NADPH–Oxidase Expression and In Situ Production of Superoxide by Osteoclasts Actively Resorbing Bone

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Abstract. Recent reports have suggested that production of superoxide or other reactive oxygen species by activated osteoclasts may play a role in the complex process of bone resorption; however, the enzyme responsible for production of superoxide by osteoclasts has not been characterized. To determine if osteoclasts express NADPH–oxidase, a superoxide-generating enzyme found in phagocytic leukocytes, immuno-histochemical studies were performed on tibia from 1-5-d-old rats using mAbs 449 and 48 and an antiserum specific for p47-phox. These antibodies recognize epitopes on the α and β subunits of cytochrome b_{558}, respectively, and the p47 cytosolic component of NADPH–oxidase. We found that osteoclasts attached to bone surfaces in tibia expressed all three components, as did mature polymorphonuclear and some mononuclear leukocytes in the bone marrow. In many adherent osteoclasts, the cytochrome b_{558} subunits were localized to the ruffled-border and bone interfaces. Studies were also performed on mature rat tibia that had undergone controlled fracture. By two weeks the healing fractures develop a callus rich in actively resorbing osteoclasts. Osteoclasts within the calluses, and attached to bone surface, also expressed the cytochrome b_{558} proteins. In addition to demonstrating the expression of NADPH–oxidase, the active production of superoxide by osteoclasts was also demonstrated in situ in freshly isolated tibia using 3,3'-diaminobenzidine (DAB)-Mn^{2+}, a histochemical method specific for superoxide localization. Osteoclasts attached to bone surfaces contained deposits of oxidized DAB which were observed by light microscopy. Nonstimulated polymorphonuclear and mononuclear leukocytes in the bone marrow did not contain DAB deposits unless stimulated with phorbol myristate acetate, a known activator of NADPH–oxidase. These findings indicate that osetoclasts contain NADPH–oxidase, and during the process of resorbing bone, are actively producing superoxide.

RESORPTION of bone by activated osteoclasts is well documented; however, how osteoclasts are formed and activated or the mechanisms by which bone is resorbed by osteoclasts remain unclear. Bone resorption is a complex process that involves the differentiation of precursor cells to osteoclasts, the release of mineral from bone and degradation of the proteinaceous bone matrix. Recent reports have suggested that the production of superoxide or other reactive oxygen species (ROS)[1] by activated osteoclasts play a role in the complex process of bone resorption

(Garret et al., 1990; Key et al., 1990; Suda et al., 1993; Bax et al., 1992; Ries et al., 1992). ROS generated from superoxide include hydrogen peroxide, hydroxyl radicals, and singlet oxygen.

Garrett et al. (1990) were the first to report that the reduction of NBT by osteoclasts in mouse calvarial cultures in response to exogenous parathyroid hormone, IL-1, TNF, and 1_α,25-dihydroxy vitamin D_3 was associated with formation and activation of osteoclasts. This group also reported that addition of superoxide dismutase (SOD) to deplete superoxide, but not catalase which removes hydrogen peroxide, blocked NBT reduction and formation of additional osteoclasts. In contrast, Suda et al. (1991) reported that addition of catalase inhibited both the number of osteoclast-like cells formed from precursor cells as well as bone resorption in mouse calvarial organ cultures stimulated with 1_α25-dihydroxy vitamin D_3. This group also found that addition of exogenous hydrogen peroxide to their cultures overcame the effect of catalase, while addition of SOD increased the number of osteoclast-like cells. A third group (Bax et al., 1992) found that exogenous hydrogen peroxide added to cultures of rat osteoclasts incubated on devitalized bovine

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1. *Abbreviations used in this paper:* KCN, potassium cyanide; MIK, modified Ito-Karnovsky; MSS, modified Susa solution; NBT, nitrobule tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase.

cortical bone was a direct stimulator of bone resorption and cell motility. It is, therefore, still unclear what the relative contributions of superoxide and hydrogen peroxide are in the formation of osteoclasts from precursor cells or in the tightly regulated activation of osteoclasts.

