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Biochemical and Biophysical Research Communications 318 (2004) 162–167
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Effects of basic fibroblast growth factor on osteoclasts and osteoclast-like cells

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Received 16 March 2004

Abstract

Mouse marrow, which contains osteoblast and osteoclast precursors, was grown in the presence of calcitriol and/or basic fibroblast growth factor (FGF-2). RAW 264.7 cells were differentiated into osteoclast-like cells in the presence of receptor activator of NF-κB-Ligand (RANK-L) and/or FGF-2. FGF-2 alone supported osteoclastogenesis in mouse marrow cultures, but not by RAW 264.7 cells alone. Although FGF-2 supported low levels of osteoclastogenesis in mouse marrow cultures, it strongly inhibited the high levels of osteoclastogenesis triggered by calcitriol. Adding excess recombinant-RANK-L to the cultures did not relieve this inhibition. After mouse marrow osteoclasts were differentiated, FGF-2 dose-dependently inhibited bone resorptive activity. FGF-2 increased the tendency of RAW 264.7 osteoclast-like cells to fuse into very large giant cells and induced reorganizations of the actin cytoskeleton in mature, RANK-L-induced RAW 264.7 osteoclast-like cells. These results suggest that FGF-2 has both direct and indirect effects on osteoclast formation and bone resorption.

Basic fibroblast growth factor (FGF-2) is a component of the bone matrix and plays a role in regulating bone remodeling [1–3]. Both osteoblasts and osteoclasts express the receptor for FGF-2 [4]. Recent studies indicate that pharmaceutical manipulation of the FGF-2 signaling pathway represents a promising approach to the treatment of bone diseases [1,3,5,6]. It has become apparent that FGF-2 has multiple effects on bone remodeling [7–10]. Some are by the stimulation of the production of receptor activator of NF-κB-ligand (RANK-L) by osteoblasts and other stromal cells [11]. Others are more elusive. For example, fibroblast growth factor receptor-1 (FGFR-1) is present on osteoclasts, but the effects of stimulation of the receptor on the activity of mature osteoclasts are not fully documented. To better understand the regulation of osteoclasts by FGF-2 we utilized mouse marrow cultures and RAW 264.7 osteoclast-like cells. Our results indicate that FGF-2 has RANK-L independent inhibitory effects on osteoclast formation and bone resorption. Direct effects on osteoclasts include increasing the tendency of osteoclast precursors to fuse into giant cells and triggering mature osteoclasts to reorganize their actin cytoskeletons. Indirectly, FGF-2 stimulates low levels of osteoclastogenesis and bone resorption but strongly inhibits the pro-resorptive activities of calcitriol (1,25-dihydroxyvitamin D_3).

Materials and methods

Materials. Unless otherwise noted, reagents were obtained from the Sigma Chemical (St. Louis, MO). Recombinant human basic-fibroblast growth factor (FGF-2) was purchased from In Vitrogen (Carlsbad, CA). Antibodies to V-ATPase subunits utilized in this study were described previously [12]. The expression vector containing a RANK-L (158–316) glutathione-*S*-transferase fusion protein construct was a kind gift of Dr. Beth S. Lee (Ohio State University, Columbus, OH) [13].

Generation of osteoclasts. Mouse marrow osteoclasts were generated as described previously [14]. Swiss-Webster mice (8–20 gram) were killed by cervical dislocation, femora, and tibia were dissected from adherent tissue, and marrow was removed by cutting both bone ends, inserting a syringe with a 25 gauge needle and flushing the marrow

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doi:10.1016/j.bbrc.2004.04.013

using αMEM plus 10% fetal bovine serum (αMEM D10). The marrow was washed twice with αMEM D10 and then plated at a density of 1×10^6 cells/cm^2 on tissue culture plates for 5 days in αMEM D10 plus 10^{-8} 1,25-dihydroxyvitamin D_3 or FGF-2 concentrations as noted. Cultures were fed on day 3 by replacing half the media per plate and adding fresh 1,25-dihydroxyvitamin D_3. After 5 days in culture, osteoclasts appeared. These were detected as giant cells which stained positive for tartrate-resistant acid phosphatase activity (TRAP, a marker for mouse osteoclasts) or V-ATPase overexpression [15,16]. The University of Florida Institutional Animal Care and Usage Committee approved this protocol. Osteoclast-like cells were differentiated from RAW 264.7 cells, a mouse hematopoietic cell line, by stimulation with recombinant RANK-L as described [13]. Once mature, the osteoclasts or osteoclast-like cells were scraped and replated on either glass coverslips or bone slices.

