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Fibronectin Inhibits Osteoclastogenesis While Enhancing Osteoclast Activity via Nitric Oxide and Interleukin-1β-Mediated Signaling Pathways

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ABSTRACT

Osteoclasts are bone-resorbing cells formed by fusion of mononuclear precursors. The matrix proteins, fibronectin (FN), vitronectin (VN), and osteopontin (OPN) are implicated in joint destruction and interact with osteoclasts mainly through integrins. To assess the effects of these matrix proteins on osteoclast formation and activity, we used RAW 264.7 (RAW) cells and mouse splenocytes differentiated into osteoclasts on tissue culture polystyrene (TCP) or osteologic™ slides pre-coated with 0.01–20 μg/ml FN, VN, and OPN. At 96 h, osteoclast number and multinucleation were decreased on VN and FN compared to OPN and TCP in both RAW and splenocytes cell cultures. When early differentiation was assessed, VN but not FN decreased cytoplasmic tartrate-resistant acid phosphatase activity and pre-osteoclast number at 48 h. OPN had the opposite effect to FN on osteoclast formation. When RAW cells were differentiated on OPN and treated by FN and OPN, osteoclast number only in the FN treated group was 40–60% lower than the control, while the total number of nuclei was unchanged, suggesting that FN delays osteoclast fusion. In contrast to its inhibitory effect on osteoclastogenesis, FN increased resorption by increasing both osteoclast activity and the percentage of resorbing osteoclasts. This was accompanied by an increase in nitric oxide (NO) levels and interleukin-1β (IL-1β). IL-1β production was inhibited using the NO-synthase inhibitor only on FN indicating a FN-specific cross-talk between NO and IL-1β signaling pathways. We conclude that FN upregulates osteoclast activity despite inhibiting osteoclast formation and that these effects involve NO and IL-1β signaling. J. Cell. Biochem. 111: 1020–1034, 2010.

KEY WORDS: BONE; OSTEOCLAST; INTEGRIN; EXTRACELLULAR MATRIX PROTEINS; FIBRONECTIN; OSTEOPONTIN

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting a large proportion of the world's population and is more prevalent amongst women [Silman and Pearson, 2002]. Abnormalities in adaptive immune responses associated with RA result in systemic manifestations and multiple organ involvement. However, synovial inflammation and subsequent progressive bone loss in the affected joints are hallmarks of the symptoms dominating RA [Martin, 2004]. The skeletal complications of RA consist of subchondral bone erosions and periarticular osteoporosis at the sites of inflammation. Joint destruction is initiated by invasive pannus formation and immune cell (T cells and macrophages) activation and infiltration. These events lead to the recruitment of the bone-resorbing cells; the osteoclasts [Bendele et al., 1999; Romas et al., 2002]. Osteoclasts are terminally differentiated multinucleated cells formed by fusion of their hematopoietic progenitors. They are characterized by their unique ability to resorb bone through forming a lysosomal-like membrane juxtaposed to bone called the ruffled border [Baron et al., 1985; Palokangas et al., 1997].

Bone loss in inflammatory diseases is the result of the uncoupling of the two events comprising bone remodeling; bone formation by osteoblasts and bone resorption by osteoclasts. Both processes are synchronized by inter- and intracellular signaling involving hormones, growth factors, and attachment receptors binding to the extracellular matrix (ECM) [as reviewed by Novack and Teitelbaum,

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2008]. In RA, the prevalence of proinflammatory signals disrupts bone homeostasis shifting the balance towards bone resorption [Hoshino et al., 2001; Takayanagi, 2009]. An increase in the number, size, and activity of osteoclasts in arthritic joints of patients with inflammatory bone diseases and in animal models have been shown in several reports [Shimizu et al., 1985; Shibutani et al., 1997; Hoshino et al., 2001; Ritchlin et al., 2003].

The three ECM proteins fibronectin (FN), vitronectin (VN), and osteopontin (OPN) are elevated in arthritic joints and are implicated in bone loss through their interactions with osteoclasts [Carsons et al., 1981; Lavietes et al., 1985; Rosenblum and Carsons, 1996; Ohshima et al., 2002; Xu et al., 2005]. These matrix proteins are adhesive macro-glycoproteins that bind cells and other matrix molecules mediating cell attachment in an integrin-dependant mechanism. While FN and VN are abundant in a soluble plasma form, they can also be found in an insoluble matrix fibril form [Hayman et al., 1983]. Physiologically, both plasma and cellular forms of FN exist in connective tissues. Bone contains only plasma VN [Seiffert et al., 1994] and both forms of FN with plasma FN being predominant [Bentmann et al., 2010]. Even though FN, VN, and OPN have multiple binding domains that confer a wide range of biological functions, the arginine–glycine–asparagine (RGD) motif represents the common integrin-binding domain among the three proteins. In addition to their role as adhesive molecules, VN and, to a larger extent, FN and OPN are now also recognized as cytokines with autocrine proinflammatory properties. This emerging concept is founded on a growing body of evidence showing a marked elevation of VN and OPN plasma levels during infections, tumor progression, and more frequently in inflammatory arthritis [Patarca et al., 1993; Seiffert et al., 1995; Oates et al., 1996; Saeki et al., 2003]. Furthermore, several of these studies reported a joint specific increase of all three ECM proteins levels during joint inflammation [Carsons et al., 1981; Lavietes et al., 1985; Rosenblum and Carsons, 1996; Ohshima et al., 2002; Xu et al., 2005]. This elevation in matrix protein levels was associated with the induction of inflammatory cytokines and proteolytic enzymes promoting joint injury [Saito et al., 1999; Xu et al., 2005] and in turn resulting in the release more matrix proteins which further contribute to this vicious cycle.

The evidence implicating ECM proteins in bone loss in arthritic joints suggests that they are directly modulating osteoclast formation and activity. While OPN's stimulatory effect on osteoclasts has been shown extensively [Rittling et al., 1998; Razzouk et al., 2002; Chellaiah et al., 2003b; Contractor et al., 2005; Rajachar et al., 2008; Ek-Rylander and Andersson, 2010], studies on FN and VN have been limited to investigating mature osteoclast attachment [Flores et al., 1992; Abu-Amer et al., 1997; Hu et al., 2008]. Here we hypothesize that FN and VN, similar to OPN, have stimulatory effects on both osteoclast formation and resorption. Thus we undertook this study to compare the effects of FN and VN to the well-established effects of OPN. Our data identify FN as a novel promoter of osteoclast activity. Despite its inhibitory effect on osteoclast formation, FN possesses the unique ability to increase osteoclast resorption through increasing the percentage of activated osteoclasts as well as enhancing their resorptive capacity. FN's effects were mediated via inducing the proinflammatory mediator; nitric oxide (NO) leading to the downstream activation of interleukin-1β (IL-1β), which was shown to be a FN-specific activation mechanism.

MATERIALS AND METHODS

MATERIALS

Human FN and recombinant human OPN were purchased from Sigma–Aldrich Ltd (St. Louis, MO) and human VN was purchased from BD Biosciences (BD Labwares, Franklin Lakes, NJ). Bovine OPN was provided by Dr. J. Sodek (University of Toronto). The RAW 264.7 (RAW) cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). Recombinant soluble receptor activator of NFκB ligand (RANKL) was made in our laboratory through bacterial expression system. Macrophage colony stimulating factor (M-CSF) was purchased from Calbiochem (EMD BioSciences, Inc., San Diego, CA). Dulbecco's modified Eagle's medium (D-MEM), α-minimum essential medium (α-MEM), antibiotics and antimycotics (penicillin/streptomycin, fungizone), and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA). Fast red violet LB salt, naphthol AS-MX, phosphatase substrate, and *p*-nitrophenol and Arg-Gly-Asp-Ser (RGDS) and Ser-Asp-Gly-Arg-Gly (SDGRG) and 4′-6-diamidino-2-phenylindole (DAPI) were obtained from Sigma–Aldrich Ltd. VNR149, an Armenian hamster anti-rat αvβ3 antibody, was generously provided by Dr. Su-Yau Mao (MedImmune, Inc., Gaithersburg, MD). Armenian hamster anti-mouse β3 antibody and goat polyclonal to Armenian hamster IgG FITC were obtained from Abcam (Cambridge, UK) and rat anti-mouse α5β1 was obtained from US Biological (Swampscott, MA). Rat anti-mouse CD44 was obtained from SouthernBiotech (Birmingham, AL). Quantikine® Mouse TRAP™ assay (TRACP 5b mouse) was obtained from Immunodiagnostic Systems IDS (Boldon, Tyne and Wear, UK). L-N^G-monomethyl Arginine (L-NMMA) was purchased from Biomol International (Plymouth Meeting, PA). CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI). Mouse IL-1 beta/IL-1F2 DuoSet ELISA was purchased from R&D Systems (Minneapolis, MN). BioCoat™ Osteologic™ bone cell culture system (16-well), pre-treated 6- and 12-well plastic Falcon™ tissue culture plates and 96-well Falcon™ microtiter plates were purchased from BD Biosciences (BD Labwares). Griess Reagent Kit for nitrite determination and rhodamine phalloidin were purchased and from Molecular Probes, Inc. (Eugene, OR).

