Estrogen Decreases Osteoclast Formation by Down-regulating Receptor Activator of NF-κB Ligand (RANKL)-induced JNK Activation

The differentiation of cells of the monocytic lineage into mature osteoclasts (OC) is specifically induced by the tumor necrosis factor-related factor, RANKL (receptor activator of NF-κB ligand; also known as OPGL, ODF, or TRANCE). Because inhibition of osteoclastogenesis is one of the main mechanisms by which estrogen (E2) prevents bone loss, it is likely that E2 may regulate either the production of, or the target cell responsiveness to RANKL. We found that E2 decreases the differentiation into OC of both murine bone marrow monocytes and RAW 264.7 cells, a monocytic line, by down-regulating the activation of Jun N-terminal kinase 1 (JNK1). Diminished JNK1 activity results in decreased nuclear levels of the key osteoclastogenic transcription factors, c-Fos and c-Jun, and lower binding of these transcriptional inducers to DNA. Thus, one novel mechanism by which E2 down-regulates osteoclastogenesis is by decreasing the responsiveness of OC precursors to RANKL.

The differentiation of osteoclast (OC) precursors of the monocytic lineage into mature OCs is induced specifically by simultaneous stimulation of bone marrow monocytes by two cytokines produced by bone marrow stromal cells, M-CSF (1–3) and a TNF-related factor known as receptor activator of NF-κB ligand (RANKL) (also known as OPGL, TRANCE, or ODF) (4–6). The formation of mature OCs is completely dependent on the presence of both RANKL and M-CSF, as demonstrated by the lack of OC development in mice lacking the expression of either factor (5, 7, 8).

It is now recognized that inhibition of osteoclastogenesis is the main mechanism by which estrogen (E2) prevents bone loss (9, 10). Moreover, considerable evidence support the hypothesis that E2 down-regulates OC formation by blunting the production of IL-1, IL-6, and TNF (9–13), cytokines that enhance the stromal cell production of RANKL and M-CSF (14–17). Additional mechanisms that serve to explain the antosteoclastogenic effects of E2 include the ability of sex steroids to stimulate the production of osteoprotegerin (OPG), a decoy RANKL receptor (18), and a direct repressive effect on the production of membrane-bound M-CSF (19, 20).

Despite the relevance of RANKL as an osteoclastogenic factor, and of the potent antosteoclastogenic effect of E2, sex steroids have not been reported to regulate the production of RANKL. The lack of such regulation is consistent with the absence of both estrogen-responsive elements and binding sequences for E2-regulated transcription factors on the RANKL promoter (21).

Binding of RANKL to its signaling receptor RANK results in the activation of Jun N-terminal kinase (JNK), a mitogen-activated protein kinase that increases the trans activation activity and the production of AP-1 transcription factors. We have recently reported that E2 down-regulates TNF-induced JNK activation in monocytic cells (22), and others have shown that OC formation is impaired in mice lacking the AP-1 factors c-Fos and c-Jun (23, 24). These data prompted us to investigate the hypothesis that an additional mechanism by which E2 down-regulates OC formation is by decreasing the responsiveness of maturing OC to RANKL.

Here we show that E2 decreases the activation of JNK leading to decreased production of the osteoclastogenic transcription factors c-Fos and c-Jun. Thus, E2 down-regulates OC formation not only by modulating the production of osteoclastogenic cytokines but also by affecting the sensitivity of maturing OC to RANKL.

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Animal Care and Use Committee of Barnes-Jewish Hospital. Unless otherwise specified, reagents and media were purchased from Sigma.

RAW 264.7 Cell and BMM Cultures—Bone marrow monocytes (BMM), defined as CD11b+ cells, were purified from the bone marrow of 5-week-old mice by positive immunoselection using MACS CD11b immunomagnetic beads (Miltenyi Biotec, Auburn, CA). RAW 264.7 cells were obtained from the ATCC. All cells were maintained in phenol red-free media and 10% charcoal-stripped serum cultured in α-MEM and treated with either E2 (10−10 M) or control vehicle. For some experiments, RAW 264.7 cells were treated with rolipaxine (10−7 M), 4-hydroxytamoxifene (10−7 M), and estrogen (10−8 M) plus ICI 182780 (10−6 M).

