Communication

Intracellular Fragmentation of Bone Resorption Products by Reactive Oxygen Species Generated by Osteoclastic Tartrate-resistant Acid Phosphatase*

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Tartrate-resistant acid phosphatase (TRAP) is highly expressed in bone-resorbing osteoclasts and activated macrophages. It has been suggested that a redox-active iron in the binuclear iron center of TRAP could have the capacity to react with hydrogen peroxide to produce highly destructive reactive oxygen species (ROS). Here we show that TRAP can generate ROS in vitro and that cells over-expressing TRAP produce higher amounts of intracellular ROS than their parent cells. We further demonstrate that these ROS can be targeted to destroy collagen and other proteins. In resorbing osteoclasts, TRAP was found in transcytotic vesicles transporting matrix degradation products through the cell, suggesting that TRAP-facilitated fragmentation of endocytosed material takes place in a specific cellular compartment. These results suggest that bone matrix degradation occurs not only extracellularly in the resorption lacunae but also intracellularly in the transcytotic vesicles. We propose that proteins containing redox-active iron could represent a novel mechanism of physiological fragmentation of organic molecules. This mechanism could be important in tissue remodeling and as a defense mechanism of phagocytosing cells.

During bone growth and remodeling, a remarkable amount of bone is degraded by osteoclasts that have developed an efficient machinery to break down inorganic and organic bone matrix (1). Crucial components of this machinery are the secretions of acid and proteinases into an extracellular resorption lacuna (2–5). Resorbing osteoclasts are highly polarized and reveal at least four different membrane domains (5). Ruffled border (RB) forms the actual resorbing organ and penetrates into bone matrix with continuous endocytosis and transcytosis of degraded matrix components (6, 7). Finally, resorption products are secreted to the extracellular space via a functional secretory domain (FSD) in the basolateral membrane (8). In addition to active acidification and production of various proteinases, osteoclasts produce high amounts of reactive oxygen species (ROS) during resorption (9). However, the source and exact role of ROS have remained unclear.

Tartrate-resistant acid phosphatase (TRAP) is widely used as a specific marker for osteoclasts (10). High amounts of TRAP are also expressed in lung macrophages and in the hairy cells of the spleen in hairy cell leukemia (11). TRAP contains a binuclear iron center (12, 13), which has been suggested to participate in the generation of hydroxyl radicals (‘OH) (14, 15). The function and subcellular localization of TRAP have remained unclear despite extensive biochemical characterization and production of knock-out animals. Aging TRAP+/− mice revealed mild osteopetrosis (excess accumulation of bone) despite having a normal number and morphology of osteoclasts (16). To learn the physiological function of TRAP, we hypothesized that TRAP could facilitate the formation of ‘OH and target those ‘OH to break down extracellular matrix components during bone resorption.

EXPERIMENTAL PROCEDURES

Detection of Hydroxyl Radicals—Hydroxyl radical formation was monitored by following the formation of malondialdehyde acetal (MDA) from deoxyribose as described earlier (17). Recombinant rat bone TRAP (18, 19) was incubated for 1 h at 37 °C in a 1.0-mL reaction mixture containing 10 mM deoxyribose, 0.1 mM ascorbate, 1 mM hydrogen peroxide, 5 μM (1 unit) of apotinin, and 1 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline, pH 7.2. Ferric EDTA (0.1 mM FeCl3, in 0.2 mM EDTA) was used as a positive control and transferrin, carbonic anhydrase II (CA II), and matrix metalloproteinase 9 (MMP-9) as negative controls. Formation of ‘OH was further studied by adding various amounts of mannitol, a scavenger of ‘OH (17), to the reaction mixture containing TRAP. Color was developed by heating for 15 min at 100 °C in thiobarbituric acid and trichloroacetic acid as described (17).

Over-expression of TRAP—The P3-TRAP plasmid was constructed by cloning reverse transcriptase-polymerase chain reaction-cloned TRAP cDNA from human osteoclast-like cells into pCI Neo mammalian expression vector (Promega, Madison, WI) at the EcoRI site. This construct was transfected into murine macrophage-like RAW-264 cells using a cationic liposome reagent (Roche Molecular Biochemicals). Transfected cells were selected with 400 μg/ml geneticin (G418, Calbiochem-Novabiochem, San Diego, CA) over 14 days. Isolated clones were maintained in 200 μg/ml geneticin.

Measurement of Intracellular Oxidized State—Intracellular ROS were detected using the fluorescent probe DCFH-DA (Molecular Probes, Eugene, OR) (20). Cells were grown on glass coverslips, incubated for 5 min in Hanks’ solution (Life Technologies Inc.) containing 10 μM DCFH-DA.

