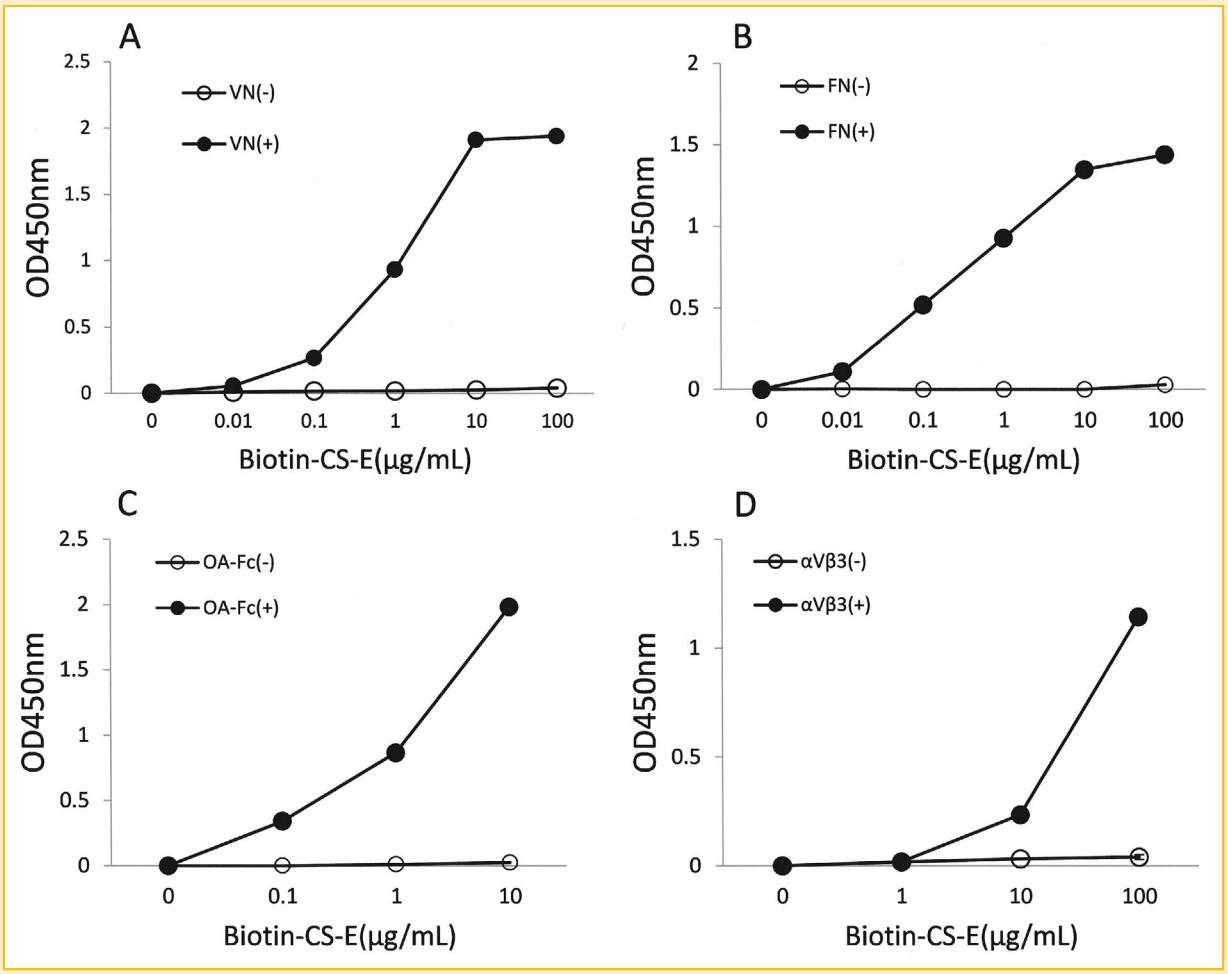

Fig. 2. Binding affinity of biotin-CS-E to integrin αVβ3 and its ligands. Each concentration of biotin-CS-E was incubated with immobilized VN (A), FN (B), OA-Fc (C), and integrin αVβ3 (D) (closed circle) under the conditions described in the materials and methods. The absorbance at 450 nm was then measured. An open circle indicates the negative control without coating of integrin αVβ3 or its ligands.

To investigate the binding affinity of the other GAGs, a competition assay using biotin-CS-E was performed. A mixture of biotin-CS-E (10 μg/mL) and ten-fold concentrations of free-GAGs (100 μg/mL) were added and incubated. The plate was then washed and horseradish peroxidase (HRP) labeled-avidin (MP Biomedicals, Santa Ana, CA) was added and further incubated. The wells were developed with TMB substrate, and the reaction was stopped by adding 1N HCl. The absorbance at 450 nm was determined with a microplate reader (Corona Electric, Hitachinaka, Japan). For VN, FN, and integrin αVβ3, the proteins were coated directly to the 96-well plate and then the same procedure was performed.

