Tumor Necrosis Factor Receptors Types 1 and 2 Differentially Regulate Osteoclastogenesis*

Received for publication, May 8, 2000
Published, JBC Papers in Press, June 28, 2000, DOI 10.1074/jbc.M003886200

Yousef Abu-Amer‡§, Jeanne Erdmann§, Lena Alexopoulou**+, George Kollias, F. Patrick Ross§, and Steven L. Teitelbaum§

From the ‡Department of Orthopedic Surgery and the §Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ¶Department of Molecular Genetics, Hellenic Pasteur Institute, Athens 11521, Greece

The potent osteoclastogenic agent, tumor necrosis factor-α (TNF), exerts its biological effects via two receptors, namely TNF receptors 1 (p55r) and 2 (p75r), each present on osteoclast precursors. Thus, we asked if p55r and p75r differentially impact the osteoclastogenic process. Marrow derived from mice expressing only p55r generates substantially more osteoclasts, in the basal state, than does wild type, while marrow expressing only p75r, produces substantially fewer. Reflecting its preferential activation of p55r, exogenous TNF stimulates osteoclast formation by p55r+/−/p75r−/−, but not p55r−/−/p75r−/−, marrow. Consistent with the fact that NF-κB is essential for osteoclastogenesis, this transcription complex is activated, relative to wild type, in p55r+/−/p75r−/− osteoclast precursors and suppressed in those expressing only p75r. Because p55r enhances, and p75r suppresses, osteoclastogenesis, we asked if their principal ligands, namely soluble and membrane-residing TNF, respectively, differentially impact basal osteoclast recruitment. We find, in contrast to the significant level of osteoclast formation in wild type marrow, osteoclastogenesis by that derived from mice expressing membrane, but not soluble, TNF, is negligible. Thus, optimal therapeutic inhibition of bone resorption may entail selective TNF receptor modulation and/or arrested cleavage of membrane TNF to its soluble form.

Bone remodeling is a lifelong process characterized by coupling of the appearance of osteoclasts and osteoblasts. Despite this anatomical relationship, the rates at which bone is resorbed by osteoclasts and synthesized by osteoblasts diverge with age. This divergence is typically characterized by a predominance of bone degradation over formation, eventuating in a net loss of skeletal mass typically initiated in males and females during their fourth or fifth decades (1). Although its rate is accelerated by loss of gonadal steroids, age-associated bone loss appears prior to menopause. Thus, its etiology cannot indicate this fact.

The pace of bone resorption may reflect either the rate at which the average osteoclast resorbs bone or the number of osteoclasts in the resorptive pool. The bulk of evidence indicates, however, the magnitude of osteoclast recruitment is the predominant regulator of the net resorptive process (2, 3). Understanding the factors governing osteoclast number, in the basal state, is therefore essential to preventing age-attended bone loss.

TNF is a pleotropic molecule, which prompts a variety of often opposing biological effects in a cell-specific manner. Among the most perplexing of these events is the cytokine’s capacity, in some circumstances, to promote cell survival and in others, apoptosis (4). The discovery that TNF recognizes two cell surface receptors, namely the type 1 or p55 and type 2 or p75 moieties, and that each is capable of mediating distinct intracellular signals (4), has added substantially to understanding the complex activities of the cytokine. As regards the skeleton, TNF-induced osteoclast recruitment is probably central to the pathogenesis of disorders of inflammatory osteolysis, such as periodontal disease (5) and peri-prosthetic bone loss (6). In fact, TNF may participate in the genesis of post-menopausal osteoporosis (2, 7).

We have shown that, in pathological circumstances, TNF exerts its osteoclastogenic effect via p55r (5, 6). In the present study, we address the role of TNF in basal osteoclast recruitment. In fact, we find that marrow cultures expressing only p55r contain more osteoclasts than do wild type animals, whereas those bearing exclusively p75r contain fewer. Consistent with the conclusion that TNF enhances basal osteoclastogenesis via p55r and suppresses it by p75r, the magnitude of nuclear compartment-residing NF-κB in osteoclast precursors expressing only one or the other receptor, mirrors osteoclast number. Finally, establishing that soluble TNF, the principal ligand for p55r, is essential for basal osteoclast recruitment, osteoclastogenesis by marrow derived from mice expressing only the membrane-residing form of TNF, which is the major activating ligand for p75r, is diminished.