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DA, briefly rinsed in Hanks’ solution, and kept at +37 °C during the measurement. Cells were excited with a 495 nm wavelength using a computer-driven filter wheel (MAC 2000, Ludi Electronic Products, New York) and a 32D neutral filter to avoid UV-induced radical production. Emitted light was collected through a dichroic mirror and an interference filter at 510 nm. Fluorescence was quantitated using an MCID image analyzer utilizing M2 software (Imaging research Inc., Brock University, Ste. Catharines, Ontario, Canada) as described (21).

**RESULTS**

**TRAP Generates ROS**—TRAP and EDTA-chelated iron were approximately equally potent in facilitating OH formation from H₂O₂, whereas transferrin did not generate OH in the deoxyribose system (Fig. 1A). Two zinc-containing osteoclast enzymes involved in bone resorption, CA II and MMP-9, were also unable to generate OH (Fig. 1B). The results shown in Fig. 1B were not affected if protease inhibitors were omitted from the reaction mixture (data not shown). The OH scavenger mannitol decreased the amount of MDA formation in the presence of TRAP (data not shown). We calculated the rate constant with the rate constant 1.0–2.0 of type I collagen incubated in the reaction mixture (data not shown). We calculated the rate constant 1.0–2.0 of type I collagen incubated in the reaction mixture (data not shown). We calculated the rate constant 1.0–2.0 of type I collagen incubated in the reaction mixture (data not shown).

**Monitoring of Collagen Breakdown**—Type I collagen (Fluka, Buchs, Switzerland) was purified from low molecular weight impurities by gel filtration using Superdex 200 HR 10/30 gel filtration column equilibrated with 10 mM Tris-HCl, pH 8.2, containing 0.3 M NaCl, using HPLC equipment (Amersham Pharmacia Biotech). 100 μg of the purified collagen was incubated for 1 h at 37 °C in a reaction mixture containing 80 nM TRAP, 0.1 mM ascorbate, 1 mM hydrogen peroxide, 5 μg (1 unit/ml aprotinin, and 1 μM phenylmethylsulfonyl fluoride in phosphate-buffered saline, pH 7.2. After the reaction, a 0.5-ml aliquot was immediately chromatographed through Superdex 200 HR 10/30 gel filtration column as described above. Similar experiments were also performed with ferric EDTA and transferrin. Controls were as indicated under “Results.”

**Intracellular Localization of TRAP in Osteoclasts**—We studied the distribution of TRAP in resorbing osteoclasts and noticed that TRAP was not localized in RB (Fig. 2, A and B). Instead, it was co-localized to intracellular vesicles containing endocytosed organic bone degradation products released from bone matrix during resorption (Fig. 2C; see Ref. 7).

**ROS Generated by TRAP Destroy Type I Collagen**—On the basis of TRAP’s capacity to facilitate ROS formation and its localization in the transcytotic vesicles, we hypothesized that ROS generated by TRAP could directly damage type I collagen and other proteins. To test the hypothesis, we used the same conditions in demonstrating the generation of hydroxyl radicals by TRAP (see Fig. 1A) and replaced deoxyribose with type I collagen. The Mᵣ of type I collagen incubated in the reaction mixture without trap was > 1000, and the Mᵣ of trap incubated without type I collagen was 32 (data not shown). When type I collagen and trap were incubated together without H₂O₂, their Mᵣ did not change (Fig. 3A). The addition of H₂O₂ resulted in fragmentation of most of the type I collagen into small fragments with Mᵣ < 10, whereas the Mᵣ of trap did not change (Fig. 3B). Experiments in which albumin was used instead of collagen revealed similar results (data not shown). Similarly to TRAP, EDTA-chelated iron efficiently destroyed collagen, whereas transferrin had no effect (data not shown), as suggested based on the deoxyribose test (see Fig. 1A).

**DISCUSSION**

Osteoclasts take up resorbed bone material, which is transported through the cell and secreted through FSD (6, 7). This transcytotic route explains how osteoclasts are able to remove large amounts of matrix degradation products and simultaneously penetrate into bone. We show here that in resorbing...
osteoclasts, TRAP is localized in transcytotic vesicles and not in RB or in the resorption lacuna. Our results are in good agreement with those published earlier by Clark and co-workers (25), who demonstrated by immunoelectron microscopy that TRAP is localized in large cytoplasmic vesicles and not in RB. We suggest that TRAP-containing vesicles from the biosynthetic pathway are fused to transcytotic vesicles transporting matrix degradation products from RB to FSD, where the enzyme is secreted together with the matrix degradation products. Based on our results, we hypothesize that the physiological function of TRAP in osteoclasts is to destroy endocytosed matrix degradation products during their transcytosis. Thus, matrix degradation would occur not only extracellularly in the resorption lacunae but also intracellularly in the transcytotic vesicles.