IMMOBILIZING ECM PROTEINS ON TISSUE CULTURE PLATES

Ninety-six-well tissue culture polystyrene (TCP) plates were precoated with 100 μl of 10 μg/ml of FN, VN, or OPN dissolved in phosphate-buffered saline (PBS) overnight (O/N) at 4°C. To increase the amount of proteins physically adsorbed onto the TCP plates, the matrix proteins were incubated for an additional 1 h at 37°C. After aspiration of the matrix proteins, the wells were subsequently blocked using 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C in a CO_2 incubator to minimize non-specific binding of serum proteins to TCP. Finally, the wells were washed 3× with 100 μl PBS and left in PBS until cells were plated to prevent denaturing of the proteins.

RAW 264.7-DERIVED OSTEOCLAST CULTURES

RAW cells were plated on the ECM-coated wells and cultured in DMEM supplemented with 10% FBS, 100 μg/ml penicillin/streptomycin, and 0.2 μg/ml fungizone and incubated at 37°C in 5% CO_2. Osteoclasts were generated using 75 ng/ml RANKL. After 96 h of incubation, multinucleated osteoclasts were observed. When early differentiation was studied, cultures were stopped at 48 h. At the end of the experiments, cultures were fixed using 4% formaldehyde for 5–6 min. To assess early differentiation, the number of mononuclear tartrate-resistant acid phosphatase positive (TRAP+) cells (pre-osteoclasts) was counted at 48 h using bright field microscopy. To determine the effect of ECM proteins on osteoclast formation and multinucleation, the number of TRAP+ cells with ≥2 nuclei (osteoclasts) and with >10 nuclei (large osteoclasts) were counted at 96 h using bright field microscopy.

For assessment of the effects of soluble ECM proteins on osteoclastogenesis, a similar experimental design was used as described above. Briefly, RAW cells were plated in the presence of 75 ng/ml RANKL. Two hours after cell attachment, soluble FN, VN, and OPN (10 μg/ml) were added to the cells. The differentiation experiments were carried out and assessed as described above.

Initial attachment was determined by counting nuclei using DAPI as a nuclear stain. Two hours after RAW cells were plated on the adsorbed ECM proteins, the cultures were washed 2× using PBS and fixed using 4% formaldehyde for 5–6 min. The cultures were subsequently stained using DAPI at a final concentration of 30 ng/ml for 3 min. Fluorescent micrographs of cultures were captured using a Leica DMIRE2 microscope and Openlab® imaging system and the number of nuclei/field was subsequently counted.

Assessment of the effects of functionally blocking integrins was carried out as follows. After 72 h of culture in the presence of 75 ng/ml RANKL on 10 μg/ml FN, VN, and OPN, RAW cell cultures were incubated with: RGDS (10, 20 μM) or DRGS (10 μM) peptide, anti-αvβ3 (VNR149) antibody, or its IgG isotype at 1 μg/ml for an additional 24 h. The cultures were fixed and stained at 96 h and the number of TRAP+ osteoclasts (≥2 nuclei) was counted.

SPLENOCYTE-DERIVED OSTEOCLAST CULTURES

Mice were sacrificed by cervical dislocation and their spleens were dissected and each spleen was placed in a sterile 50 ml Falcon tube with 5 ml of α-MEM. Spleens were crushed separately through a sterile mesh into 2 ml of supplemented media (α-MEM + 10% FBS + 1× antibiotic) in a sterile glass dish. After transferring the cell suspension from the dish into a clean 50 ml Falcon tube, the dish was washed 2× using 1 ml supplemented media. All cell suspensions from different washes were pooled into one tube. To increase cell number, the previous steps were repeated with several spleens and cell suspensions from different animals were combined into the same tube. The cell suspension was centrifuged at 200*g* for 5 min after which the supernatant was discarded and the pellet was resuspended in 20 ml FBS per spleen with 5 ml RBC lysis buffer subsequently added per spleen. RBC lysis buffer was prepared in advance as follows: (1) Solution A: 0.16 M NH_4Cl was prepared fresh before each experiment. (2) Solution B: 0.17 M Tris, pH 7.65. Both solutions were filter sterilized and 90 ml of Solution A were mixed with 10 ml of Solution B immediately prior to using. After 10 min of adding the RBC lysis buffer to the cell suspension, 10 ml of supplemented media was added per spleen. The cell suspension was centrifuged at 200*g* for 5 min and were washed 3× with 10 ml PBS. Finally, the cells were resuspended in 5 ml supplemented media. Cells were plated in a 96-well plate at a density of 10,000 cells/well. The cells were incubated with 100 ng/ml RANKL and 50 ng/ml M-CSF for 3 days. On day 3, the media were changed and cells were incubated with 75 ng/ml RANKL and 10 μg/ml FN, VN, and OPN. Cultures were stopped 48 and 72 h after matrix proteins were added and TRAP staining was used to identify osteoclasts and pre-osteoclasts.

TARTRATE-RESISTANT ACID PHOSPHATASE (TRAP) STAINING

TRAP staining was carried out according to the protocol described in BD Biosciences Technical Bulletin #445. Briefly, cell cultures were washed 3× with PBS, fixed with 4% formaldehyde for 5–6 min, and incubated in TRAP staining solution (50 mM acetate buffer, 30 mM sodium tartrate, 0.1 mg/ml naphthol AS-MX phosphate, 0.1%, w/v, Triton X-100, and 0.3 mg/ml Fast Red Violet LB stain) for 10 min until the desired staining intensity was reached. The TRAP staining solution was aspirated and the cells were washed 3× with dH_2O.

TRAP ACTIVITY ASSAY

TRAP activity assay was used to assess TRAP enzyme levels in RAW cell cultures. The assay was adapted from the Sigma protocol as described by [Voronov et al., 2005]. Briefly, after culture media were aspirated, cultures were washed 2× with PBS. Subsequently, cells were lysed using cold citrate lysis buffer (90 mM sodium citrate and 10 mM sodium chloride, pH 4.8) containing 0.1% (w/v) Triton X-100 by adding 100 μl of the buffer to the 96-well plate. After cells were resuspended in the lysis buffer by pipetting up and down 3–4 times, a 20 μl aliquot of the cell lysate was incubated with the 50 μl of phosphatase substrate (4 mg/ml) and 50 μl 40 mM tartrate acid buffer (40 mM tartrate acid in citrate buffer, pH 3.9) in a 96-well plate for 30 min at 37°C. The reaction was stopped using 80 μl of cold 2 N NaOH. Absorbance was measured at 405 nm using a plate reader, and activity calculated from a standard curve generated using *p*-nitrophenol standards.

CELL VIABILITY ASSAY

The CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega) was used to measure viability and proliferation of RAW cells on the different matrices according to the manufacturer's instructions. Briefly, 20 μl of the CellTiter 96 AQ_{ueous} One Solution Reagent was added to 100 μl culture media for 2 h at 37°C in a humidified, 5% CO_2 incubator and colorimetric changes were measured at 409 nm.

SECRETED TRAP5b ACTIVITY ASSAY

The mouse TRAP™ Assay was carried out according to the manufacturer's instructions. To first adsorb the primary antibody to the plates, 100 μl of mouse anti-TRAP antibody was added to each well with continuous shaking (950 rpm) for 60 min at room temperature (RT). The wells were subsequently washed 4× using 300 μl of washing buffer and 100 μl of calibration solution and controls were added to each well. Seventy-five microliters of 0.9%

NaCl was mixed with 25 μl of conditioned media samples and was also added to the wells. Release agent (25 μl) was subsequently added to all wells and the samples were incubated in the microplate wells while shaking (950 rpm) for 60 min at RT. After the incubation period, the washing step was repeated as described previously and 100 μl freshly prepared substrate solution was added to each well. The microplate was covered and incubated for 2 h at 37°C. The reaction was stopped with 25 μl 2 N NaOH and absorbance was measured at 405 nm.

NITRITE AND NITRATE MEASUREMENTS

Conditioned media from RAW-derived osteoclast cultures on FN, VN, and OPN were analyzed for nitrite and nitrate levels at 24 and 48 h using Griess reagent (Molecular Probes, Inc.) as an indirect method of measuring NO levels according to the manufacturer's protocol. Briefly, Griess reagent (100 μl of sulfanilic acid, 100 μl of *N*-(1-naphthyl)ethylenediamine dihydrochloride) was incubated with 100 μl of conditioned media in a microplate for 30 min at RT. A standard curve was generated using 1 mM sodium nitrite in deionized water. Absorbance was measured at 548 nm.

RESORPTION STUDIES

Osteoclastic resorption was studied using 16-well osteologic slides (BD Biosciences) using a similar experimental design and culture conditions described above for osteoclast differentiation. Briefly, ECM matrix proteins (FN, VN, and OPN) at 20 μg/ml were physically adsorbed onto the hydroxyapatite (HA)-coated slides and RAW cells were plated and differentiated onto the coated discs for 72 h in the presence of 75 ng/ml RANKL. To determine both resorption and osteoclast number for the same experiment, two slides were used in parallel for each experiment and all procedures were identical for both slides. Following the 72-h culture period, cells on one slide were detached from the surface of osteologic slides by adding 200 μl of bleach solution (6% NaOCl, 5.2% NaCl) to each well before removing media and agitating for 5 min. Wells were washed 2× with 150 μl dH_2O and then examined under the light microscope to ensure complete removal of cells. The contrast between non-resorbed areas and resorption pits was visualized using von Kossa staining as described in Gramoun et al. [2007]. The perimeters of the resorption pits were traced manually and their area was measured using Leica DMIRE2 microscope and Openlab imaging system. Cells on the second slide were fixed and stained for TRAP activity at the same time point. The number of osteoclasts was determined using bright field microscopy. To assess actin ring formation at the same time, rhodamine phalloidin at a titer 1:50 was added after TRAP staining. Images were acquired using Leica DMIRE2 microscope and Openlab imaging system. The total number of osteoclasts and actin rings was determined.