MATERIALS AND METHODS

Reagents—Recombinant murine TNF (specific activity 1.1 × 10^8 units/mg) and soluble p75r were purchased from R&D Systems (Minneapolis, MN). In all experiments involving TNF, 10 ng/ml cytokine was used (5). ECL kit was obtained from Amersham Pharmacia Biotech. All other chemicals were obtained from Sigma.

Mice—Transgenic mice in which the p55r gene has been deleted (8) were provided by Dr. Warner Lesslauer (Hoffman-LaRoche, Basel, Switzerland). Wild type and p75r gene deleted mice (8) were provided

1 The abbreviations used are: TNF, tumor necrosis factor; mTNF, transmembrane TNF; sTNF, soluble TNF; LPS, lipopolysaccharide; TRAP, tartrate-resistant acid phosphatase.

Printed in U.S.A.
p55r and p75r differentially regulate basal osteoclastogenesis. A, equal number of marrow cells isolated from wild type (WT), p55r−/−, or p75r−/− mice were maintained in osteoclastogenic conditions. Eight days later, the cells were stained for TRAP activity (red reaction product) as a manifestation of osteoclastogenesis. The nuclei were then resuspended in neutral extraction buffer B (20 mM HEPES (pH 7.8), 420 mM NaCl, 1.2 mM MgCl, 0.2 mM EDTA 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 5 μg/ml pepstatin A, 5 μg/ml leupeptin), vortexed for 30 s, and rotated for 30 min in 4 °C. The samples were then centrifuged, the nuclear proteins in the supernatant were transferred to fresh tubes, and protein content was measured using standard BCA kit (Pierce). Nuclear extracts (10 μg) were incubated with an end-labeled double-stranded oligonucleotide probe containing the sequence 5′-AAA CAG GGG GCT TTC CCT CCT C-3′ (11) derived from the κB3 site of the TNF promoter. The reaction was performed in a total of 20 μl of binding buffer (20 mM HEPES (pH 7.8), 100 mM NaCl, 0.5 mM dithiothreitol, 1 μg of poly(dI-dC), and 10% glycerol) for 15–20 min at room temperature. After incubation with the labeled probe for 30 min, samples were fractionated on a 4% polyacrylamide gel and visualized by exposing dried gel to film.

RESULTS

p55r and p75r Differentially Regulate Osteoclastogenesis—Our first exercise was to determine the impact of each of the two TNF receptors on basal osteoclastogenesis. To this end we turned to mice expressing only p55r (p55r+/− or p55r−/−) or p75r (p55r−/− p75r+/−). Equal numbers of marrow cells derived from these mutants, as well as their wild type counterparts, were placed in osteoclastogenic conditions (5). After 8 days, the cultures were stained for TRAP activity as a marker of osteoclastogenesis. Eight days later, the cultures were terminated at day 6 instead of standard day 8. Our rationale for this exercise was based upon preliminary experiments showing little basal, or TNF-stimulated, osteoclast formation in wild type marrow at this early time point. As seen in Fig. 2, in the presence or absence of cytokine, day 6 wild type and p55r−/− or p75r−/− mice contain less TRAP activity than wild type marrow. The number of multinucleated osteoclasts (Fig. 1B) reflects the intensity of TRAP staining, in each circumstance. Thus, in the basal state, the presence of p55r enhances the number of committed osteoclast precursor cells, which is reduced, in turn, by p75r.