In addition to the formation and activation of osteoclasts, strong evidence supporting a direct role for superoxide or one of its metabolites in the resorption of bone by osteoclasts has also been reported. Key et al. (1990) demonstrated by transmission electron microscopy that the reduction of nitroblue tetrazolium (NBT) in phorbol myristate acetate-stimulated mouse calvarial organ cultures was localized to formazan granules within the osteoclasts as well as to the ruffled border-bone interface near indentations in the bone. The reduction of NBT by osteoclasts and the resorption of bone, measured as the release of ^{45}Ca from labeled-calvaria, was decreased by the addition of a manganese-based superoxide dismutase-mimic, desferal-Mn^{3+} (Ries et al., 1992). Although NBT reduction histochemistry is not specific for superoxide, the decrease in NBT reduction to formazan in response to desferal-Mn^{3+} strongly supports that formazan formation is due to production of either superoxide or one of its metabolites.

Osteoclasts from several species have been reported to be the source of superoxide (Garrett et al., 1990; Ries et al., 1992) and hydrogen peroxide (Suda et al., 1993), however, the enzyme(s) involved in production of these ROS has not been characterized. Mechanistically, production of superoxide and hydrogen peroxide in phagocytic leukocytes proceeds by means of an activatable enzyme called NADPH–oxidase, which, upon appropriate triggering, catalyzes transfer of electrons from NADPH via cytochrome b_{558} to oxygen (Babior et al., 1973; Rossi, 1986). Cytochrome b_{558} is a unique low-potential cytochrome component of NADPH–oxidase (Segal and Jones, 1978) reported to have two subunits, a 22–23-kD protein (α subunit) and a 75–90-kD glycoprotein (β subunit) (Segal, 1987; Parkos et al., 1987; Rotrosen et al., 1992). The production of superoxide by NADPH–oxidase is dependent upon cytosolic factors, including two specialized proteins, p47-phox and p67-phox (for review see Segal and Abo, 1993). No evidence exists at present to indicate that osteoclasts have an NADPH–oxidase system. However, the development of specific antibodies to the cytochrome b_{558} subunits (Nakamura et al., 1987; 1988; Verhoeven et al., 1989) has enabled investigators to investigate the presence of NADPH–oxidase in cells other than phagocytic leukocytes, including B lymphocytes, mesangial cells, and fibroblasts (Maly et al., 1989; Radeke et al., 1991; Meier et al., 1993).

In the present study, mAbs 449 and 48 recognizing the unique cytochrome b_{558} of NADPH–oxidase (Verhoeven et al., 1989), and antiserum specific for the cytosolic component p47-phox (Verhoeven et al., 1993), identified the superoxide-generating system in rat osteoclasts as NADPH–oxidase. In addition, the production of superoxide in situ by activated osteoclasts was demonstrated in noncultured tibia freshly isolated from 1–5-d-old rats, in the absence of exogenous stimulators of bone resorption. Superoxide production was established using the DAB-Mn^{2+} histochemical method developed by Briggs et al. (1986), under conditions we have extensively shown to be specific for superoxide (Steinbeck et al., 1993).

Materials and Methods

4β-Phorbol-12-myristate-13-acetate, superoxide dismutase (SOD type I from bovine erythrocytes), 3,3'-diaminobenzidine tetrahydrochloride (DAB), Hepes, sucrose, $MnCl_2 \cdot 4 H_2O$, deferroxamine mesylate (desferal), MnO_2, 3-amino-1,2,4-triazole, sodium azide, and catalase (10,000–25,000 U per mg protein from bovine liver, thymol-free) were obtained from Sigma (St. Louis, MO). Potassium cyanide (KCN) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). All other buffers, salts, and chloroform were from Fisher Scientific (Fairlawn, NJ).