IL-1β ELISA

Conditioned media samples from RAW-derived osteoclast cultures on FN, VN, and OPN (10 μg/ml) were analyzed for IL-1β levels at 24 and 48 h using the Quantikine Mouse IL-1_/IL-1F2 Immunoassay (R&D Research). The assay was carried out exactly according to the manufacturer's instructions.

FLOW CYTOMETRY ANALYSIS OF INTEGRIN EXPRESSION

Surface expression of integrins αvβ3 and α5β1 in cultures differentiated on FN, VN, and OPN (10 μg/ml) was analyzed using flow cytometry. RAW cells were plated and differentiated on ECM protein-coated 60 mm culture dishes in the presence of 75 ng/ml RANKL. At 72 h, the cells were washed 2× using PBS–Ca–Mg before they were incubated with 0.06% EDTA for 45 min at 37°C in a humidified, 5% CO_2 incubator. The cells were subsequently scrapped and centrifuged for 5 min at 200*g* and 4°C. The cells were resuspended and incubated in a blocking solution (0.5% BSA in PBS). After a 1-h incubation period, the cells were incubated with the primary antibodies (hamster anti-mouse β3 and rat anti-mouse α5β1) or their IgG isotypes at a titer 1:20 for 1 h on ice. This was followed by a 30-min incubation period with the secondary antibodies on ice. Finally, the cells were washed and analyzed using an Epics Altra Beckman Coulter flow cytometer.

GENERATION OF FN CONDITIONAL KNOCKOUT MICE

Transgenic Mice. Transgenic mice carrying loxP-flanked (floxed) FN (FN-fl/fl) have been reported by Sakai et al. [2001]. Mice possessing a construct of the inducible Mx promoter driving cre recombinase expression were used to delete FN in hepatocytes hematopoietic cells in mice homozygote for the floxed FN gene [Sakai et al., 2001; Bentmann et al., 2010]. Induction of Mx was performed as described [Sakai et al., 2001]. Success of the deletion was confirmed by measuring the level of plasma FN by ELISA as previously described [Kawelke et al., 2008].

HISTOMORPHOMETRY

Tibiae were fixed and embedded in methylmethacrylate. Masson–Goldner staining was performed as follows: sections were deplasticized, treated with hematoxylin (Gill II), acid fuchsin/ponceau xylidine, and phosphomolybdic acid/orange G to stain the cells and osteoid. Light green was used at the end to stain the mineralized matrix. Primary cancellous bone was defined as the 120 μm band below the growth plate. Cancellous bone was defined as the remaining trabecular area that extends down 2 mm. The ASBMR nomenclature was used [Parfitt et al., 1987]. The following measurements of the proximal tibia were performed using ImageJ: bone surface (BS), osteoclast number (Oc.N), and osteoclast surface (Oc.S).

STATISTICS

Statistical evaluation was carried out using SPSS 12.0 for Windows using one-way analysis of variance (ANOVA) and Dunnett T3 test. *P*-values <0.05 were considered statistically significant.

RESULTS

FN REDUCES OSTEOCLAST FORMATION WITHOUT AFFECTING RAW CELL PROLIFERATION OR INITIAL ATTACHMENT

Several extracellular proteins and cytokines contribute to the process of osteoclastogenesis. The aim of this study was to ask if the matrix proteins FN, VN, and OPN affect osteoclast formation. RAW cells were differentiated on increasing concentrations of FN, VN,

and OPN (0.1, 1, 10, and 20 μg/ml) physically adsorbed on TCP dishes. After 96 h, fewer osteoclasts were formed on FN and VN than on OPN and TCP (Fig. 1A). These effects were not due to differences in initial attachment as the number of cells attached to FN, VN, OPN, and TCP 2 h after initial plating were not significantly different (Fig. 1B). However, there seemed to a non-significant trend of enhanced attachment on FN and VN compared to TCP. The differences in osteoclast number were also not due to differences in viability or proliferation since all three ECM proteins did not alter the metabolic activity of RAW cells as shown by the MTS assay (Fig. 1C). Lastly, there were also no differences in total protein content up to 96 h on 10, 20, and 50 μg/ml of adsorbed matrix proteins (data not shown). These data show that both FN and VN reduce osteoclastogenesis without affecting initial attachment or viability.

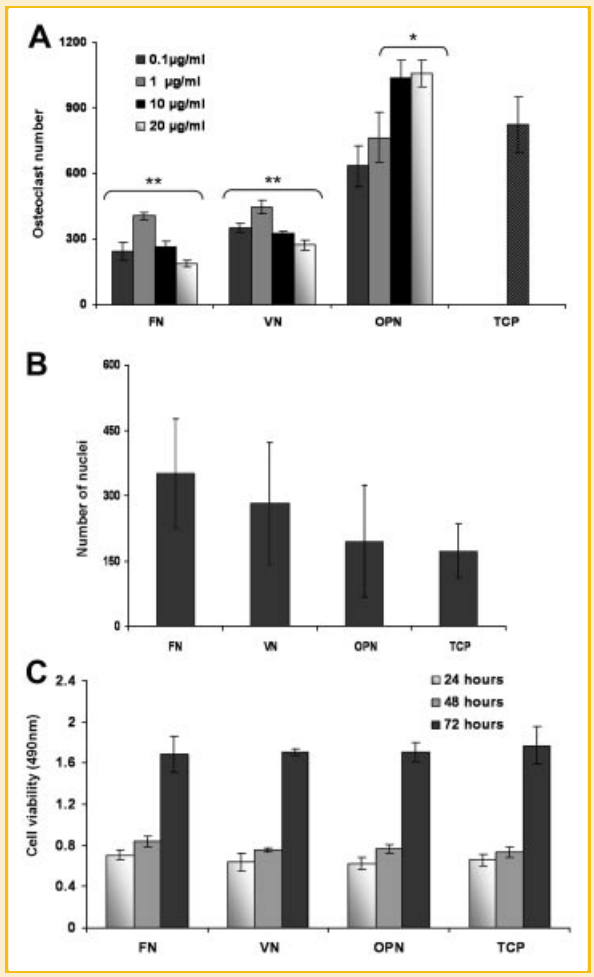

Fig. 1. FN and VN decrease osteoclastogenesis compared to OPN without affecting initial attachment or proliferation. A: RAW cells were plated on TCP pre-coated with FN, VN, and OPN at 0.1, 1, 10, and 20 μg/ml. RAW cells were differentiated into osteoclasts for 96 h in the presence of 75 ng/ml RANKL. The cultures were fixed and stained for TRAP activity and the number of TRAP+ osteoclasts was counted. Each data point represents the pooled results from four wells per treatment and is expressed per well (means ± SD). B: RAW cells were plated on TCP pre-coated with 10 μg/ml FN, VN, and OPN and cultured in the presence of RANKL (75 ng/ml) for 2 h. Cells were stained with DAPI and the total number of nuclei in eight fields per well was counted. Each data point represents the pooled results from three dishes per treatment and is expressed per field (means ± SD). C: RAW cells were plated on TCP dishes pre-coated with FN, VN, and OPN at 10 μg/ml. RAW cells were cultured in the presence of RANKL (75 ng/ml) for 24, 48, and 72 h. Cell proliferation was indirectly measured at 24, 48, and 72 h using an MTS assay. Each data point represents the pooled results from six wells per treatment and is expressed per well (means ± SD). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the TCP group. Similar results were obtained in three separate experiments.

FN INHIBITS PRE-OSTEOCLAST FUSION AND/OR MIGRATION BUT NOT PRE-OSTEOCLAST RECRUITMENT

We next asked which step of osteoclastogenesis was affected by the matrix proteins. Osteoclast formation is composed of two sequential steps. The first step is the recruitment and differentiation of hematopoietic cells that do not stain for TRAP activity (TRAP−) cells into mononuclear cells which stain positive for TRAP activity (TRAP+) known as pre-osteoclasts or prefusion osteoclasts. Subsequently, pre-osteoclasts fuse into mature multinucleated osteoclasts. To assess both steps, early (48 and 72 h) and late (96 h) time points were studied in RAW cell cultures. Cytoplasmic TRAP has been shown to be proportional to the total number of TRAP+ cells regardless of their size when normalized to the number of nuclei [Trebec et al., 2007]. At 48 and 72 h, cytoplasmic TRAP was not different between FN and TCP, indicating that FN did not alter the rate of RAW cell differentiation (Fig. 2A). Even though TRAP levels on VN and OPN were reduced at these time points. Nonetheless, at 96 h there were no differences between all groups (Fig. 2A). This indicates that the amount of TRAP was not affected by the matrix proteins despite initial differences at early time points and suggests that ECM VN and OPN delay but do not inhibit pre-osteoclast recruitment.

We next determined the number of pre-osteoclasts (mononuclear TRAP+ cells), osteoclasts (TRAP+ cells with ≥2 nuclei), and large osteoclasts (TRAP+ cells with ≥10 nuclei) at 48 and 96 h after induction of differentiation. In agreement with cytoplasmic TRAP levels at 48 h, VN and OPN decreased pre-osteoclast numbers whereas FN did not affect these numbers compared to TCP (Fig. 2B). However, the number of osteoclasts and large osteoclasts was significantly lower on FN and VN than on OPN and TCP at 96 h (Fig. 2C). Due to the large number of TRAP+ mononuclear cells at 96 h, counting cells was not feasible. Therefore, cytoplasmic TRAP levels were used to assess osteoclastogenesis at this time point.