In other experiments the MEKI/ERK pathway inhibitor PD 98059 (New England Biolabs, Beverly, MA) was added (50 μM) to control and...
E2-treated RAW 264.7 cells 1 h before stimulation with RANKL for 30 min.

**OC Generation and Characterization**—OC were generated by culturing BMM with recombinant soluble RANKL (20 ng/ml) and M-CSF (10 ng/ml) for 7 days as described (4, 6). OC were also generated by culturing RAW 264.7 cells with RANKL (20 ng/ml) without M-CSF as described (25). Both culture systems generate large numbers of TRAP multinucleated cells, which express typical OC markers including calctotonin and vcatenoretin receptors and positivity for pp60-src and cathepsin K (4, 6, 25).

At the end of each experiment, cells were then fixed and stained for tarization of phosphatase activity (TRAP). Expression of calctotonin receptors and the ability to form resorption pits in vitro were assessed as previously described (26). Immunohistochemical staining for pp60-src and cathepsin K was conducted as described (27). More than 98% of the TRAP+ cells with three or more nuclei showed specific binding of labled calcitonin and expression of pp60-src and cathepsin K. These cells were capable of forming resorption pits in vitro. Therefore, TRAP+ cells with three or more nuclei were defined and counted as OC.

**JNK Activity Assay and Western Blot Analysis of Total and Active JNK**—RAW 264.7 cells were treated with E2 (10−8 M), ICI 182780 (10−6 M), estrogen + ICI, 4-hydro-tamoxifen (10−4 M) or raloxifene (10−7 M), or vehicle for 24 h and then stimulated with RANKL (200 ng/ml) for 5–30 min. JNK activity was determined in cell extracts as described (22). Briefly, cell extracts were precluled with 10−15 μl of protein A-Sephasore bead. The resin was then removed by centrifugation and the supernatant incubated with 1 μg of anti-JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h on ice. The immunocomplexes were then precipitated using protein A-Sephasore beads. The beads were washed, resuspended in 25 μl of kinase buffer containing 50 μM ATP and 5 μCi of [γ-32P]ATP, and incubated for 25 min at 30 °C with 1 μg of recombinant c-Jun (28, 29). The reactions were boiled in Laemmli loading dye and resolved by 12% SDS-PAGE. Phosphorylated c-Jun-GST protein was detected by autoradiography as described previously (30). For some experiment, JNK activity was determined in cell extracts using a JNK assay kit (Cell Signaling Technology, Beverly, MA) as described by the manufacturer. Briefly, JNK was precipitated by incubating cell extract overnight with c-Jun fusion protein beads. Beads were resuspended in 50 μl of kinase buffer supplemented with 100 μM ATP and incubated for 30 min at 30 °C. The reaction was boiled in SDS sample buffer, resolved on 4–20% Tris-glycine gel, and electrottransferred to nitroceullose membrane. The appropriate primary and secondary antibodies were used for detection of phosphorylated c-Jun on the membrane followed by a standard chemiluminescence reaction using Lumino (Cell Signaling Technology).

The levels of dephosphorylated and phosphorylated species of JNK1 and JNK2 (total JNK1/2) and those of the active (phosphorylates) forms of JNK1 and JNK2 (active JNK1/2) were measured using whole cell lysates from control and E2-treated RAW 264.7 cells either unstimulated or stimulated with RANKL for 30 min. Cell lysates (50 μg) were resolved on 12% SDS-PAGE and transblotted onto nitrocellulose. Total and phosphorylated JNK1/2 were detected using antibodies specific for either total JNK1 and JNK2 or phosphorylated JNK1 and JNK2 (Santa Cruz Biotechnology) using an ECL detection system (Amersham Pharmacia Biotech).

**Western Blot Analysis of Total and Active MKK4**—Western blot studies were conducted using whole cell lysates from control and E2-treated RAW 264.7 cells either unstimulated or stimulated with RANKL for 30 min. Cells were washed twice with ice-cold phosphate-buffered saline and then lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 (v/v), 1 mM NaF, 10 μg/ml leupeptin, 2 μM Na3VO4, and 1 μM phenylmethylsulfonyl fluoride) as described (28). Proteins were boiled in Laemmli loading dye, resolved on 12% SDS-PAGE, and transblotted onto nitrocellulose. The levels of total and phosphorylated MKK4 were detected using antibodies (New England Biolabs) specific for phosphorylated (active) and total (dephosphorylated plus phosphorylated) MKK4 using an ECL detection system (Amersham Pharmacia Biotech).