TRAP knock-out mice show disrupted endochondral ossification and mild osteopetrosis (16). If our hypothesis were correct, TRAP would not have any effect on the attachment of osteoclasts to bone in the normal bone remodeling cycle. Nor would TRAP affect development of the RB, acidification of the resorption lacuna, degradation of bone matrix in the resorption lacunae, or endocytosis of dissolved bone material. The only effect would be on the processing of the endocytosed matrix components during transcytosis. Thus, inactivation of TRAP would most probably result in only a small decrease in bone resorption, which would be in good agreement with the observed mild osteopetrosis in TRAP knock-out mice.

Bone resorption produces various breakdown products of bone matrix components into serum and urine (26). Clinical studies indicate that stimulated bone resorption leads to increased levels of TRAP in the serum (23, 27, 28), suggesting that osteoclasts would secrete TRAP into the circulation during bone resorption, where it is found intact in a large calcium-containing complex (28). Our results show that TRAP-facilitated ROS formation leads to fragmentation of collagen and other proteins. However, TRAP molecules themselves are not targets for this fragmentation. This result is in accordance with the in vivo findings that matrix proteins such as collagen, but not TRAP, are found as small fragments in the circulation (26, 28).

Our results clearly demonstrate that TRAP is capable of generating ROS that can destroy proteins in vitro. This activity of TRAP was not inhibited by protease inhibitors, and a similar activity was not observed in the same conditions for transferrin, an iron-containing protein without redox-active iron, and for CA II and MMP-9, two zinc-containing osteoclast enzymes involved in bone resorption. Instead, a similar activity was observed for EDTA-chelated redox-active iron. These observations suggest that the redox-active iron in TRAP would be active in generating ROS. The fact that the TRAP over-expressing cells produce high amounts of ROS further supports this hypothesis, although it is possible that some amount of the ROS in the cells is generated because of cell damage.

Iron-catalyzed ‘OH formation from hydrogen peroxide by the Fenton reaction is well known to cause DNA damage (29). Hydroxyl radicals also affect proteins by destroying the peptide backbone and by attacking some amino acid side chains, resulting in the fragmentation of proteins and the formation of aggregates (30, 31). Instead, ‘OH in combination with the superoxide anion (O2·−) cause protein fragmentation without the formation of aggregates (30). Our results demonstrate the fragmentation of proteins by ‘OH generated by TRAP without aggregate formation, suggesting that O2·− would also be present in the reaction mixture. When the redox-active iron in the binuclear iron center of TRAP is in the ferrous form, it can react with H2O2 by the Fenton reaction to produce a ferric ion and ‘OH: Fe2++ + H2O2 → Fe3++ + OH− + ‘OH. The newly formed ferric ion is still redox-active and able to react with H2O2 to form O2·− and a ferric ion: Fe3++ + H2O2 → Fe3++ + 2 H+ + O2·−. The formed ferrous ion is again able to react by the Fenton reaction etc. Thus, a sequence of reactions generating both ‘OH and O2·− occurs with continuous oxidation and reduction of the redox-active iron, making it possible for one enzyme molecule to generate a high number of these ROS as long as H2O2 is available.

In addition to osteoclasts, activated macrophages such as alveolar macrophages of the lung express high amounts of TRAP in normal human tissues (11). We have reported earlier
The role of ROS in the physiological degradation of the extracellular matrix and in tissue remodeling is unknown. Our results demonstrate that in specific cellular compartments of osteoclasts, TRAP-facilitated ROS production can be targeted to destroy extracellular matrix proteins. We propose that the family of proteins containing redox-active iron could represent a novel mechanism of physiological fragmentation in organic molecules. This mechanism could be important in tissue remodeling and as a defense mechanism of phagocytosing cells.

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FIG. 3. HPLC Superdex 200 gel filtration was used to study the ability of OH formed by recombinant TRAP to destroy type I collagen. A, when type I collagen and TRAP were incubated together without H2O2, type I collagen eluted in the void volume, and trap eluted in fractions corresponding with Mr 32,000. B, the addition of H2O2 resulted in fragmentation of most of the type I collagen to small fragments with Mr < 10, whereas TRAP still eluted as described in A.