Soluble FN, VN, and OPN at 10 μg/ml had the same effects on RAW cell differentiation as the physically adsorbed proteins (Supplementary Fig. 1A,B vs. Fig. 2B,C). To confirm the results obtained with RAW cells in primary cell cultures, these experiments were repeated using mouse spleen cells. Mouse spleen cells were plated on TCP dishes in the presence M-CSF for 3 days. On day 3, both RANKL and M-CSF were used to differentiate attached cells in the presence of soluble FN, VN, or OPN. FN and VN reduced osteoclast numbers and multinucleation at 48 and 72 h compared to OPN and TCP (Supplementary Fig. 1C,D). These results agree with the data obtained from both the physically adsorbed and soluble ECM proteins in RAW cells.

Based on these results, it appears that FN inhibits osteoclast formation by diminishing pre-osteoclast fusion and/or migration.

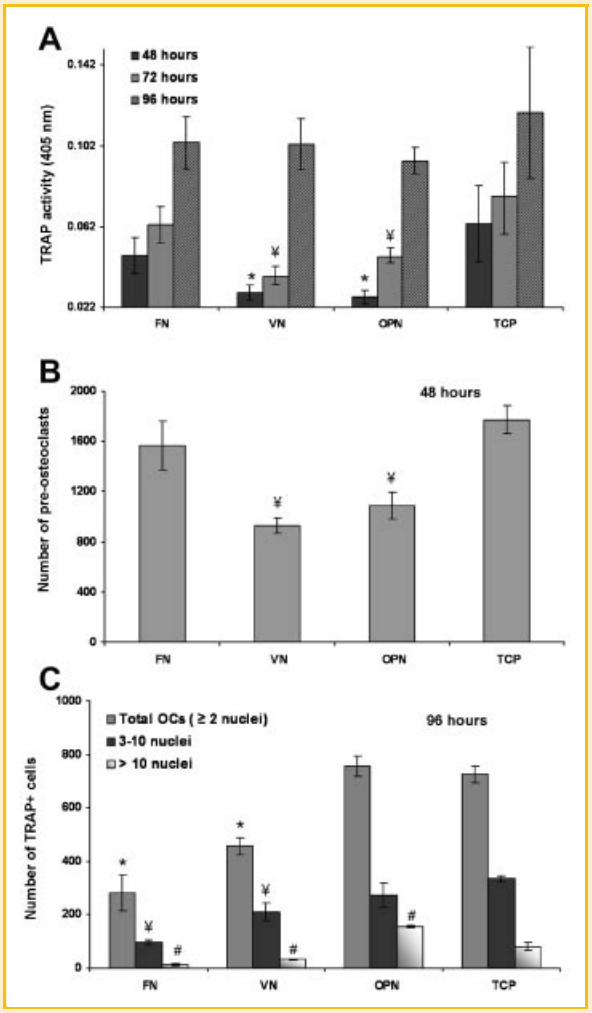

Fig. 2. VN delays early pre-osteoclast formation while both VN and FN decrease osteoclast multinucleation. A: RAW cells were plated on TCP dishes pre-coated with 10 μg/ml FN, VN, and OPN and cells were differentiated into osteoclasts with 75 ng/ml RANKL. Experiments were stopped at 48, 72, and 96 h and cytoplasmic TRAP activity was measured using an enzymatic TRAP assay. Each data point represents the pooled results from six wells per treatment and is expressed per well (means ± SD). B,C: RAW cells were plated on TCP dishes pre-coated with FN, VN, and OPN at 10 μg/ml. The cells were differentiated into osteoclasts with 75 ng/ml RANKL for 48 and 96 h. TRAP+ pre-osteoclasts (mononuclear cells) at 48 h and osteoclasts at 96 h were counted. Each data point represents the pooled results from four dishes per treatment and is expressed per well (means ± SD). $^{*,¥,\#}P < 0.05$ versus the respective TCP control group. Similar results were obtained in three separate experiments.

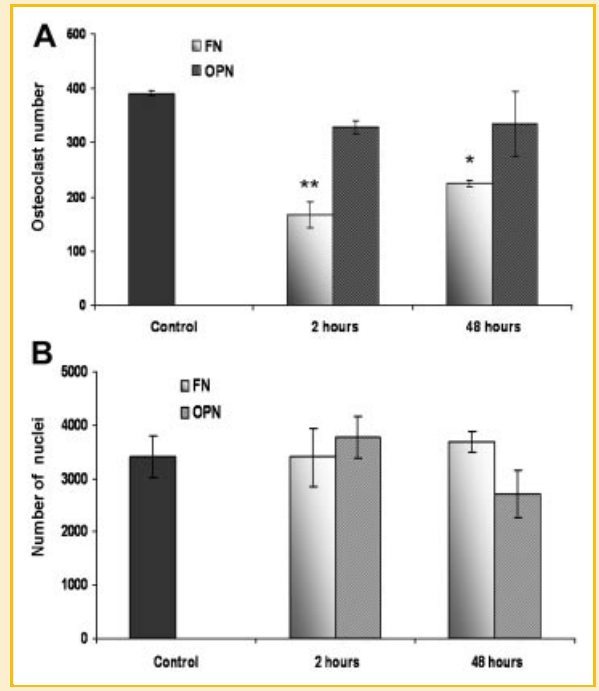

Fig. 3. Soluble FN decreases migration/fusion of pre-osteoclasts formed on physically adsorbed OPN. A: RAW cells were plated on TCP dishes pre-coated with 10 μg/ml of OPN in the presence of 75 ng/ml RANKL. Media supplemented with or without 20 μg/ml FN and OPN were added to the cultures at 2 and 48 h after the cells were plated. At 96 h the number of TRAP+ osteoclasts was counted. Each data point represents the pooled results from four dishes per treatment and is expressed per well (means ± SD). B: DAPI was subsequently used as a nuclear stain and the total number of nuclei in eight fields per well was counted using epi-fluorescent microscopy. Each data point represents the pooled results from four dishes per treatment and is expressed per field (means ± SD). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group. Similar results were obtained in three separate experiments.

This is in contrast with OPN which increases the number of osteoclasts by enhancing what appears to be the multinucleation of formed osteoclasts. We therefore asked if soluble FN added during differentiation can inhibit the formation of osteoclasts on OPN. RAW cells were plated on OPN (10 μg/ml) coated dishes and primed with RANKL. At 2 and 48 h following cell plating, soluble FN and OPN (20 μg/ml) were added, and then cultures were stopped at 96 h. Addition of FN at 2 and 48 h resulted in 40–60% reduction in osteoclast numbers compared to cells differentiated only on adsorbed OPN (control), but did not affect the total number of nuclei at either time points. In contrast, addition of soluble OPN affected neither osteoclast numbers nor total number of nuclei (Fig. 3A,B). These results show that FN and OPN have opposite effects on the steps of osteoclastogenesis and that FN can inhibit OPNs stimulatory effects.

ASSESSMENT OF OSTEOCLAST FORMATION IN AN FN CONDITIONAL KNOCKOUT MOUSE MODEL

FN total knockout mice cannot be studied since it is embryonically lethal. Complete deletion of FN causes death at E8.5 due to defects in cell migration and mesoderm formation [George et al., 1993]. Therefore, to study the effects of FN on osteoclasts in vivo, conditional knockouts of FN in hepatocytes were examined. Deletion of plasma FN produced in hepatocytes and hematopoietic stem cells (and hence in osteoclasts) using the inducible Mx promoter was chosen as this significantly reduced ECM FN levels [Bentmann et al., 2010]. The osteoclast histomorphometric parameters Oc.N/BS and Oc.S/BS (Table I) show a trend for higher osteoclast number and larger cell area in the Mx conditional knockout line; however, the results are not significant and thus

TABLE I. Histomorphometric Osteoclast Parameters in the FN Conditional Knockout (cKO) Mx Mouse Line

Parameter	CT (n = 10)	cKO (n = 10)	*P*-value
Oc.S/BS %	2.5 ± 1.2	3.3 ± 0.8	0.10
Oc.N/mm BS	0.38 ± 0.19	0.50 ± 0.16	0.14

inconclusive ($P = 0.14$ and 0.10, respectively). Since FN was not completely deleted in osteoblasts, some FN might have been available to osteoclasts. A complete FN knockout in all bone marrow cells might be needed to reach a conclusive answer about FN's effect on osteoclasts in vivo. Alternatively, a more rapid bone remodeling system, such as the hind limb unloading model or an ovariectomy model, might be necessary to produce a phenotype, as was the case with the OPN KO mice [Rittling et al., 1998; Yoshitake et al., 1999; Saeki et al., 2003].