**Extraction of Nuclear Protein and Electrophoretic Mobility Shift Assays**—Electrophoretic mobility shift assays were performed by incubating nuclear extracts prepared from control and E2-treated RAW 264.7 cells stimulated with RANKL (120 ng/ml) for 3 h with a 32P-labeled double-stranded probe (5′ AAAGAGGAGCCGGAGCTATGATGAC 3′) as described previously (22). Mononuclear antibodies directed against members of the Jun and Fos families of nuclear proteins were used to supershift the relevant proteins.

The probe was end-labeled with T4 polynucleotide kinase according to manufacturer's instructions (Promega, Madison, WI). The annealed probe was incubated with nuclear extract on ice for 30 min prior to separation of the DNA-protein complexes on 4% nondenaturating PAGE, pre-run for 1 h in 0.25× TBE running buffer. The gels were dried and exposed to Kodak XAR-5 film for the appropriate length of time. For band supershifting, 200 ng of the appropriate antibody was added to the reaction mixture on ice for 40 min before adding the oligonucleotide probe.

**Western Blot Analysis of Jun and Fos**—Western blot analysis was performed using antibodies specific for either c-Jun, JunD, JunB, c-Fos, FosB, Fra-1, or Fra-2 (Santa Cruz Biotechnology) as described (22).

**Statistical Analysis**—Group mean values were compared by two-tailed Student’s t test or one-way analysis of variance as appropriate. Subsequent mean comparison tests were performed by the Fisher protected least significant difference test.

**RESULTS**

**E2 Decreases RANKL-induced Osteoclastogenesis**—The discovery that stimulation with RANKL and M-CSF is sufficient to induce the differentiation of BMM into OC in the absence of stromal cells (4, 5) has made it possible to investigate whether E2 has direct effects on maturing hematopoietic OC precursors. Triplicate experiments revealed that E2 (10−8 M) decreases by ~40% the ability of soluble recombinant RANKL and M-CSF to induce the differentiation of primary murine BMM into mature OC (Fig. 1A), defined as TRAP+, calcitonin receptor, pp60src, and cathepsin K+ multinucleated cells capable of resorbing bone in vitro.

Inhibition of either M-CSF- or RANKL-induced signals could account for the ability of E2 to blunt the differentiation of BMM into OC. Because we wished to examine the role of E2 in modulating RANKL-induced signals, we utilized the property of RAW 264.7 cells, a mononuclear macrophage, to differentiate into OC when treated with RANKL in the absence of M-CSF (25). Quadruple experiments (Fig. 1B) revealed that, similar to its effect on primary BMM, E2 decreases by ~40% the differentiation of RAW 264.7 cells into OC induced by RANKL. The selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene were as effective as estrogen in suppressing
Estrogen Decreases RANKL-induced Osteoclastogenesis

**Fig. 2.** E2 decreases JNK activity and the ability of RANKL to increase the level of active (phosphorylated) JNK1 in RAW 264.7 cells. A, time course experiments revealed that RANKL increases JNK activity 10-fold, peaking at 15 min. B, RANKL-induced JNK activity was repressed by raloxifene (10⁻⁷ M), and 4-hydroxytamoxifen. The inhibitory effects of estrogen were reversed by the estrogen antagonist ICI 182780 (ICI) (10⁻⁷ M). C, E2 decreases the level of active (phosphorylated) JNK1 in RANKL-stimulated RAW 264.7 cells. Active (phosphorylated) JNK2 was not detectable in either unstimulated or RANKL-stimulated RAW 264.7 cells. D, E2 does not regulate the level of total (dephosphorylated + phosphorylated) JNK1 and JNK2. The figure shows representative data from one of three replicate experiments.

**Fig. 3.** A, E2 blunts the ability of RANKL to increase the level of active (phosphorylated) MKK4 in RAW 264.7 cells. B, RANKL and E2 do not regulate the level of total (dephosphorylated + phosphorylated) MKK4. The figure shows representative data from one of three replicate experiments.

RANKL-induced differentiation of both BMM and Raw 264.7 cells into OC. Moreover, the inhibitory effects of estrogen were reversed by the estrogen antagonist ICI 182780 (31), a finding that suggests sex steroids have a genomic effect on OC precursors.