The binding affinity of GAGs to integrin ligands was further investigated using fluoresceinamine-labeled GAGs. The 96-well black plate (Nunc) was coated with VN (10 μg/mL in PBS) at room temperature. The plate was washed with T-PBS, and a series of fluoresceinamine-labeled GAGs were then added to each well and incubated. After washing the plate, 0.3 M Tris–HCl (pH 9.1) was added to each well. The fluorescence intensity was measured using a fluorescence plate reader (Twinkle LB970, Berthold Japan, Tokyo, Japan) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

BINDING ASSAY OF OA TO INTEGRIN

The 96-well plate was first coated with integrin αVβ3 (10 μg/mL) and blocked under the same conditions described above. Recombinant mouse OA-Fc (10 μg/mL) was added and incubated at room temperature. For the inhibition assay, a mixture of OA-Fc and CS-E (1 μg/mL) was added to the integrin αVβ3-coated plate, which was pretreated with CS-E. After washing the plate, HRP-labeled anti-mouse IgG (Jackson ImmunoResearch Inc. West Grove, PA) was added and further incubated. The plate was washed and the wells were developed with TMB substrate, and the reaction was stopped by adding 1N HCl. The absorbance at 450 nm was subsequently determined with a microplate reader.

BINDING ASSAY OF OA TO RAW264 CELLS

RAW264 cells cultured in the growth medium were harvested by PBS containing 0.05% EDTA. The cells were washed with PBS and fixed using

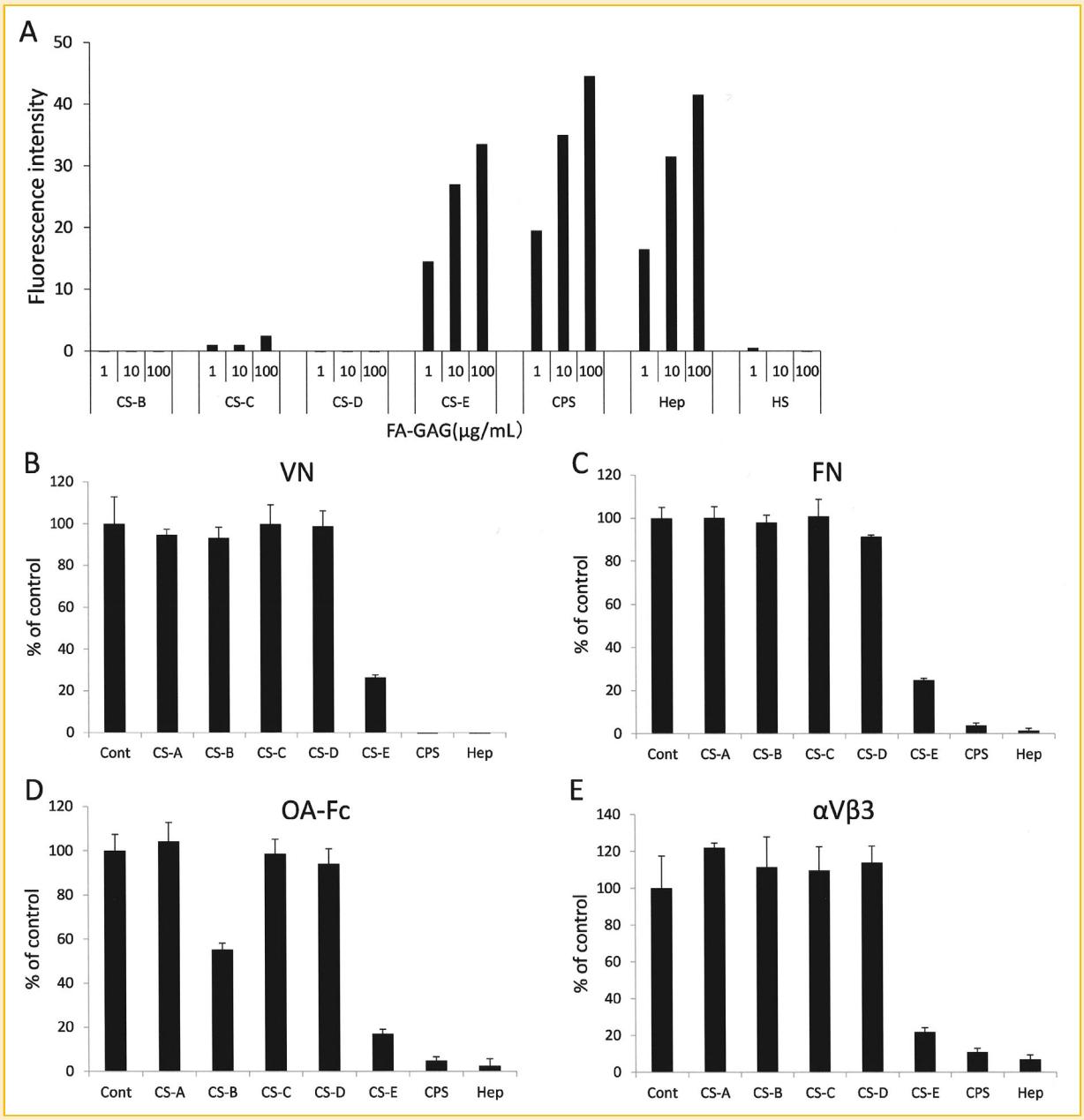

Fig. 3. Binding affinity of sulfated GAGs to integrin αVβ3 and its ligands. A: The binding affinity of fluoresceinamine-labeled sulfated GAGs to VN. Each concentration of fluoresceinamine-labeled sulfated GAG was incubated with immobilized VN under the conditions described in the materials and methods and then measured for fluorescence intensity. B–E: The binding affinity of sulfated GAGs to integrin αVβ3 and its ligands. The binding affinity to VN (B), FN (C), OA-Fc (D), and integrin αVβ3 (E) was evaluated in a competition assay using biotin-CS-E as described in the materials and methods. Data are shown as the percentage of control (Mean ± SD, $n = 3$).