To determine whether, similar to the basal state, p75r suppresses osteoclastogenesis stimulated by exogenous cytokine, marrow of mice deleted of either receptor was placed in osteoclastogenic conditions in the presence or absence of TNF. To optimize the potential pro-osteoclastogenic impact of p75r deletion, the cultures were terminated at day 6 instead of standard day 8. Our rationale for this exercise was based upon preliminary experiments showing little basal, or TNF-stimulated, osteoclast formation in wild type marrow at this early time point. As seen in Fig. 2, in the presence or absence of cytokine, day 6 wild type and p55r−/− or p75r−/− mice exhibit little evidence of osteoclastogenesis. In contrast, p55r−/− p75r−/− marrow, in basal conditions, generates more osteoclasts, a phenomenon enhanced by TNF. Thus, p75r decreases the magnitude and rate of basal and TNF-stimulated osteoclastogenesis.

Soluble p75r Blunts TNF-induced Osteoclastogenesis—The external domain of p75r enjoys a higher ligand affinity than does p55r (12). Moreover, the external domain of p75r is shed into medium, and in this state, competes for soluble ligand (13). Thus, the possibility exists that the dampening effect of p75r on osteoclastogenesis reflects buffering of soluble TNF. To explore
this possibility, wild type marrow cultures exposed to an osteoclastogenic concentration (10 ng/ml) of TNF were maintained in the presence or absence of soluble p75r (14). As seen in Fig. 3, the soluble receptor virtually eliminates the abundant osteoclastogenesis extant in TNF-containing wells. Thus, medium-residing p75r blunts p55r-mediated osteoclast recruitment.

\[ p55r \text{ and } p75r \text{ Differentially Regulate NF-kB} \]

The data, thus far, indicate that basal and stimulated osteoclastogenesis are enhanced by p55r and inhibited by p75r. If this holds true, one would expect intracellular signals essential for osteoclast differentiation to parallel the magnitude of osteoclastogenesis. Activation of the transcription complex, NF-kB, manifest as cytoplasm to nuclear translocation, is one such signal (15). To assess NF-kB activation, nuclear extracts were prepared from wild type, p55r\(^{+/+}\)/p75r\(^{-/-}\), or p55r\(^{-/-}\)/p75r\(^{+/+}\) macrophages isolated from marrow maintained for 5 days in osteoclastogenic conditions. As positive controls, wild type cells were treated with either TNF or LPS known to activate NF-kB. Mobility shift assays were performed using an NF-kB consensus response element as probe (Fig. 4). Reflecting their stimulatory effect on osteoclast formation, TNF, LPS, and deletion of p75r prompt NF-kB nuclear translocation. In contrast, nuclear extract derived from cells lacking p55r, in which osteoclast recruitment is dampened, contains no detectable NF-kB binding activity.

**Expression of Only Membrane-residing TNF Blunts Osteoclastogenesis**—TNF is expressed as a transmembrane 26-kDa protein, which is cleaved to a 17-kDa soluble form (16). Soluble TNF (sTNF) is the major activating moiety of p55r and the ligand species, which promotes osteoclastogenesis. The transmembrane form of TNF (mTNF) is also biologically functional and the major activating form of p75r (17). Since p55r-mediated signals promote, and p75r-derived signals blunt, osteoclastogenesis, it follows that osteoclast recruitment might be diminished in marrow expressing only mTNF. To address this issue, we turned to TNF\(^{-/-}\) mice overexpressing mTNF, mutated to resist cleavage to sTNF (18, 19). Marrow from these animals exclusively expressing mTNF (mTNF\(^{+/+}\)/sTNF\(^{-/-}\)), or wild type mice (mTNF\(^{-/-}\)/sTNF\(^{+/+}\)), was placed in osteoclastogenic conditions. As seen in Fig. 5, osteoclast differentiation by marrow derived from mice expressing only mTNF is markedly curtailed.