Thus, the inhibitory effect of E2 on RANKL-induced signals is sufficient to block OC formation, and RAW 264.7 cells represent an appropriate model for dissecting the molecular mechanisms involved.

**E2 Down-regulates MKK4-induced JNK Activation**—A key signaling event induced by the binding of RANKL to RANK is the activation of JNK (25). To investigate whether E2 decreases JNK activity, RAW 264.7 cells were treated with either E2 or control vehicle for 24 h, a length of time sufficient to induce genomic effects, and then stimulated with RANKL for 0–30 min. Nuclear extracts were then immunoprecipitated using an antibody that recognizes the two members of the JNK family, JNK1 and JNK2. Equal amounts of proteins were recovered and incubated with recombinant GST-c-Jun (a JNK substrate) and γ³²P]ATP. Phosphorylated material was resolved by SDS-gel and visualized by autoradiography. These studies revealed (Fig. 2A) that RANKL increases JNK activity 10-fold, peaking at 15 min. At all time points E2 treatment blunted RANKL-induced JNK activity, as compared with cells treated with RANKL plus E2 vehicle. Peak inhibitory activity (5-fold inhibition compared with vehicle-treated controls) was found at 15 min. The inhibitory effect of E2 on peak JNK activity was reversed by the estrogen antagonist ICI 182780 (Fig. 2B). Raloxifene and tamoxifen blocked RANKL-induced JNK activity in a manner similar to E2.

Western blot analysis of RAW 264.7 cells extracts with an antibody specific for the phosphorylated (active) form of JNK1 and JNK2 showed that unstimulated cells have low levels of active JNK1 and undetectable levels of active JNK2. RANKL induced the production of active JNK1 but not of JNK2 (Fig. 2C). However, at all time points phosphorylated JNK1 was ~50% lower in E2-treated cells than in cells treated with vehicle. E2 also had a small inhibitory effect on the levels of phosphorylated JNK1 in unstimulated cells. In contrast, Western blot analysis by an antibody recognizing both the dephosphorylated and the phosphorylated species of JNK1 and JNK2 (total JNK1/2) revealed the presence of both JNK species in unstimulated cells. However, neither RANKL nor E2 altered the level of total JNK1 and JNK2 (Fig. 2D). Together, these findings demonstrate that E2 represses the ability of RANKL to induce the activation of JNK1.

MKK4 is a kinase known to activate JNK1 by inducing its phosphorylation (32). Analysis of the effects of E2 on MKK4 using an antibody specific for the phosphorylated (active) form of this kinase revealed (Fig. 3A) that RANKL increases by ~3-fold the level of phosphorylated MKK4 and that E2 decreases by ~50% the activation of MKK4 induced by RANKL. E2 also had a small inhibitory effect on the levels of phosphorylated MKK4 in unstimulated cells. In contrast, Western blot studies conducted using an antibody that recognizes both the dephosphorylated (inactive) and the phosphorylated species of MKK4 revealed that neither RANKL nor E2 modulates the total levels of MKK4 (Fig. 3B). Thus, although the signaling pathway linking RANKL to MKK4 remains to be determined, the data demonstrate that E2 blunts MKK4 and JNK activation, thus leading to diminished JNK activity.

MKK4 is activated by MEKK1, a kinase that also leads to the phosphorylation of ELK-1 through the MEK1/ERK pathway (33, 34). Thus, a regulatory effect of E2 on MEKK1 could lead to blunted ERK activity and decreased ERK-induced production of c-Fos. The hypothesis that E2 blunts nuclear levels of c-Fos via inhibition of the MEK1/ERK pathway was excluded by demonstrating that PD 98059, a compound that blocks MEK1 activation (35, 36), does not abolish the ability of E2 to decrease nuclear levels of c-Fos (Fig. 4).

**E2 Decreases the Nuclear Levels of c-Jun and c-Fos and the Binding of These Proteins to DNA**—Because RANKL-induced
JNK activation results in enhanced production of Jun and Fos, we examined the effects of E2 on the nuclear levels of these proteins. Western blot studies conducted using RAW 264.7 nuclear extracts and monoclonal antibodies specific for individual members of the Jun and Fos families revealed that RANKL increased c-Jun levels and induced c-Fos production, peaking at 30 min. (Fig. 5). Moreover, in RANKL-stimulated cells, the levels of both c-Jun and c-Fos were ~3-fold lower in those treated with E2 than in those treated with control vehicle. Neither RANKL nor E2 affected the levels of JunB, JunD, FosB, Fra-1, and Fra-2 (not shown).