4% paraformaldehyde. The cells were then washed with PBS and treated with 0.3% H_2O_2 in methanol at room temperature to block endogenous peroxidase. After washing the cells with pure water, the cells were blocked with Blockace containing 10% goat serum for 30 min. The cell density for staining was 1×10^6 cells/tube. The cells and recombinant human OA-Fc (10 μg/mL) were pre-incubated separately with CS-C, CS-E, and HS (100 μg/mL), and then mixed and further incubated for 2 h at room temperature. Alternatively, the cells were pre-incubated with heparinase III before the addition of OA-Fc. After washing the cells with PBS, HRP-labeled anti-human IgG (Bethyl Laboratories, Montgomery, TX) was added and incubated for 1 h. After washing the cells with PBS, the color was developed by mixing the cells with TMB solution and stopped with 1N HCl. The supernatant of each tube was collected after centrifugation and then the absorbance at 450 nm was measured using a microplate reader.

STATISTICAL ANALYSIS

The results were expressed as the mean ± standard deviation (SD). Statistical analysis of differences was performed with a Student's *t*-test or Kruskal-Wallis test followed by Dunnett's multiple comparison test.

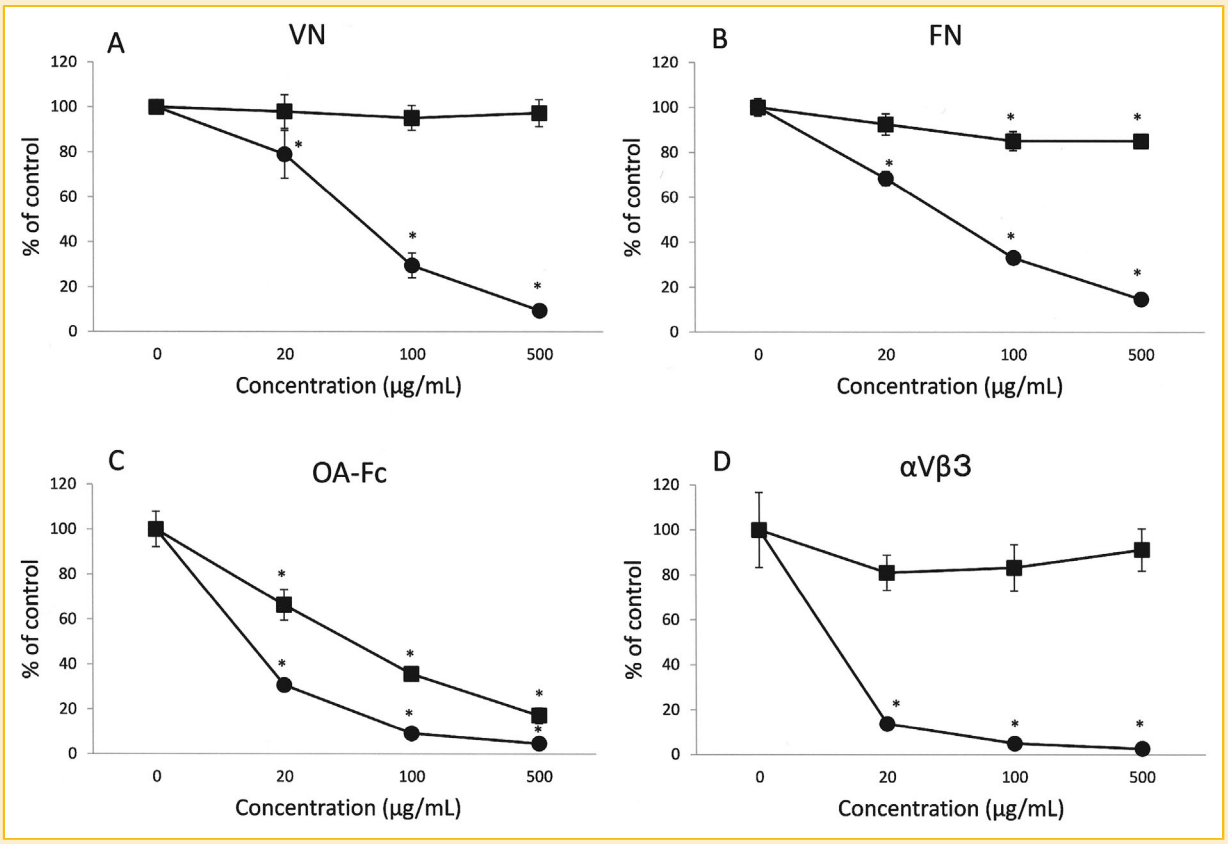

Fig. 4. Binding affinity of HS to integrin αVβ3 and its ligands. A: The binding affinity of HS to VN (A), FN (B), OA-Fc (C), and integrin αVβ3 (D) was evaluated with a competition assay using biotin-CS-E. ●: inhibition of biotin-CS-E binding by CS-E, ■: inhibition of biotin-CS-E binding by HS. Data were shown as the percentage of control (Mean ± SD, $n = 3$). *: $P < 0.05$.

RESULTS

EFFECTS OF SULFATED GAGS ON THE DIFFERENTIATION OF MDBMS INTO OSTEOCLASTS

We first examined the effect of sulfated GAGs on the differentiation of MDBMs into osteoclasts after stimulation with M-CSF and RANKL. CS-B, CS-E, CPS, and Hep clearly inhibited the appearance of TRAP-positive large multinucleated cells (Fig. 1A). The area of TRAP-positive cells significantly decreased on the treatment of CS-B, CS-E, CPS, and Hep (Fig.1B). However, the number of TRAP-positive multinucleated cells decreased after treatment with CPS and Hep (Fig. 1C). We then analyzed the bone resorption activity of osteoclasts using the pit assay. CS-A, CS-B, CS-E, CPS, and Hep inhibited pit formation, whereas CS-C and CS-D had no effect (Fig. 1D).