Bone resorption assays. Assays for bone resorption were performed as described previously [14]. Sperm whale teeth were obtained from the United States Department of Fisheries, and 100-μm-thick sections with surface area of about 1 cm were cut using a low speed diamond saw (Buehler, Lake Bluff, IL). Slices were washed by agitation in 50 ml of sterile PBS and then stored in αMEM D10 days prior to assays, dentine slices were transferred to 24-well plates and incubated in αMEM D10. Mouse bone marrow cells were cultured in tissue culture plates for 5 days and then scraped free using a disposable cell scraper (Costar, Cambridge, MA), washed with αMEM D10 three times, and plated on bone slices at a concentration of 1×10^6 total cells/well. Cells were maintained on the dentine wafers for 5 days; medium was replaced after 3 days. Dentine slices were then rinsed with 1% SDS to remove cells and debris, fixed with 2.5% glutaraldehyde, dehydrated through an ethanol series, air-dried, sputter coated with gold, and examined using a Hitachi H-400 scanning electron microscope (Tokyo, Japan) operated at 15 kV. For quantitation, photos of slices were taken at 100× with no tilt angle. Overlays that divided micrographs into 42-μm grid spaces were placed over photos, and grid spaces with or without pits were counted to determine the surface area resorbed.

Histochemistry. Osteoclasts were fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with 10 μg/ml rhodamine phalloidin. Cells were observed under a Zeiss Axiomat fluorescence microscope.

Westerns. RAW 264.7 cells were treated with vehicle, RANK-L, FGF-2 or RANK-L and FGF-2 in combination for 7 days, cells were washed with PBS and harvested in SDS–PAGE sample buffer. Dilutions of samples were separated by SDS–PAGE and visualized by staining with Coomassie brilliant blue. These gels were used to standardize the samples based on expression of actin. Samples from the different treatment groups standardized to actin were separated by SDS–PAGE, blotted to nitrocellulose, and probed with anti-B2 and anti-E and anti-a3 polyclonal antibodies. The antibodies were detected by an HRP-linked chemiluminescence system (Pierce).

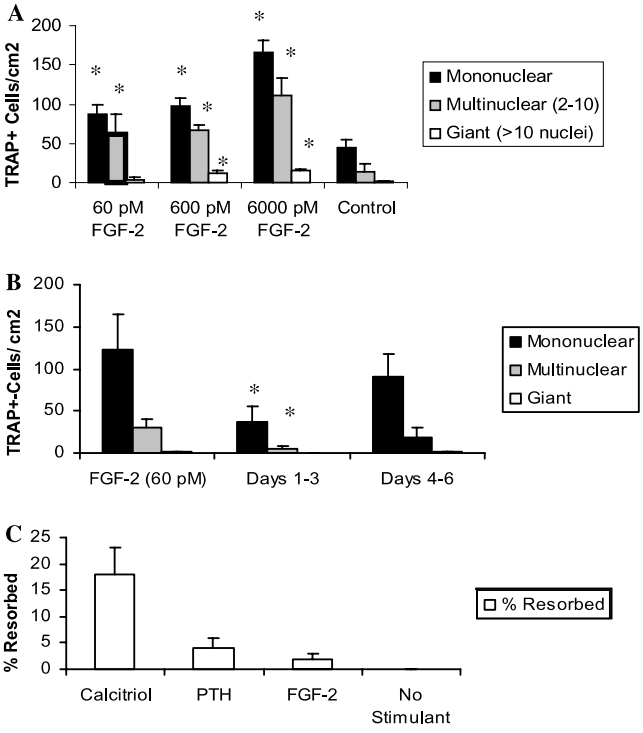

Fig. 1. FGF-2-stimulated mouse stimulates osteoclastogenesis and bone resorption by mouse marrow cultures. (A) Mouse marrow cultures were treated with the concentrations of FGF-2 noted for 7 days, the cells were fixed and stained for TRAP activity, and mononuclear, multinuclear, and giant cells were counted. (B) Mouse marrow cultures were treated with 60 pM FGF-2 for 6 days (control) or from days 1 to 3 or 4 to 6 of a 6 day culture. FGF-2 was required for late differentiation but not early differentiation. (C) Mouse marrow cultures were treated for 6 days in the presence of 10 nM cacitriol, 10 nM PTH or 60 pM FGF-2 and assayed for bone resorption by scanning electron microscopy. Asterisks indicate $p < 0.05$ from control by Student's t test.