Jun and Fos exert their transcriptional effects by binding to the AP-1 consensus sequence(s) in the promoter region of target genes. The binding of these nuclear protein to DNA is proportional to their nuclear concentration and phosphorylation status. To determine the effects of E2 on the ability of Jun and Fos proteins to bind to DNA, nuclear extracts from control and E2-treated RAW 264.7 cells were incubated with a labeled oligonucleotide corresponding to the AP-1 consensus sequence. Samples from all cells generated a single DNA-protein complex, which was confirmed as AP-1 by the ability of 50-fold molar excess AP-1 consensus oligonucleotide to displace the complex. RANKL increased by ~3-fold the binding of AP-1 to its binding motif (Fig. 6A). Moreover, in samples from RANKL-treated cells, a specific anti-c-Jun antibody led to the almost complete disappearance of the AP-1 band, whereas anti-JunD and anti-JunB antibodies had no effects. The AP-1 complex was also supershifted by anti-c-Fos antibody (Fig. 5B), whereas antibodies against Fos-B, Fra-1, and Fra-2 had no effect. Together, these findings demonstrate that RANKL increases the binding to DNA of c-Jun/c-Fos and JunD/c-Fos heterodimers. These experiments also revealed that E2 completely blocks the ability of RANKL to increase the binding of c-Jun/c-Fos and JunD/c-Fos heterodimers to DNA (Fig. 6, A and B).

**DISCUSSION**

It is now recognized that E2 prevents bone loss via multiple and complex effects on bone marrow and bone cells, which results in decreased OC formation, increased OC apoptosis, and decreased capacity of mature OCs to resorb bone (9). E2 modulates OC apoptosis and OC activity both directly (37, 38) and indirectly via regulation of growth factors and prostaglandins (39, 40). Conversely, inhibition of OC formation in vivo results, in part, from the ability of E2 to block the production of the pro-osteoclastogenic cytokines IL-1, IL-6, and TNF (9–13). Another relevant effect of E2 in vivo is its capacity to block the production of soluble M-CSF via an IL-1- and TNF-dependent mechanism (16, 17, 41). *In vitro* studies have also revealed that E2 directly block the differentiation of OC precursors into mature OC (42), although the relevance of this phenomenon in *vivo* remains to be determined.

Although IL-1, IL-6, and TNF induce the formation of OCs in *vivo* and in complex culture systems containing stromal cells, osteoblasts, and OC precursors, these cytokines (alone or in combination) are not capable of inducing the formation of OCs when added to purified OC precursors. Moreover, osteopetrosis is not a feature of mice lacking the capacity to produce and/or respond to either IL-1, IL-6, or TNF (11–13). These observations are consistent with the notions that IL-1, IL-6, and TNF enhance OC formation by increasing the production of M-CSF (43, 44) and RANKL or by potentiating the osteoclastogenic effects of RANKL (45).

Binding of RANKL to RANK results in a cascade of intracellular events, including the activation of the transcription factor NF-κB and the stimulation of the protein kinase JNK (25, 46), which, in turn, increases transcription of the *c-jun* and *c-fos* genes (33, 47). This is because the transcription of *c-Jun* is regulated by two AP-1 binding sites present in its promoter.
Estrogen Decreases RANKL-induced Osteoclastogenesis

regions (33, 48). These sites are constitutively occupied by Jun/ATF2 heterodimers (33, 48), which generate a basal rate of gene autostimulation. However, the magnitude of the autostimulation of the c-jun gene by Jun/ATF2 heterodimers is enhanced by JNK-dependent phosphorylation of two serine residues within the N-terminal activation domain of c-Jun (33). JNK is also responsible for the phosphorylation of ternary complex factors (such as Elk-1) that are constitutively bound to the serum response element in the c-Fos promoter (33). Because JNK-dependent phosphorylation of ternary complex factors stimulates the transcription of the c-fos gene, JNK activation factors result in increased c-fos gene expression (33).

The relevance of the RANK/TRA6/JNK/AP-1 signaling pathway is demonstrated by the fact that osteopetrosis is a feature of TRAF6 (49)- and pathway is demonstrated by the fact that osteopetrosis is a feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 (49)- and feature of TRAF6 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