BINDING AFFINITY OF SULFATED GAGS TO INTEGRIN AND INTEGRIN LIGANDS

Using biotin-CS-E, the binding affinity of CS-E to integrin and its ligands were investigated. The biotin-CS-E bound to immobilized VN, FN, and OA-Fc in a dose-dependent manner (Fig. 2A–C). The biotin-CS-E also bound to integrin αVβ3; however, the binding affinity was weaker than that of the integrin ligands (Fig. 2D). We next evaluated the binding affinity of other sulfated GAGs to integrin and its ligands. Using fluoresceinamine-labeled GAGs, the binding affinity of GAGs to VN was investigated. Fluoresceinamine-labeled CS-E, CPS, and Hep showed binding affinity to VN (Fig. 3A). However, the binding of CS-C to VN was very weak. CS-E, CPS, and Hep also showed affinity to VN, FN, and integrin αVβ3 in the competition assay using biotin-CS-E (Fig. 3B, 3C, 3E, respectively). The direct binding assay using fluoresceinamine-labeled GAG correlated well with the biotin-CS-E competition assay. On the other hand, OA-Fc bound to not only CS-E, CPS, and Hep, but CS-B as well (Fig. 3D). We found that HS bound OA-Fc and FN, but not to VN and integrin αVβ3 (Fig. 4A–D). In addition, the binding affinity of HS to FN was very weak compared to the affinity of CS-E.

EFFECTS OF OA, FN, AND VN ON THE ADHESION AND DIFFERENTIATION OF RAW264 CELLS

We next investigated the effect of integrin and its ligands on the differentiation of RAW264 cells into osteoclastic cells after stimulation with RANKL. When integrin αVβ3 was pre-coated on a CaP plate, pit formation was inhibited (Fig. 5A). Conversely, pre-coating with OA-Fc enhanced pit formation on the CaP plate. The RANKL-induced pit formation was significantly inhibited by adding a polyclonal neutralizing antibody against OA. The effect was

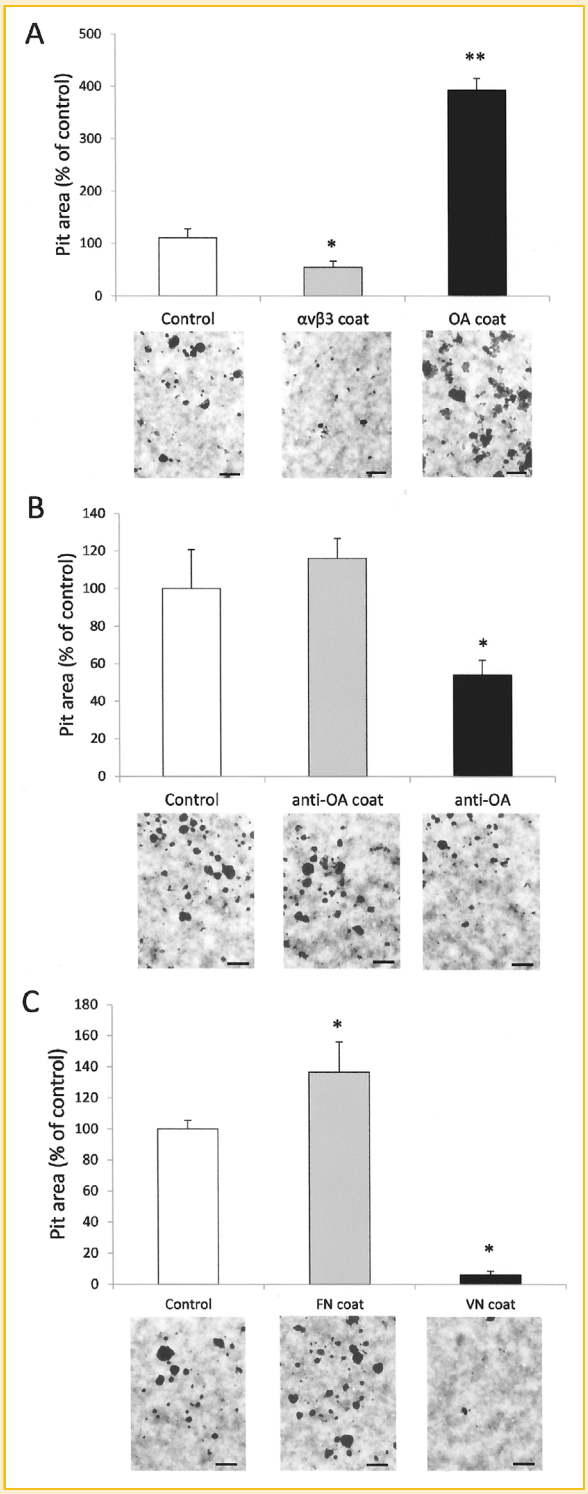

Fig. 5. Effect of OA on the osteoclastic differentiation of RAW264 cells. A: Pit formation of the RAW264 cells cultured on the CaP plate pre-coated with integrin αVβ3 or OA-Fc. B: Pit formation of the cells on the CaP plate pre-coated with a polyclonal neutralizing antibody to OA or in the presence of the same antibody in the medium. C: Pit formation of the cells on the CaP plate pre-coated with FN or VN. Cells were cultured for 6 days in the presence of RANKL. Bars = 500 μm. Data are shown as the percentage of the RANKL-containing control (Mean ± SD, $n = 3$ or 4). *: $P < 0.05$, **: $P < 0.001$.

cancelled when the antibody was pre-coated to the CaP plate (Fig. 5B). Moreover, the pit formation of RAW264 cells induced by RANKL was enhanced by pre-coating with FN, whereas it was inhibited by pre-coating with VN (Fig. 5C).