Results

FGF-2 stimulates osteoclast formation, late differentiation, and bone resorption in mouse marrow

Mouse marrow was cultured with various concentrations of FGF-2 for 7 days. The cells were then fixed, stained for TRAP activity, and counted. The number of TRAP+ cells increased dose-dependently (Fig. 1A). Mouse marrow cultures were then incubated with FGF-2 on days 1–3 or days 4–6 of a 6 day culture and TRAP+ cells were counted. FGF-2 had no detectable influence on TRAP+ cells during the first 3 days of culture but stimulated osteoclast formation during the later 3 days (Fig. 1B). Mouse marrow cultures were incubated for 7 days on tissue culture plates, scraped, loaded on bone slices, and allowed to resorb for 5 days in the presence of FGF-2. FGF-2 stimulated the formation of pits as detected by SEM (Fig. 1C). Resorption by cultures stimulated with calcitriol and PTH is presented for comparison.

FGF-2 inhibits calcitriol stimulated osteoclastogenesis and bone resorption by mouse marrow

Mouse marrow cultures were stimulated with calcitriol-alone or with calcitriol in combination with varying concentrations of FGF-2. FGF-2 inhibited osteoclast formation triggered by calcitrol (Fig. 2A). At the highest doses of FGF-2 the number of osteoclasts formed was similar to the number of osteoclasts formed in cultures stimulated with FGF-2 alone. Addition of high levels of RANK-L failed to restore osteoclastogenesis in the

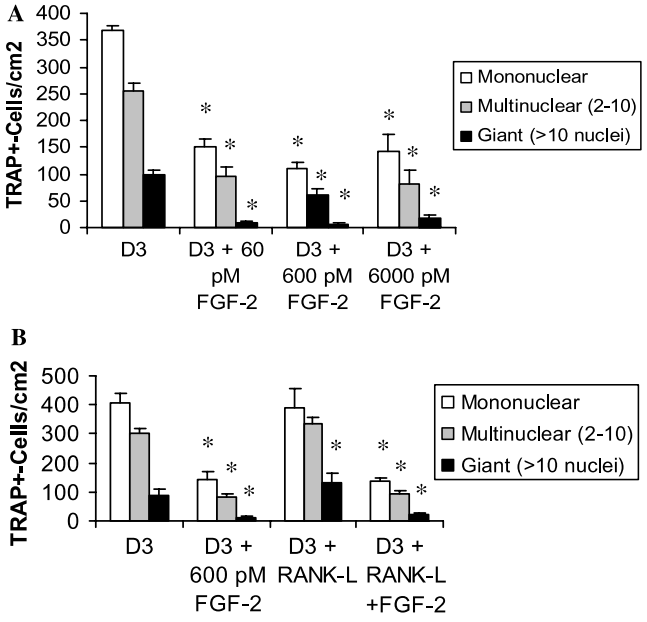

Fig. 2. FGF-2 inhibits calcitriol-stimulated osteoclastogenesis in marrow cultures in a RANK-L independent manner. (A) Mouse marrow cultures were stimulated for 6 days with 10 nM calcitriol (D3) or 10 nM calcitriol in combination with increasing concentrations of FGF-2. TRAP+ cells were counted in three groups, mononuclear cells, cells with from 2 to 10 nuclei, and giant cells—those with more than 10 nuclei. (B) Mouse marrow cultures were treated with 10 nM calcitriol (D3) alone or in combination with 600 pM FGF-2, 10 nM recombinant RANK-L as indicated. TRAP+ cells were assayed. Asterisk indicates $p < 0.05$ compared with calcitriol-alone controls.