FN INCREASES RESORPTION BY INCREASING BOTH THE RESORPTIVE ACTIVITY PER OSTEOCLAST AND THE PERCENTAGE OF RESORBING OSTEOCLASTS

We next investigated the effect of ECM proteins on RAW cell derived osteoclast activity. At 72 h, FN increased resorption by 60%, 70%, and 87% compared to OPN, VN, and uncoated HA controls, respectively (Fig. 4A). The same trend was seen when the number of pits was counted (Fig. 4C). While osteoclast number on FN and VN remained ~40% diminished compared to OPN and HA (Fig. 4B), this

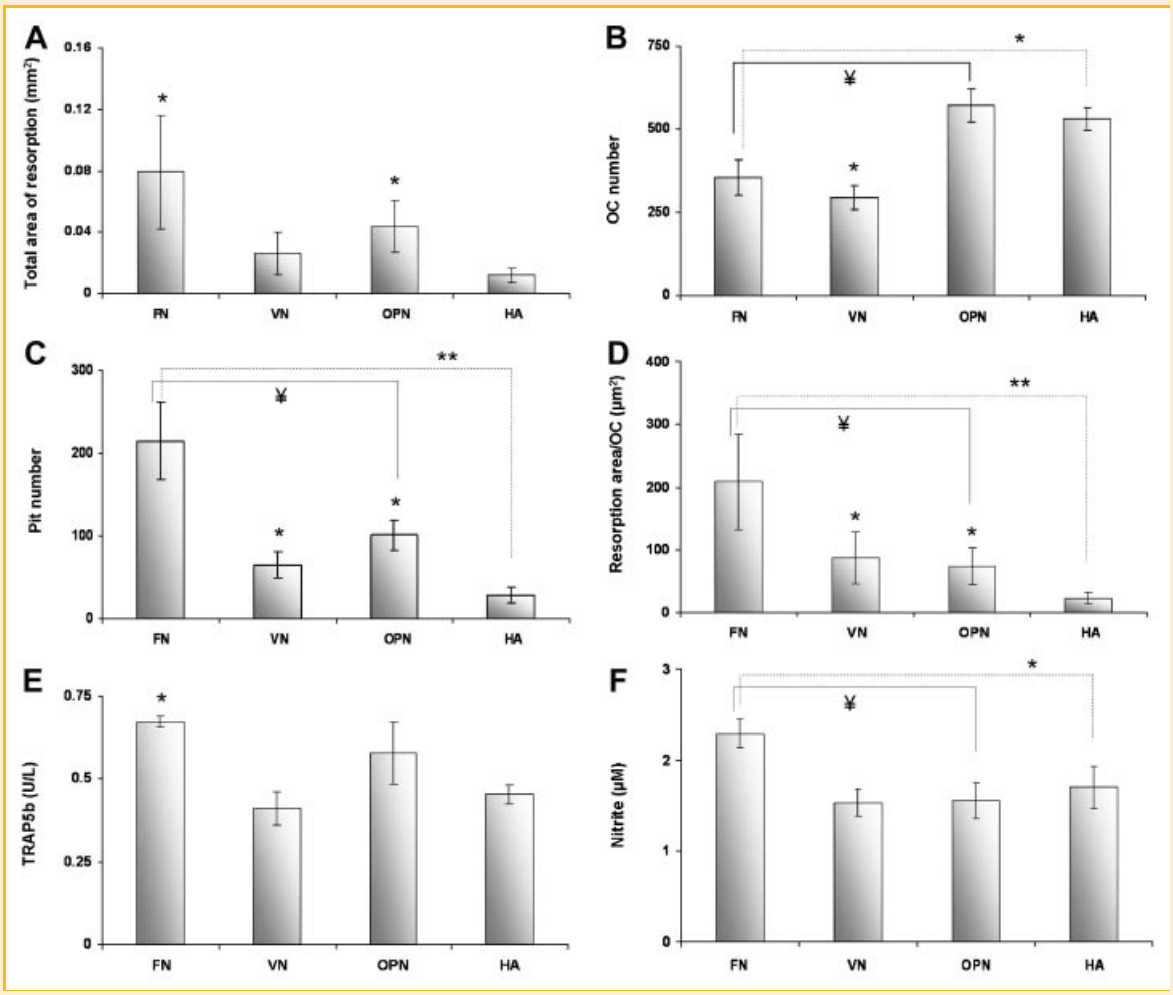

Fig. 4. FN increases resorptive parameters and NO production. RAW cells were plated on osteologic (hydroxyapatite coated) slides pre-coated with FN, VN, and OPN at 20 µg/ml. RAW cells were differentiated into osteoclasts for 72 h in the presence of 75 ng/ml RANKL. To determine both resorption and osteoclast number for the same experiment, two slides were used in parallel for each experiment and all experimental procedures for both slides were identical. On one slide the cells were detached and non-resorbed areas were visualized using von Kossa staining. The perimeter of the resorption pits was traced manually and the area was measured using Openlab software system (A). The number of pits was also counted (C). On the second slide the cultures were fixed and stained for TRAP and the number of TRAP+ osteoclasts was counted (B). The resorption area/osteoclast was calculated from the previously obtained measurements (D). The conditioned media from both slides were collected and a TRAP5b capture ELISA and Griess reagent were used to measure levels of the secreted TRAP5b enzyme (E) and NO nitrites (F), respectively. Each data point represents the pooled results from four discs per treatment and is expressed per well (means ± SD). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the HA group. $^{¥}P < 0.05$ versus the OPN group. Similar results were obtained in four separate experiments.

decrease in osteoclast numbers was accompanied by a significant increase in resorptive activity per osteoclast (Fig. 4D). Secreted TRAP5b in the conditioned medium, another indicator for osteoclast activity, was also elevated on FN (Fig. 4E).

As NO affects osteoclast resorption and is implicated in arthritic joint destruction [McCartney-Francis et al., 1993; Brandi et al., 1995; Miesel et al., 1996; Yasuda et al., 2004; Yasuda and Nakamura, 2007], we asked if FN's stimulatory effect on osteoclast resorption correlated with NO levels. NO was elevated on FN but not on VN or OPN (Fig. 4F), supporting a relationship between NO synthesis and FN's stimulatory effects on osteoclast activity.

Osteoclast activity was also assessed by evaluating sealing zone formation on ECM proteins. The sealing zone is an adhesion structure only seen in an actively resorbing osteoclast and can be visualized by staining for actin rings using rhodamine phalloidin. We found a trend for increased sealing zone formation on all ECM proteins but this increase was only significant on FN where multiple actin rings per osteoclast could be seen (Fig. 5A,B). The increase in sealing zones on FN indicates a higher number of actively resorbing osteoclasts and supports the data on increased resorption by FN.

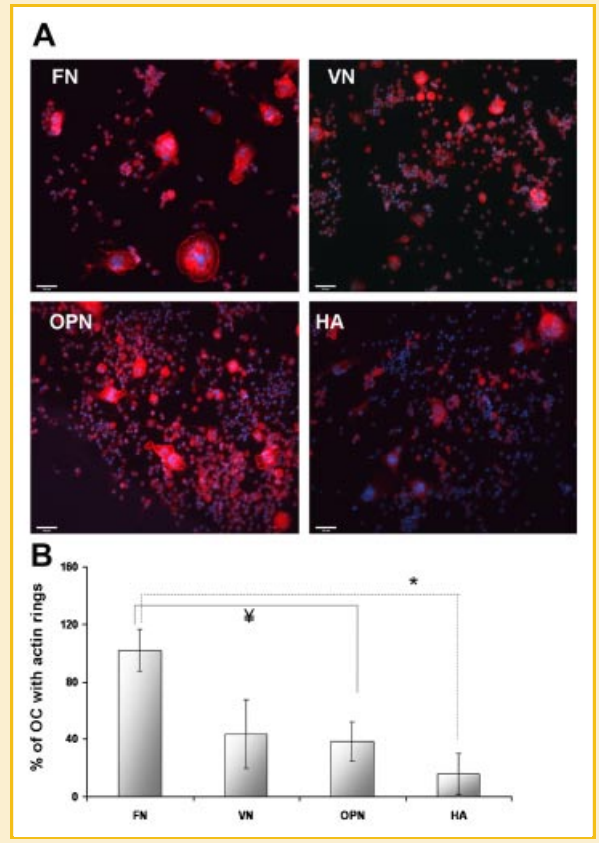

Fig. 5. Osteoclasts on FN-coated osteologic discs have more sealing zones RAW cells were plated on osteologic (hydroxyapatite coated) slides pre-coated with FN, VN, and OPN at 20 μg/ml. RAW cells were differentiated into osteoclasts for 72 h in the presence of 75 ng/ml RANKL. The cultures were fixed, permeabilized, and stained for F-actin using rhodamine phalloidin (red) to visualize actin rings and DAPI (blue) for nuclei (A). The number of actin rings and total number of osteoclasts in eight fields per treatment group were counted. The percentage of osteoclasts exhibiting actin rings per field was calculated (B). Each data point represents the pooled results from four discs per treatment and is expressed per well (means ± SEM). $^{*}P < 0.05$ the HA group. $^{¥}P < 0.05$ versus the OPN group. Similar results were obtained in three separate experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FN INCREASES IL-1β IN A NO-DEPENDANT MANNER

Levels of NO and IL-1β were assessed in RAW cell derived osteoclast cultures, as both NO and IL-1β are proinflammatory mediators, affected by ECM proteins, with cross-talking signaling pathways [Yasuda, 2006; Schmidt and Kao, 2007; Trebec et al., 2007]. NO in conditioned media was elevated on FN at 24 and 48 h at a level higher than VN and OPN (Fig. 6A). Only FN caused an increase of IL-1β at 48 h in the same conditioned media (Fig. 6B). To test the interaction between NO and IL-1β the NO-synthase inhibitor L-NMMA (0.1 and 0.5 μM) was added at 24 h of culture and the cells were incubated with the inhibitor for an additional 24 h before the levels of NO and IL-1β were measured on FN and TCP. L-NMMA decreased NO levels in a dose-dependant fashion on FN and TCP (Fig. 6C) and inhibited IL-1β production on FN but not on TCP (Fig. 6D). This suggests that FN induction of IL-1β is NO dependent.