RAW264 cells also detached and aggregated on the CaP plate after stimulation with RANKL (Fig. 6A). However, when the CaP plate was pre-coated with OA-Fc, FN, or VN, self-aggregation was blocked and cell adhesion to the CaP plate was enhanced (Fig. 6B–D).

INHIBITION OF OA BINDING TO INTEGRIN αVβ3 BY CS-E

We next investigated the binding affinity of OA-Fc to integrin αVβ3 and found that the two proteins could interact (Fig. 7A). Importantly, the binding of OA-Fc to integrin αVβ3 was inhibited by CS-E when the two proteins were pre-incubated separately with CS-E (Fig. 7B). Furthermore, the pit formation induced by OA-Fc was significantly inhibited by pre-incubation with CS-E (Fig. 7C).

EFFECTS OF HS ON OA BINDING AND OSTEOCLAST DIFFERENTIATION

In addition to CS-E, HS also inhibited the pit formation of RAW264 cells induced by RANKL (Fig. 8A). The inhibitory activity of HS was stronger than that of CS-E. Treatment of RAW264 cells with an HS degradation enzyme, heparinase III, significantly inhibited pit formation (Fig. 8B). In addition, the binding of OA-Fc to RAW264 cells was inhibited by the presence of CS-E and HS, but not CS-C (Fig. 8C). Furthermore, OA-Fc binding to RAW264 cells was inhibited by treatment of the cells with heparinase III (Fig. 8D).

DISCUSSION

In addition to the major mono-sulfated CS structure variants, CS-A, CS-B (DS), and CS-C, several oversulfated structures, such as CS-E and CS-H, are also known to exist in vertebrates. These forms of oversulfated CS have potent biological activities that regulate differentiation of the cells by interacting with growth factors and matrix proteins [Deepa et al., 2002; Furutani et al., 2005]. CS-E, CS-H, and CPS, which are those that possess a 4,6-disulfated structure of GalNAc, and Hep/HS, have the capacity to enhance osteoblast differentiation [Miyazaki et al., 2008]. On the other hand, CS-B, CS-E, CPS, and Hep inhibit the osteoclastic differentiation of RAW264 cells [Miyazaki et al., 2010]. In the present study, these CS variants, as well as Hep, exhibited inhibitory activity on the osteoclast differentiation of MDBMs in both the TRAP staining and pit assays (Fig. 1). The inhibitory activity of these sulfated GAGs on MDBMs seemed to be similar to that of RAW264 cells [Miyazaki et al., 2010]. These results suggest that sulfated GAGs have inhibitory effects on pre-osteoclastic cells in general.

A previous report suggested that the inhibition of osteoclast differentiation by CS-E might be due to blocking of the interaction between integrins and their respective ligands [Miyazaki et al., 2010]. In the present study, sulfated GAGs were found to bind immobilized VN, FN, OA-Fc, and integrin αVβ3 in a competition assay using biotin-CS-E. This approach was a simple assay for analyzing the binding affinity of proteins and GAGs. The results showing the interaction between sulfated GAGs and VN using a

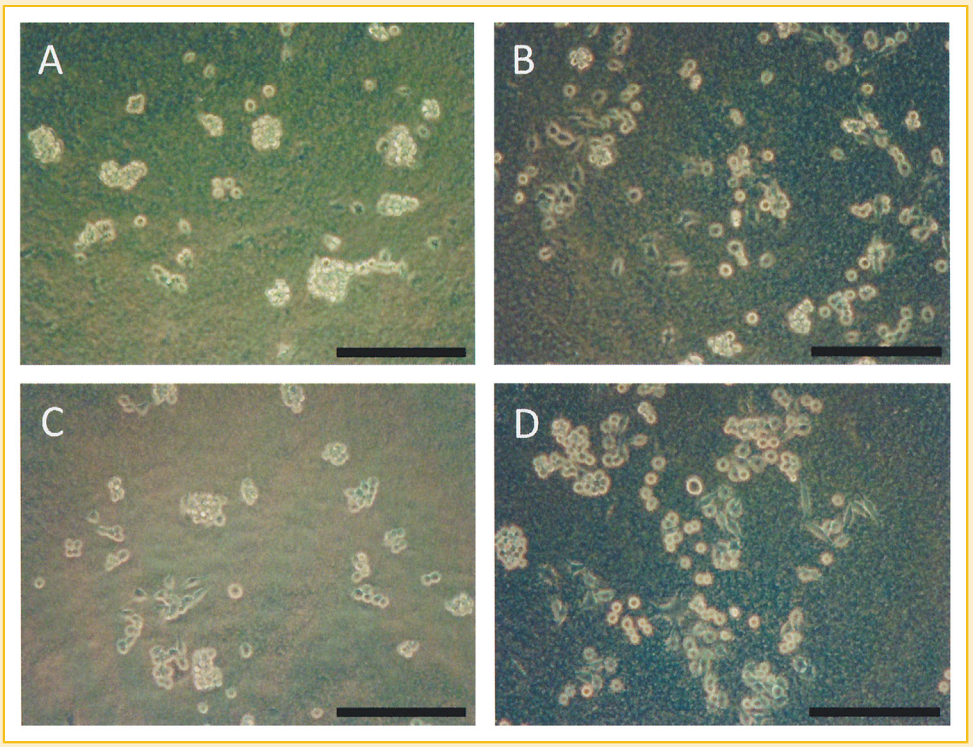

Fig. 6. Effect of integrin ligands on the adhesion of RAW264 cells. RAW264 cells were cultured in the presence of RANKL on CaP plates that were non-coated (A) or pre-coated with OA-Fc (B), FN (C), or VN (D). After 48 h, cells were photographed using a phase contrast microscope. Bars = 200 μm.