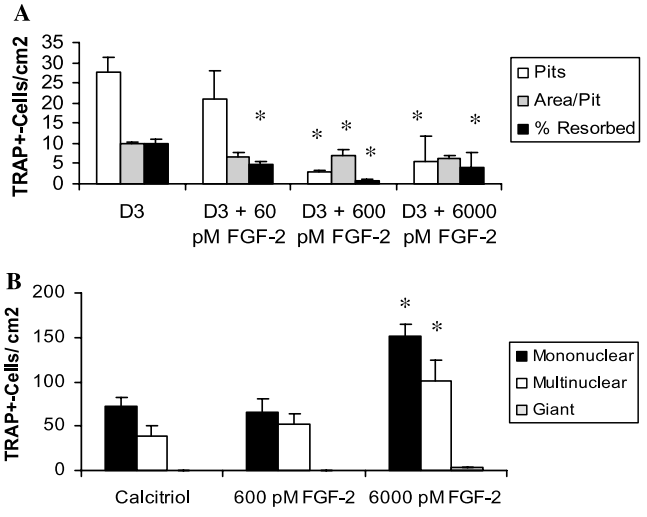

Fig. 3. FGF-2 dose-dependently inhibits bone resorption be mature osteoclasts, without decreasing osteoclast number. (A) Mouse marrow cultures in tissue culture plates were stimulated with 10 nM calcitriol (D3) for 6 days to generate osteoclasts. The cells were scraped, loaded onto bone slices, and cultured for 5 additional days in the presence of 10 nM calcitriol alone or with increasing concentration of FGF-2 as indicated. Resorption was assayed by SEM. (B) In parallel with the experiment in (A), other bone slices were assayed for the presence of TRAP+ cells. Asterisk indicates $p < 0.05$ compared with calcitriol-alone controls.

calcitriol-stimulated cultures containing FGF-2 (Fig. 2B).

Marrow cultures were stimulated with calcitriol for 5 days until osteoclasts matured and then loaded onto bone slices in the presence or absence of FGF-2. After 5 additional days of culture, bone resorption was assayed. FGF-2 inhibited bone resorption in a dose-dependent manner (Fig. 3A). In other cultures the number of TRAP+ cells were assayed. FGF-2 did not reduce the number of TRAP+ cells that were observed compared with cultures grown with calcitriol alone (Fig. 3B).

FGF-2 does not support differentiation of RAW 264.7 cells into osteoclast-like cells but does trigger increased fusion

RAW 264.7 cells were stimulated with vehicle, recombinant RANK-L, FGF-2, or RANK-L plus FGF-2. TRAP+ osteoclast-like cells did not develop in control cultures or in cultures stimulated with FGF-2-alone. In cultures stimulated with RANK-L many TRAP+ giant cells as well as smaller unfused TRAP+ mononuclear cells TRAP+ cells with only a few nuclei developed. When cells were cultured with both FGF-2 and RANK-L more Giant cells and fewer mononuclear and smaller TRAP+ cells were counted (Fig. 4).

FGF-2 stimulates changes in the actin cytoskeleton of RAW 264.7 osteoclast-like cells

RAW 264.7 cells were stimulated for 3 days with RANK-L alone. On day 4 the cells were incubated with RANK-L plus FGF-2. Within 18 h significant alterations in the arrangement of the actin cytoskeletal were detected (Fig. 5). This involved disruption of actin belts, formation of irregular protrusions, generation of isolated podosomes, and formation of structures that resemble the actin rings of resorbing osteoclasts.

FGF-2 does not effect the expression of V-ATPase genes

In RAW 264.7 cells, V-ATPase subunit expression levels increased in response to RANK-L [17]. FGF-2 did not affect the levels of expression of three subunits of V-ATPase (Fig. 6).

Discussion

FGF-2 is incorporated into the protein matrix of bone [10,18,19]. It has been shown to have anabolic effects on bone and is being considered as a possible pharmaceutical agent. FGF-2 is reported to either inhibit [20,21] or stimulate [8,22,23] osteoclast formation and resorption. Our results indicate that FGF-2 can do both depending upon the context. In mouse marrow