BLOCKING αvβ3 AND α5β1 HAS DIFFERENT EFFECTS ON OSTEOCLAST NUMBER

The adhesion receptor αvβ3 has a central role in osteoclast attachment and function; blocking αvβ3 compromises osteoclast attachment and decreases their number [Gramoun et al., 2007; reviewed by Nakamura et al., 2007]. The effects of blocking αvβ3 on osteoclasts pre-formed on FN, VN, and OPN were examined using two methods. The first technique involved non-specifically blocking all integrins on the surface of RAW cell derived osteoclasts differentiated for 72 h on the ECM proteins using an RGDS peptide (10–20 μM) or a reverse DGRS peptide (10 μM) as a control with osteoclast numbers determined after a 24-h incubation period. As expected, osteoclast numbers decreased on VN, OPN, and TCP. Interestingly, they were increased on FN in response to RGD blockade in a dose-dependant manner (Fig. 7A). These results were confirmed using the specific αvβ3 blocking antibody VNR149 (1 μg/ml) which produced similar results. VNR149 treatment caused a 65% increase in osteoclast number on FN compared to its IgG isotype control. A 60%, 40%, and 20% decrease in osteoclast numbers on VN, OPN, and TCP was seen, respectively, with VNR149 compared to the IgG control (Fig. 7B). When surface expression of αvβ3 was measured using flow cytometry, cells on FN were found to have the highest expression (Fig. 7C). The increase in osteoclast number on FN by blocking αvβ3 could be related to the integrin's upregulation on FN.

To determine which integrin modulates the interaction between osteoclasts and FN, a series of blocking antibodies was used. In contrast to blocking αvβ3, blocking α5β1 on FN decreased osteoclast number while no change on TCP was detected (Fig. 8A). The anti-CD44 antibody had no effect on osteoclasts on both FN and TCP. Assessing simultaneous expression of β3 and α5β1 on cells cultured on FN revealed a ~20% decrease in β3-positive cells (Fig. 8B) and a 5% increase in α5β1-positive cells (Fig. 8C) on FN compared to TCP. Nevertheless, the expression per cell for both β3

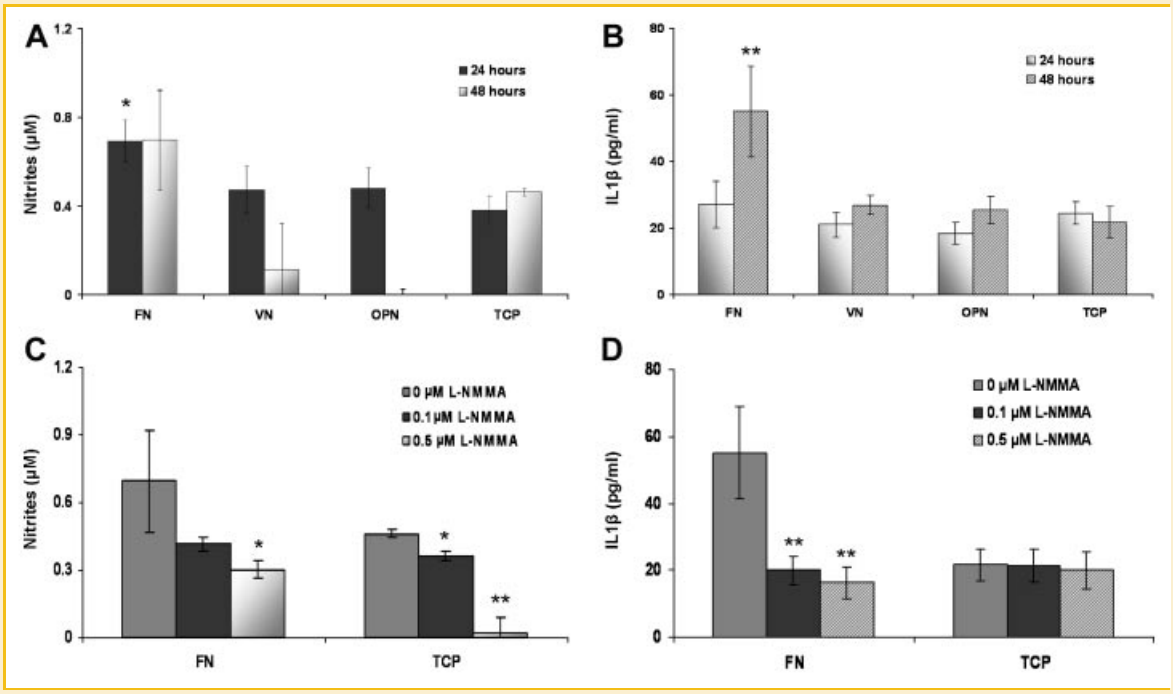

Fig. 6. IL-1β and NO production is increased on FN. Inhibition of IL-1β using the NO synthase inhibitor L-NMMA suggests that NO is upstream of IL-1β. RAW cells were cultured with 75 ng/ml RANKL in pre-coated dishes with 10 μg/ml FN, VN, and OPN. The media were changed after 24 h and the NO-synthase inhibitor L-NG-monomethyl arginine (L-NMMA) was added (0.1 and 0.5 μM) to the FN and uncoated controls for an additional 24 h. NO was measured indirectly using Griess reagent at 24 and 48 h (A,C). IL-1β was measured by ELISA at 24 and 48 h (B,D). Each data point represents the pooled results from five dishes per treatment and is expressed per well (means ± SD). A,B: $^{*}P < 0.05$ and $^{**}P < 0.01$ versus TCP control group. C,D: $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the respective untreated control. Similar results were obtained in three separate experiments.

and α5β1 was elevated on FN (Fig. 8D). These results suggest that attachment of osteoclasts to FN is mediated through α5β1and demonstrate that FN alters the integrin profile of osteoclasts.

DISCUSSION

The prevalence of ECM proteins during inflammatory bone diseases and their effects on these conditions implicate them as osteoclastogenic promoters. Nonetheless, their effect on osteoclastogenesis has not been thoroughly investigated. To delineate the role of matrix proteins in osteoclast formation, we studied the effects of physically adsorbed FN and VN on differentiating RAW cells and compared these effects with these on OPN. OPN was chosen as a reference for the other two proteins since OPN's effects on osteoclast function have been extensively studied [Rittling et al., 1998; Chellaiah and Hruska, 2003; Chellaiah et al., 2003b; Contractor et al., 2005]. FN, VN, and OPN will each be discussed separately in the following sections.

Physically adsorbed or soluble FN had the most pronounced inhibitory effect on osteoclastogenesis in both RAW and primary splenocytes cultures. This result is in accord with the observation that adhesive matrix proteins, in particular FN, increase cell growth, proliferation, and spreading while reducing cell differentiation [Ruoslahti and Pierschbacher, 1987; Schwartz et al., 1989].

To better understand FN's effects on osteoclastogenesis, we assessed two time points that coincide with the two main steps of osteoclast formation. At 48 h the commitment of hematopoietic precursors into pre-osteoclasts was assessed while at 72 and 96 h the fusion of these pre-osteoclasts into multinucleated cells was examined. Pre-osteoclast commitment on FN was similar to that on TCP and higher than that on OPN and VN at 48 h. At 96 h, osteoclast number and their multinucleation were diminished while cytoplasmic TRAP levels were unaffected. These results suggest that equal number of prefusion osteoclasts were present on FN, OPN, and TCP but only a small percentage of these committed cells fused to form osteoclasts on FN. These findings led us to hypothesize that FN inhibits pre-osteoclast fusion and/or migration while OPN promotes these processes. To test this, we asked whether FN could inhibit OPN's stimulatory effect on multinucleation. Treating cells differentiated on physically absorbed OPN with soluble FN resulted in a 60% decrease in osteoclast number without affecting the total number of nuclei. These results further support the hypothesis that FN's inhibitory effect on osteoclast formation is via reducing pre-osteoclast fusion and/or migration.

To assess FN's effect on osteoclasts in vivo, histomorphometrical measurements were performed on conditional knockout mice in which FN was deleted in the circulation and hematopoietic cells using the Mx promoter to drive cre expression. These mice have a significant decrease in the amount of FN in the bone matrix [Bentmann et al., 2010], while mice in which FN was deleted in