biotin-CS-E competition assay correlated well with the results from a direct binding assay using a series of fluoresceinamine-labeled GAGs (Fig. 3A and B). Interestingly, CS-B bound OA-Fc, and the binding affinity of GAGs to OA-Fc correlated well with inhibition of osteoclastogenesis. This competition assay also provided evidence that HS binds to OA-Fc and FN (Fig. 4B and C). Although FN, OA, and VN possess a heparin binding domain, the binding affinity to sulfated GAGs was different, and VN was the only protein of the series that did not bind HS. The interaction of integrin ligands and GAGs is complicated. For example, FN is known to possess several heparin binding domains, but only the Hep II domain of FN is able to bind to HS [Tumova et al., 2000]. We found that the binding affinity of HS to FN was weak (Fig. 4B), suggesting that CS-E not only binds the Hep II domain, but also to other heparin binding domains of FN. On the other hand, CS-E and HS had a similar binding affinity to OA-Fc. Since OA has one heparin binding domain [Shikano et al., 2001], CS-E, HS, and CS-B are thought to bind this domain. In addition, CS-E also bound integrin αVβ3. It has been reported that Hep binds to integrin αVβ3 and α5β1 [Faye et al., 2009], and therefore CS-E may also bind to other types of integrins, such as α5β1. We used the CS-E polymer in the present study, which has a molecular weight of approximately 50 kDa. Since the CS-E oligosaccharide has been shown to inhibit osteoclast differentiation [Miyazaki et al., 2010], further investigation is required to determine the relationship between the size of CS-E and OA binding.

Among integrin families, integrin αVβ3 has been the main protein studied for involvement in osteoclast formation [Boissy et al., 1998]. There are several reports about the effect of OA on bone metabolism. OA enhances bone regeneration in a calvarial model [Bateman et al., 2012], and the overexpression of OA promotes osteoclast formation and bone loss [Sheng et al., 2012]. In addition, the expression of OA is up-regulated by RANKL stimulation, and osteoclastogenesis is inhibited by the presence of a neutralizing antibody against OA [Sheng et al., 2008]. Since OA co-immunoprecipitates with integrin β1 and β3 subunits in bone marrow-derived osteoclasts [Sheng et al., 2008], integrin αVβ3 was considered as one of the receptors of OA. Indeed, the present results showed that OA-Fc bound to immobilized integrin αVβ3 (Fig. 7A). RAW264.7 cells also express both OA and integrin αVβ3 [Jung et al., 2012; Sheng et al., 2012], and the resorption activity of RAW264 cells was inhibited by the addition of a neutralizing antibody against OA in the medium (Fig. 5B). Furthermore, the coating of OA-Fc to the CaP plate significantly enhanced pit formation, whereas integrin αVβ3 inhibited its formation. We hypothesize that the coated integrin αVβ3 binds to its ligand on the cell surface, such as OA, and inhibits the interaction of the two proteins. We demonstrated the CS-E bound both OA-Fc and integrin αVβ3, and the binding of OA-Fc to integrin αVβ3 was significantly inhibited by the addition of CS-E (Fig. 7B). In this inhibition assay, both integrin αVβ3 and OA-Fc were pre-incubated with CS-E independently before initiating the reaction, since OA-Fc is presumed to bind to integrin αVβ3 indirectly through the CS-E chain. Furthermore, the enhancement of resorption activity induced by OA-Fc was inhibited by pre-incubation of OA-Fc with CS-E (Fig. 7C). These results indicate that