**DISCUSSION**

Osteoclasts are polykaryons derived from monocyte/macrophage precursors (20). This realization prompted development of experimental models permitting meaningful exploration of the factors governing osteoclastogenesis. It has long been known, for example, that commitment of precursor cells to the osteoclast phenotype requires the presence of marrow stromal cells or osteoblasts (21). The recent discovery that macrophage colony-stimulating factor and receptor-activator of NF-kB ligand are essential osteoclastogenic factors provides the molecular foundation of this observation (22).

Because the bulk of available evidence indicates that states of accelerated bone resorption, as occurs in post-menopausal osteoporosis, primarily reflect enhanced osteoclast recruitment (23), identifying the molecules governing this process is essential for averting pathological bone loss. In fact, recent evidence suggests that estrogen prevents post-menopausal osteoporosis by dampening the increase in macrophage colony-stimulating factor synthesis, by stromal cells, which attends loss of ovarian function (2, 3).

TNF, which is a potent osteoclastogenic agent (5, 6), is not only synthesized in sites of inflammatory osteolysis (6), but also appears to participate in the pathogenesis of post-menopausal osteoporosis (2, 7). This conclusion rests on the observation that administration of TNF-binding protein (24) or overexpression of soluble p55r (25) blunts bone loss, in the oophorectomized mouse. These observations imply that circulating TNF in non-inflammatory (i.e., basal) conditions impacts skeletal degradation. The role of constitutively expressed TNF in other circumstances also indicates it may be physiologically relevant. For example, normal levels of the cytokine induce massive hepatic apoptosis in Rel-A-deficient mice (26).
Thus, selective targeting of membrane or soluble TNF, and not mTNF, is necessary for optimal basal osteoclastogenesis. This observation is in keeping with our conclusion that sTNF, but which predominantly recognizes p75r, would be resistant to (28, 32). This being the case, a reasonable hypothesis would be that p75r is the membrane-residing form of the cytokine (17). For vivo (5). On the other hand, the principal activating ligand of p55r is the membrane-associating form of the cytokine (17). For events such as T cell activation, thymocyte proliferation, and p75r is markedly diminished, is novel and unexpected. Al- though the mechanism by which this predominantly pro-survival receptor diminishes osteoclast number is not in hand, TRIP, which associates with the p75r signaling complex, and inhibits its activation of NFκB (30), is a reasonable candidate molecule.

NFκB activation is central to the anti-apoptotic signals generated by p55r and TNF administered to osteoclast precursors activates the transcription factor (10). Furthermore, specific NFκB subunits are essential for osteoclast formation (15) and dominant/negative blockade of IκB phosphorylation, which prevents nuclear translocation of the transcription complex, hinders osteoclastogenesis (31). Thus, the finding that untreated marrow macrophages of p55r−/−p75r+/+ mice contain a paucity of activated NFκB, is in keeping with the diminished osteoclastogenic capacity of these cells. Conversely, and again mirroring osteoclastogenesis, the quantity of activated transcription complex is increased, relative to wild type, in p55r+/−p75r−/− osteoclast precursors.

sTNF is the major activating moiety of p55r, and we show that its addition, to wild type marrow cultures, promotes osteoclastogenesis. Furthermore, we find p55−/−p75+/+ marrow incapable of mounting an osteoclastogenic response to TNF, at any concentration, in vitro, or following LPS administration, in vivo (5). On the other hand, the principal activating ligand of p75r is the membrane-residing form of the cytokine (17). For example, mTNF is superior to sTNF in inducing, via p75r, events such as T cell activation, thymocyte proliferation, and granulocyte/macrophage colony-stimulating factor production (28, 32). This being the case, a reasonable hypothesis would be that marrow derived from mice expressing only mTNF, which predominantly recognizes p75r, would be resistant to osteoclastogenesis and, in fact, we find such to be so. This observation is in keeping with our conclusion that sTNF, but not mTNF, is necessary for optimal basal osteoclastogenesis. Thus, selective targeting of membrane or soluble TNF, and their respective predominant receptors, may optimize antinecrotic resorptive therapy.

Acknowledgment—We thank Dr. Robert D. Schreiber for helpful discussions.

REFERENCES