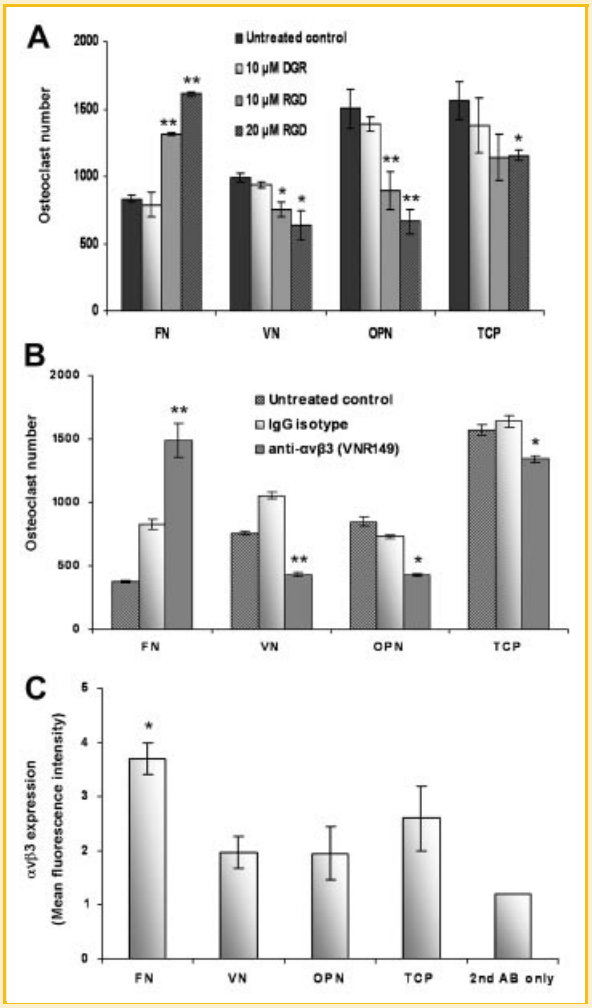

Fig. 7. Exclusive blockade of αvβ3 in osteoclasts differentiated on FN increases osteoclast number. The surface expression of αvβ3 in osteoclasts is the highest on FN. RAW cells were plated on TCP dishes pre-coated with FN, VN, and OPN at 10 μg/ml and differentiated in the presence of 75 ng/ml RANKL. After 72 h the cells were incubated with the following: RGDS (10 and 20 μM) or DRGS (10 μM) peptide (A), anti-αvβ3 (VNR149) antibody or its IgG isotype control at 1 μg/ml (B). The cultures were fixed and stained for TRAP and the number of TRAP+ osteoclasts was counted. Alternatively, after 72 h the cells were detached and immuno-stained using an anti-αvβ3 antibody and subsequently with a FITC-labeled secondary antibody. Flow cytometry was used to measure the fluorescence intensity (C). Each data point represents the pooled results from four dishes per treatment and is expressed per well (means ± SD). A: $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the respective untreated control. B: $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the respective IgG control. C: $^{*}P < 0.05$ versus the TCP control group. Similar results were obtained in three separate experiments.

osteoblasts had normal amounts of FN in the bone matrix [Bentmann et al., 2010]. This is surprising at first glance, since osteoblasts lay down the matrix containing FN which later becomes bone after mineralization. Considering that circulating FN infiltrates the bone matrix, it was not totally surprising to see an effect on FN content in bone when it was deleted. There was a trend towards an increase in osteoclast number and osteoclast surface area between the FN conditional knockouts and their controls; however, the difference between the two groups was not statistically significant. These results point at a possible effect of FN on osteoclast formation and function in vivo that could be partially masked in this mouse model due to the presence of FN originating from osteoblasts. This is however an interesting effect that needs to be further explored in a double FN knockout in both osteoblasts and hematopoietic cells. Additionally, FN deletion needs to be examined in high bone turnover states where osteoclast numbers are markedly elevated, since it might reveal significant differences.

As bone erosion and periarticular osteoporosis responsible for joint destruction in RA require increased activation of osteoclastic resorption, we next examined how FN affects resorption parameters. Despite its inhibitory effect on osteoclast formation, FN increased total resorption, resorption area per osteoclast, pit number, TRAP5b and NO levels. The increase in total area of resorption on FN is the result of the increase in both the number of resorption pits and the area resorbed per osteoclast. The increase in pit number suggests that FN stimulates osteoclast migration on HA.

TRAP5b is used as a bone turnover marker [Halleen et al., 2000]; however, it is controversial whether it correlates with osteoclast number or activity [Halleen et al., 2001; Janckila et al., 2001; Alatalo et al., 2004; Rissanen et al., 2008, 2009]. Here the induction of TRAP5b on FN correlated with the increase in resorptive activity, not the number of mature osteoclasts. We propose that on FN the correlation is between number of the mononuclear pre-osteoclasts which may be contributing to the elevated TRAP5b levels and potentially to bone resorption as well as the increased activity of osteoclasts.

Another important parameter reflecting osteoclast activity is sealing zone formation. Although all three matrices promoted sealing zone formation, the highest percentage was seen on FN indicating it has the highest percentage of active osteoclasts. Furthermore, osteoclasts on FN often had more than one sealing zone suggesting that they possessed more efficient resorbing machinery and explaining the increased resorptive activity per osteoclast we reported. Finally, the proinflammatory mediator NO was elevated in conditioned media only on FN. These data indicate that FN is a proinflammatory molecule capable of activating osteoclasts and enhancing their resorptive efficiency and that NO is involved in this process.

One possible mechanism for the induction of osteoclasts on HA could be related to conformational changes of adsorbed FN. FN can be found in a dimeric globular form that can undergo conformational changes to form a polymeric fibrillar network exposing certain cryptic domains in the molecule under specific conditions [Baneyx et al., 2001; Mao and Schwarzbauer, 2005]. Although this process of FN activation is mainly cell/integrin mediated, it was also seen in vitro in the absence of cells when FN was adsorbed onto HA [Pellenc et al., 2006; Dolatshahi-Pirouz et al., 2009]. However, the role of FN fibrillogenesis and FN's cryptic motifs in osteoclast function are not known. Another possible mechanism for increased osteoclast activation on FN involves extracellular pH regulation. Extracellular pH of osteoclasts on FN was indeed lower than cultures on TCP, VN, and OPN [Gramoun et al., 2010]. As low extracellular pH increases osteoclast resorption in vitro, the low pH resulting from FN offers plausible mechanism for the enhancement

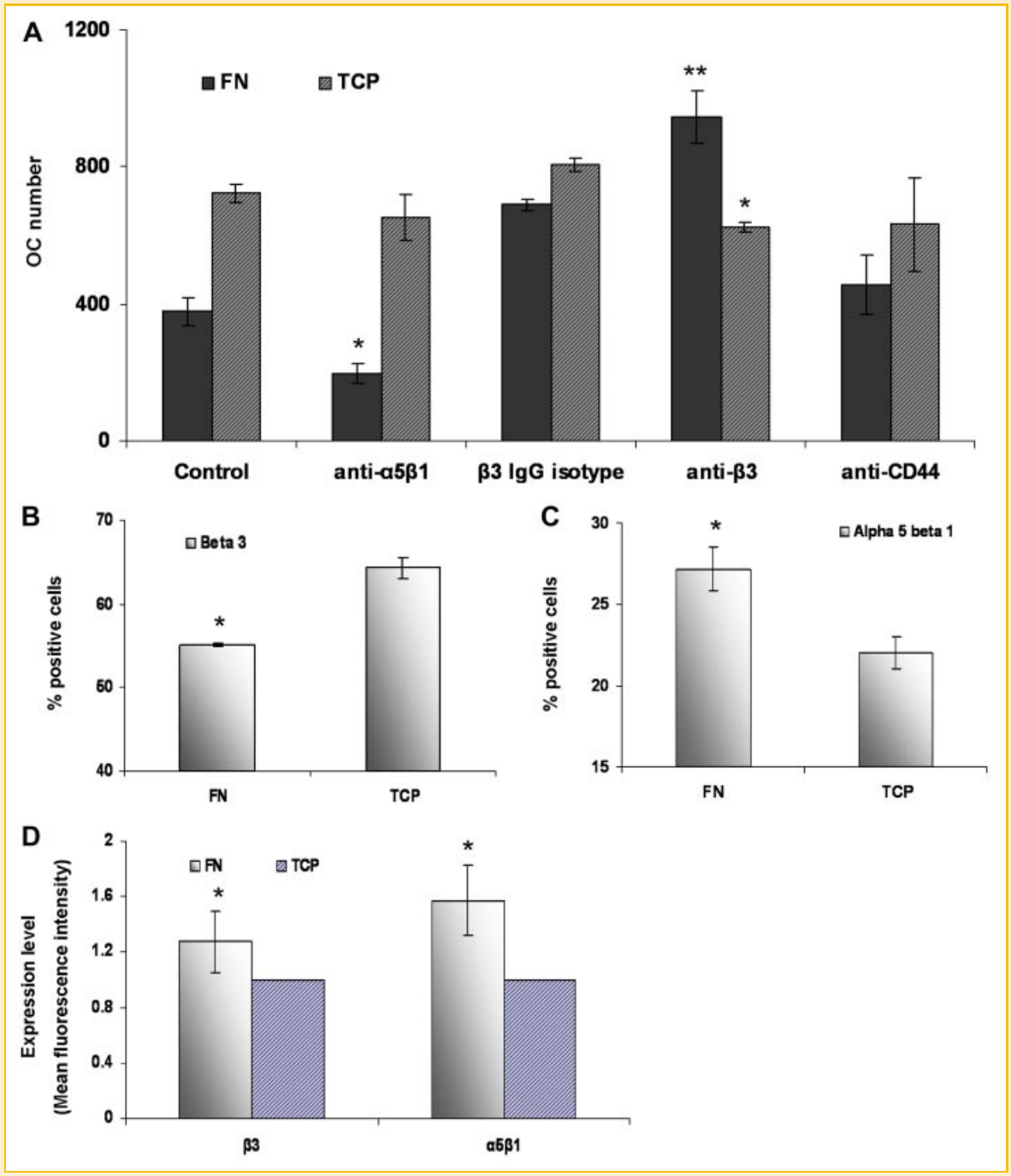

Fig. 8. Blocking $\alpha5\beta1$, but not $\alpha v\beta3$ or CD44, decreases osteoclast number on FN and its expression is highest on FN. RAW cells were plated on TCP dishes pre-coated with FN at 10 μg/ml and differentiated in the presence of 75 ng/ml RANKL. After 72 h the cells were incubated with the following: anti-$\alpha v\beta3$, anti-$\beta3$, $\alpha v\beta3/\beta3$ IgG isotype, anti-CD44, and anti-$\alpha5\beta1$at 1 μg/ml. The cultures were fixed and stained for TRAP and the number of TRAP+ osteoclasts was counted (A). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the respective control. Similar results were obtained in three separate experiments. Alternatively, after 72 h the cells were detached and immuno-stained with an anti-$\beta3$ and antibody and anti-$\alpha5\beta1$ or their IgG isotype control. Subsequently, the cells were incubated with FITC and Cy5-labeled secondary antibodies. Flow cytometry was used to measure the percentage of $\beta3$ (B), $\alpha5\beta1$ (C) positive cells. $\beta3$ and $\alpha5\beta1$ mean fluorescence intensity was also determined (D). Each data point represents the pooled results from three different experiments with three dishes per treatment and is expressed per experiment (means $\pm$ SD). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the TCP control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

of osteoclast activity [Carano et al., 1993; Shibutani and Heersche, 1993; Arnett et al., 1994; Nordstrom et al., 1997].