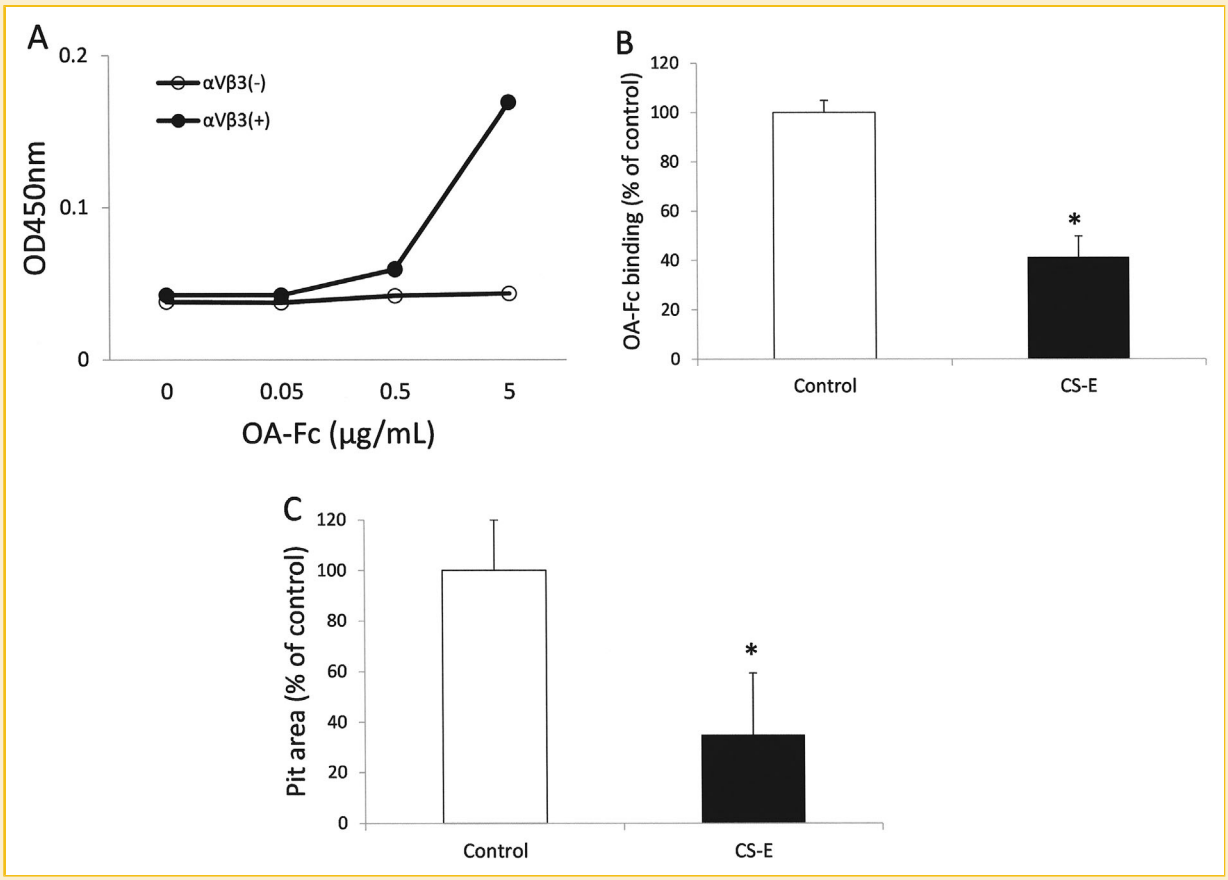

Fig. 7. Effect of CS-E on the binding of OA to integrin αVβ3. A: Binding of OA-Fc to immobilized integrin αVβ3. Each concentration of OA-Fc was incubated with integrin αVβ3 (closed circle) and then the absorbance at 450 nm was measured (open circle: integrin αVβ3 non-coating control). B: Inhibition of OA-Fc binding to immobilized integrin αVβ3 by CS-E. Data are shown as the percentage of the OA-Fc binding control. C: The inhibition of OA-Fc-induced pit formation of RAW264 cells by CS-E. RAW264 cells were cultured on the CaP plate pre-coated with OA-Fc, CS-E, or both OA-Fc and CS-E together for 6 days in the presence of RANKL and the pit area was then measured. Since the pre-coating of CS-E alone affects pit formation, the inhibition activity was evaluated as follows: Control: (pit area in the presence of RANKL and OA-Fc) − (pit area in the presence of RANKL), CS-E: (pit area in the presence of RANKL, OA-Fc, and CS-E) − (pit area in the presence of RANKL and CS-E). Data are shown as the percentage of control (Mean ± SD, $n = 3$). *: $P < 0.01$.

CS-E binds to both OA and integrin αVβ3 on the cell surface and inhibits the resorption activity of osteoclasts induced by the OA and integrin interaction.

In this study, it was clear that CS-E and HS inhibit osteoclast differentiation and that the inhibitory activity of HS is stronger than CS-E (Fig. 8A). It has been previously shown that when cells are treated with chondroitinase ABC to remove CS chains on the cell surface, osteoclast differentiation is not affected [Miyazaki et al., 2010]. On the other hand, treatment of the cells with heparinase III, which removes heparan sulfate on the cell surface, significantly inhibited the resorption activity (Fig. 8B). These results suggest that the cell surface HS chain, but not the CS chain, may work on osteoclast differentiation. Furthermore, the binding of OA-Fc to the cells was inhibited by pretreatment with CS-E and HS as well as by digesting the cells with heparinase III (Fig. 8C and D). The cytoplasmic domain of HSPG, such as syndecan-4, has the ability to induce signal transduction [Kwon et al., 2012]. DC-HIL binds to T cells via an HS chain of HSPG and mediates negative co-regulatory function of the cell [Chung et al., 2013]. Taken together, these results suggest that CS-E inhibits OA-induced osteoclast differentiation by inhibiting both OA-integrin αVβ3 and OA-HSPG interactions on the cell surface.