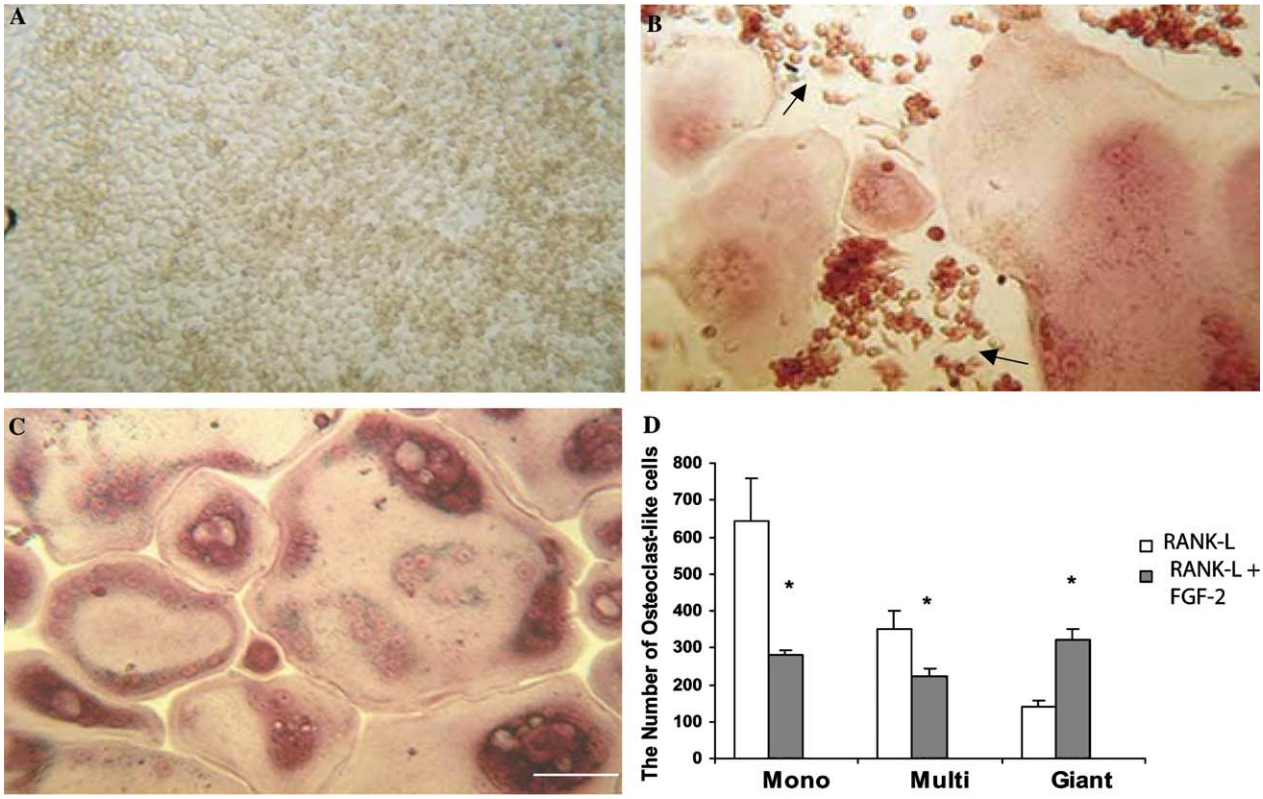

Fig. 4. FGF-2 does not support the formation of osteoclast-like cells from RAW 264.7 cells but increases their tendency to fuse into giant cells. In all experiments RAW 264.7 cells were plated at a concentration of 4×10^4 cells/cm^2, cultured for 7 days, and fixed and stained for TRAP activity. (A) Cells were cultured in the presence of 600 pM FGF-2. (B) Cells were cultured with 10 nM RANK-L. Arrows indicate clusters of mononuclear cells; (C) Cells were cultured with 600 pM FGF-2 and 10 nM RANK-L. Note the low levels of mononuclear cells. (D) Figure shows quantitatively that FGF-2 promotes the formation of Giant cells at the expense of TRAP+ cells with fewer nuclei. Bar equals 50 microns. Asterisk indicates $p < 0.05$.