As both NO and IL-1β are key factors implicated in joint destruction, and as IL-1β induces NO in other cells, we asked whether there is a correlation between their pattern of induction. IL-1β was also a prime candidate for investigation as FN regulates the release of IL-1β by rat macrophages [Beezhold and Lause, 1987] and has the second strongest stimulatory effects on IL-1β, after

collagen [Schiffer et al., 1999]. Furthermore, IL-1β was shown to stimulate osteoclast resorption [Trebec et al., 2007]. Here we show that NO levels were elevated on FN at both 24 and 48 h, however, only at 24 h was it significantly higher than VN, OPN, and TCP. IL-1β levels, on the other hand, peaked at 48 h while no changes were detected on any of the other substrates at either time points. The sustained elevation of NO at 24 and 48 h followed by the increase in IL-1β suggests that the NO signaling cascade is upstream of IL-1β. This is not consistent with other studies showing that in chondrocytes and synoviocytes, IL1β induces NO [Charles et al., 1993; Miyasaka and Hirata, 1997]. Furthermore, avian osteoclast-like cell cultures also had a marked increase in NO through iNOS when treated by several ILs and proinflammatory agents [Sunyer et al., 1996]. These contradictory findings could be reconciled on the basis that both NO and IL-1β are part of a positive feedback loop that auto-amplifies inflammation and induces the production of each other. Importantly, we show that the general NO inhibitor L-NMMA decreased both NO and IL-1β levels in a dose-dependent manner only on FN. This corroborates our hypothesis that IL-1β induction is downstream of NO and that this effect is mediated by FN.

An interesting finding of this study was the stimulatory effect of integrin blockers on osteoclast formation on FN. Integrin blockers, such as integrin-specific antibodies, peptides, and disintegrins, allosterically block the interaction between the integrin αvβ3 and its ligand thus impairing osteoclast attachment and/or migration [reviewed in Nakamura et al., 2007]. When osteoclast cultures were treated with anti-αvβ3 antibody or with an RGD peptide, an increase in osteoclast number was only seen on FN while the expected inhibitory effect was seen on VN, OPN, and TCP.

To address this unforeseen result, we asked which receptors were involved in the interaction between FN and osteoclasts mediating their attachment using a series of receptor-specific antibodies. Consistent with our previous experiments, an anti-β3 antibody increased osteoclast number on FN, whereas osteoclast attachment was diminished in the presence of an anti-α5β1 antibody on FN, but not on TCP. The αvβ3 and α5β1 are known to interact with the RGD motif on FN while CD44 binds to FN's heparin-binding domain [Jalkanen and Jalkanen, 1992]. These data suggest that osteoclast attachment on FN is mediated by the integrin α5β1 and that this interaction is substrate specific to FN. CD44 did not affect osteoclast number on either FN or TCP. Similar results by Hofmann et al. [2001] show that attachment of the pre-osteoclastic leukemia cell line FLG 29.1 on FN was not mediated by αvβ3 and that only the α5β1 blocking antibody affected cell adhesion. These effects were substrate specific since the anti-α5β1 did not affect cell adhesion on VN. Furthermore, Hu et al. [2008] showed that rat osteoclast adhesion on FN was inhibited using an anti-β1 antibody but not with an anti-β3 antibody.

To explain the stimulatory effect of FN we propose a model based on the competition between two major signaling pathways in osteoclast; αvβ3 and the CD44 signaling pathways. FN is a ligand for both αvβ3 and CD44 [Jalkanen and Jalkanen, 1992], both present in osteoclasts. CD44 is a surface receptor mediating pre-osteoclast and macrophage fusion [Sterling et al., 1998; Vignery, 2000] while αvβ3 mediates mature osteoclast attachment. We show that osteoclast attachment on FN is mediated through α5β1 and not αvβ3. Furthermore, our data suggest that FNs interaction with αvβ3 inhibits osteoclast formation by preventing pre-osteoclast fusion. Therefore, we propose that when the FN/αvβ3 interaction is blocked, stimulatory signals from CD44 promote the fusion of pre-osteoclasts and increases osteoclast formation.

Because of the distinct functions of the integrins αvβ3 and α5β1 on FN, we quantified their expression levels. Although the percentage of αvβ3-positive cells on FN cultured cells was 20% lower than TCP, αvβ3 and α5β1 expression per osteoclast were elevated. This agrees with Hofmann et al.'s [2001] results showing that a pre-osteoclastic leukemia cell line growing on FN had increased αvβ3 expression. Upregulation of αvβ3 could also explain the difference in behavior of osteoclasts on FN when αvβ3 is blocked. An increase in αvβ3 could increase pre-osteoclast attachment and hence decrease their mobility and subsequent fusion. Blocking this interaction could decrease their attachment promoting motility and pre-osteoclast fusion.

The results from the matrix protein VN indicate that the protein acts mainly as an adhesive molecule and has no cytokine-like effects. This finding does not confirm our initial hypothesis that elevated VN levels in joints and plasma of arthritis patients contributes to joint damage through increasing osteoclast resorption. VN decreased osteoclast number by reducing both pre-osteoclast recruitment and subsequent fusion. VN's effects on osteoclast activity were in line with its inhibitory effects on osteoclast differentiation in that it did not stimulate total resorption compared to FN or OPN and only slightly increased pit formation and resorbed area/osteoclast. The slight increase in pit formation and resorption per osteoclast correlates with our observation that VN increases the ratio of osteoclasts with a migratory morphology [Gramoun et al., 2010].

The highly phosphorylated matrix protein OPN is one of the most abundant non-collagenous glycoproteins in the matrix. Not only is OPN an adhesive protein regulating cell adhesion and migration, but it is also a signaling molecule with cytokine-like properties that regulates immune responses and tumor progression [Oates et al., 1996; Wang and Denhardt, 2008]. Many of its diverse effects are related to the degree of its post-translational modifications (PTM) specifically phosphorylation, which differs between tissues [Kazanecki et al., 2007]. The role of OPN and its PTMs is influential in bone homeostasis and osteoclast adhesion, migration, and resorption [Yoshitake et al., 1999; Ishijima et al., 2001; Razzouk et al., 2002; Ek-Rylander and Andersson, 2010]. However, OPN's mechanism of regulating these processes remains controversial. Here we used the most phosphorylated form of OPN from bovine milk. In agreement with our initial hypothesis, we demonstrated that OPN is a positive regulator of osteoclastogenesis when coated on TCP. Even though OPN initially delayed pre-osteoclast recruitment, it stimulated osteoclast formation and promoted multinucleation. These data suggest that OPN can increase pre-osteoclast migration. This is compatible with reports from several groups demonstrating that OPN promotes macrophage and osteoclast migration through a CD44-Rho kinase-dependant mechanism that requires OPN's phosphorylation and TRAP processing activity [Chellaiah and Hruska, 2003; Chellaiah et al., 2003a; Al-Shami et al., 2005; Ek-Rylander and Andersson, 2010]. Furthermore, Chellaiah et al.

[2003b] found that osteoclasts derived from OPN null bone marrow macrophages have impaired motility which could be rescued by exogenous OPN. In contrast, Rajachar et al. [2008] showed that $OPN^{-}/^{-}$ bone marrow-derived macrophages formed more osteoclasts on TCP than $OPN^{+}/^{+}$ macrophages, a finding consistent with previous in vitro and in vivo investigations of OPN null mice [Rittling et al., 1998; Chellaiah et al., 2003b]. Although we found no difference in osteoclast number on OPN compared to HA, all resorption parameters were elevated except for sealing zone formation. This increased resorptive capacity in the presence of OPN, with the exception of one study [Contractor et al., 2005], matches the rest of the literature demonstrating that OPN is required for proper bone resorption both in vivo and in vitro [Razzouk et al., 2002; Chellaiah et al., 2003b; Rajachar et al., 2008].

Taken together, these results highlight the important contribution of matrix proteins in modulating bone remodeling. FN, VN, and OPN exerted different effects on osteoclast formation and resorption by modifying different aspects of cell signaling, cytokine production, and receptor expression. We also demonstrate that integrins have multiple functions which are substrate specific. Given the prevalence of FN in inflamed joints in RA, an important outcome of this study is the stimulatory effect of FN on osteoclast function.

Increased osteoclast activity in RA results in irreparable joint destruction leading to permanent incapacitation and increased risk of fractures and implant failure, increasing the RA's morbidity and reducing patients' quality of life [Yasuda et al., 2005]. This investigation highlights FN as a novel target that could be exploited to modify the progression of bone loss in inflammatory diseases.

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