There is a general consensus that integrin ligands enhance cell adhesion and spreading on a variety of cell types. In the present study, pre-coating of FN, VN, or OA-Fc enhanced cell adhesion and inhibited the aggregation of RAW264 cells (Fig. 6). The coating of CS-E or HS to the CaP plate also enhanced cell adhesion and spreading (data not shown). On the other hand, the pre-coating of VN on the CaP plate significantly inhibited pit formation, whereas OA-Fc and FN pre-coating enhanced its activity. Therefore, the resorption activity may not simply correlate with cell adhesion to the CaP plate. Regarding the difference between FN and VN, a previous report indicated that FN and VN inhibit osteoclastogenesis, while FN enhances resorption activity via nitric oxide and interleukin-1β-mediated signaling pathways [Gramoun et al., 2010]. The study also indicated that osteoclast attachment on FN is mediated through integrin α5β1, but not integrin αVβ3. Therefore, these results suggest that a detailed analysis of the type

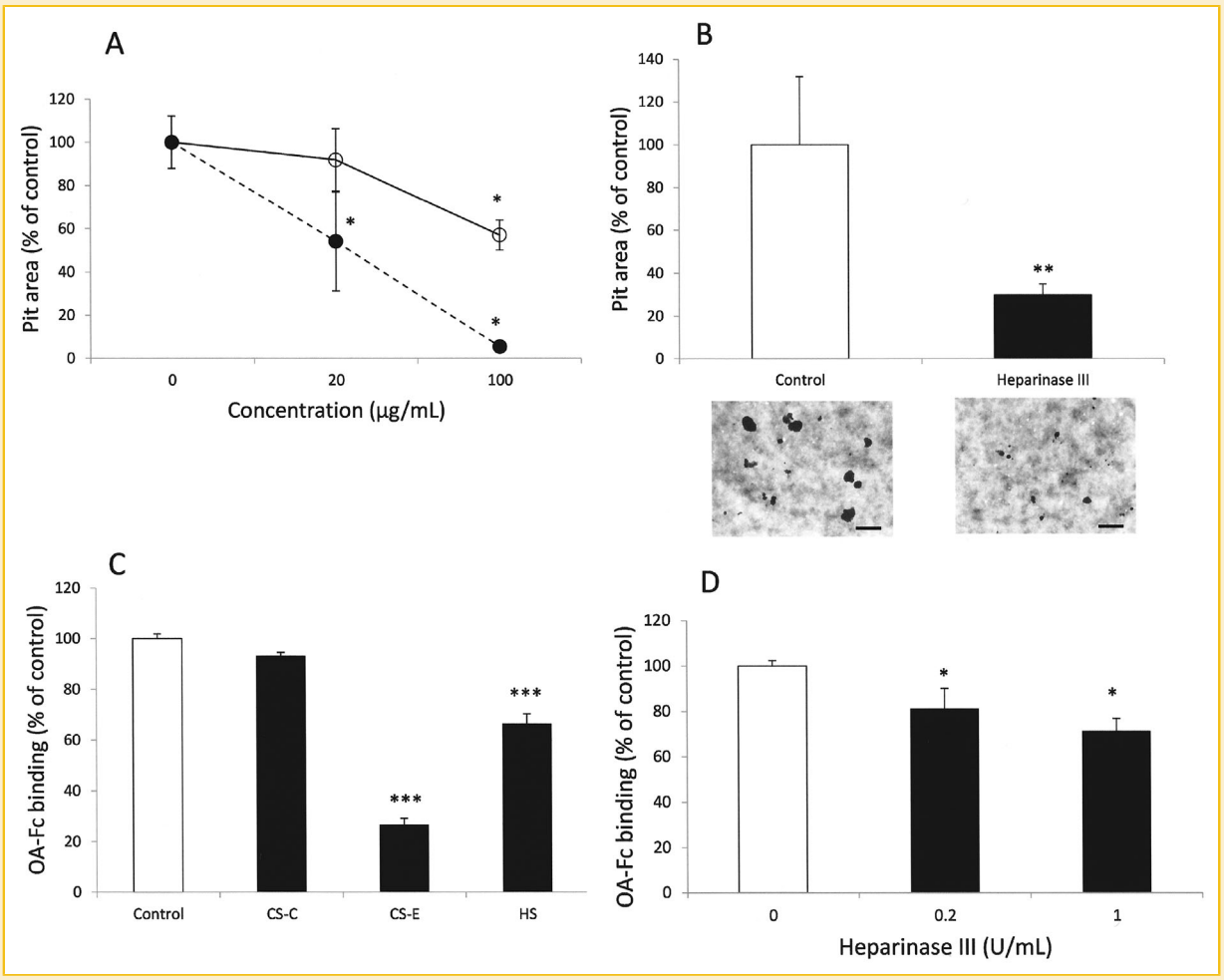

Fig. 8. Effect of HS and heparinase III on osteoclastic differentiation. A: RAW264 cells were cultured on the CaP plate in the presence of HS (closed circle) and CS-E (open circle) for 6 days while in the stimulation with RANKL and then the pit area was measured. Data are shown as the percentage of the RANKL-containing control. B: RAW264 cells were cultured on the CaP plate in the presence of heparinase III (1U/mL) for 6 days and in the presence of RANKL and the pit area was then measured. Data are shown as the percentage of the RANKL-containing control. Bars = 500 μm. C: The binding of OA-Fc to RAW264 cells in the absence or presence of CS-C, CS-E, and HS was measured as described in the materials and methods. D: The binding of OA-Fc to RAW264 cells in the presence of heparinase III was measured as described in the materials and methods. Data are shown as the percentage of control (Mean ± SD, $n = 3$ or 4). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

of integrin present on the cell surface is required to clarify the differences in interactions with ligands. It is possible that the difference in the action among integrin ligands can be attributed to their respective binding affinities to different types of HSPGs on the cell surface.

In conclusion, the present study demonstrated that CS-E binds both OA and integrin αVβ3 and inhibits OA-induced osteoclast differentiation by blocking the interaction of OA with integrin αVβ3 and HSPGs on the cell surface. Our data provide a possible mechanism for these observations and underscore the importance of CS structure on the interaction with integrins in relation to the osteoclastogenesis.

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