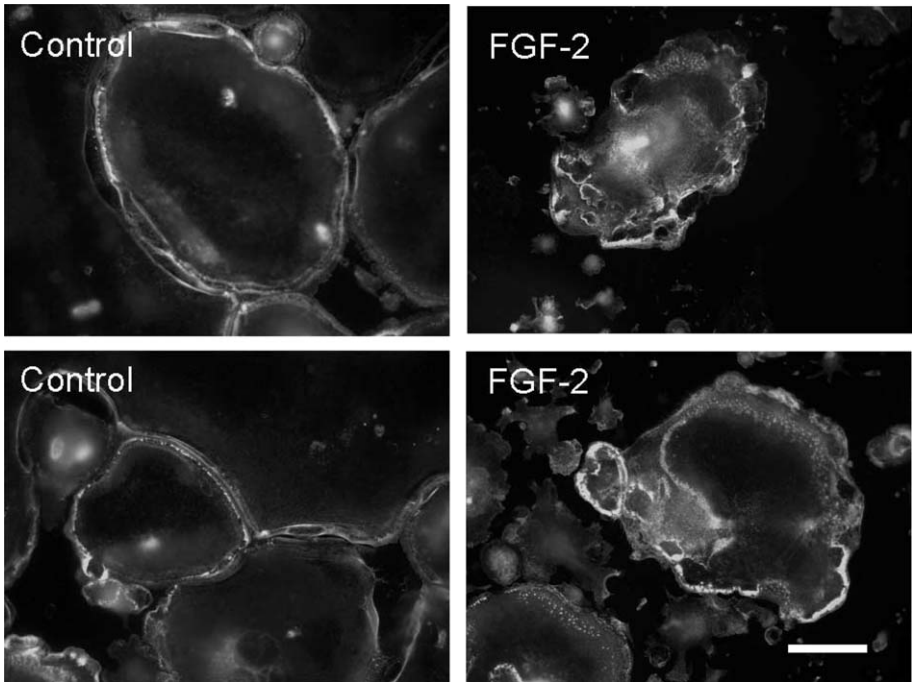

Fig. 5. FGF-2 triggers reorganization of actin cytoskeleton when used to treat RAW 264.7 osteoclast-like cells. RAW 264.7 cells were incubated for 3 days with 10 nM RANK-L to induce osteoclast-like cell formation. The cells were then treated with vehicle or 600 pM FGF-2 for 18 h, fixed and stained with phalloidin. Actin belts were disrupted in many of the FGF-2 treated cells. Bar equals 50 microns.

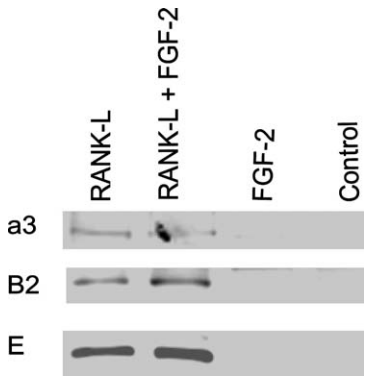

Fig. 6. FGF-2 does not effect expression levels of V-ATPase subunits. RAW 264.7 cells were treated with vehicle, 10 nM RANK-L, and 600 pM FGF-2 as indicated. The samples were loaded so that each contained the same amount of actin, blotted to nitrocellulose, and probed with antibodies to a3, B2, and E subunits of V-ATPase.

cultures FGF-2 was found by one group to inhibit calcitriol-stimulated bone resorption by reducing the levels of RANK-L expressed by osteoblasts [20]. Our findings agree with that report and show that FGF-2 is a potent inhibitor of both osteoclastogenesis and bone resorption stimulated by calcitriol. However, we found that providing excess RANK-L did not restore osteoclastogenesis, and thus another mechanism must explain the inhibition.

We found that FGF-2 inhibited bone resorption by osteoclasts that had been predifferentiated from mouse marrow cultures. The mechanism by which FGF-2 inhibits bone resorption by the fully differentiated osteoclasts does not involve reduction in the number of osteoclasts. Instead it seems that FGF-2 reduces the activity of osteoclasts. One possible mechanism for this would be by altering the organization of the actin cytoskeleton to prevent the formation of the actin ring structures that are required for bone resorptive activity [24–27]. Our studies indicate that direct action of FGF-2 alters the organization of the cytoskeleton of RAW 264.7 osteoclast-like cells. Further studies will be required to pinpoint the signaling events that underlie FGF-2 triggered reorganization of the cytoskeleton.

FGF-2 also directly triggered an increased tendency for RAW 264.7 cells to fuse into giant cells. Whether this relates to osteoclast activity is not clear. The underlying mechanism is also not known. A recent article that indicates levels of Wiscott–Aldrich Syndrome protein, an important regulator of the actin cytoskeleton [28], correlates with the eventual size of osteoclast points to a link between actin cytoskeletal dynamics and the propensity of osteoclast precursors to fuse [29]. This could be consistent with the alterations in the osteoclast cytoskeleton in response to FGF-2 that we observed.

FGF-2 did not have a direct effect on the expression of TRAP activity or on the expression of three V-ATPase components. Our data are consistent with direct effects of FGF-2 modulating the activity of the mature osteoclast, but not directly influencing differentiation of the osteoclast from precursors.

This study, taken together with other reports, indicates that the ultimate effects of FGF-2 on bone remodeling in vivo reflect a balance of positive, negative, direct, and indirect effects. The net result on bone remodeling is likely to be highly context specific. While this makes sorting out the influences of FGF-2 on bone resorption challenging, this work may lead to novel regulatory pathways that could potentially be mined for bone active agents.

Acknowledgments

This work was supported by an Arthritis Investigator Award from the Arthritis Foundation and AR-47959 (NIAMS/NIDDK) to L.S.H.

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