Bone Fracture

Bone fractures were performed according to the technique of Selye (1957) on male Sprague-Dawley rats weighing 100–200 g (35–42-d-old). The animals were anaesthetized by intraperitoneal injection of sodium pentobarbital at 50 mg/kg. The animal was prepared by shaving and prepping the right hind limb and inguinal region with 70% alcohol. A 3–4-cm incision was made over the ventral aspect of the right knee joint and the proximal third of the tibia was freed of the surrounding muscles. A curved probe was placed between the tibia and fibula in order to isolate the tibia for fracture. A littauer bone cutter was used to make a controlled fracture at the midpoint of the tibia. The skin was closed with a simple interrupted stitch using 5.0 ethilon suture. All rats received a single subcutaneous injection of 150,000 U of procaine penicillin to prevent infection. None of the animals developed problems postoperatively; and discomfort was limited because in the rat, the distal end of the fibula is rigidly connected to the tibia by a synostosis; consequently, the fibula acts as a natural splint which maintains the two ends of the tibial fracture in position. On day 14, after sacrificing the rat, the fracture calluses were dissected with a razor blade and one-quarter of each callus was fixed overnight at 25°C and simultaneously decalcified in modified Susa solution (50 ml of saturated zinc chloride in 0.6% NaCl, 6.0% glacial acetic acid, and 2.0% formalin) for subsequent histological examination. The remaining pieces of callus were fixed overnight at 4°C in modified Ito-Karnovsky fixative (0.05% glutaraldehyde, 4.0% paraformaldehyde, and 15% saturated picric acid in 0.1 M Cac, pH 7.2) and then decalcified for one week in several changes of 5.0% EDTA, 0.2 M sucrose, pH 7.2. After being embedded in paraffin, sections of 4-μm thickness, were stained with hematoxylin-eosin or used in immunohistochemical studies. The presence of osteoclasts in healing fractures has been previously reported (Gonzales and Karnovsky, 1961).

Preparation of 1–5 day old rat tibia. Tibia were removed from 1–5-d-old rat pups following euthanasia with an overdose of ether. The surrounding tissue and muscle were removed and the tibia were cut longitudinally and washed in several changes of ice-cold Ringer's, fixed at room temperature in modified Susa solution (MSS) or on ice in modified Ito-Karnovsky (MIK) fixative for 60 min, and then embedded in paraffin. After being embedded in paraffin, sections of 4-μm thickness were stained with Harris hematoxylin or used in immunohistochemical studies.

Preparation of macrophages and polymorphonuclear leukocytes. Peritoneal macrophages were elicited from male Sprague-Dawley rats (400–500 g) (CD strain; Charles River, Wellesley, MA) using 10 ml 4% sterile thioglycollate (DIFCO Laboratories, Detroit, MI). The cells were prepared by the method of Badwey et al. (1983) and were further purified over Ficoll-Paque 1.077 gm/ml (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) to ∼94 ± 0.8% macrophages, washed twice and resuspended in PBS without calcium and magnesium (138 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 7.5 mM D-glucose, pH 7.35). Elicited polymorphonuclear leukocytes from male Sprague-Dawley rats (400–500 g) were prepared by the method of DePierre and Karnovsky (1974), using 9% sterile sodium caseinate (Fisher Scientific). The cells were further purified over Ficoll-Paque (1.077 gm/ml) to ≥95% neutrophils, washed twice and resuspended in PBS. Viability of both cell preparations were always ≥90% as determined by trypan blue exclusion. According to the procedure of Verhoeven et al. (1989) macrophages and polymorphonuclear leukocytes were preincubated for 10 min at 37°C in a 5.0% CO_2 atmosphere in incubation medium containing 132 mM NaCl, 6.0 mM KCl, 1.2 mM KPO_4, 1.0 mM $MgSO_4$, 20 mM Hepes, and 1.0 mM EGTA, 0.5% human serum albumin, pH 7.4, or in incubation medium minus EGTA and plus 1.0 mM $CaCl_2$ and 1.0 μM fMLP. The only variation was that the cells were incubated on glass slides instead of in suspension. Unstimulated and fMLP-stimulated cells were then permeabilized by adding 15 μM digitonin, washed with incubation medium, and fixed in 0.5% paraformaldehyde and incubation medium for 5 min at room temperature (Verhoeven et al., 1989). The slides were washed and stored in 0.9% normal saline at 4°C until use.

Tartrate-resistant acid phosphatase activity assay. This method was performed as reported by Cole and Walters (1987) on MIK- or MSS-fixed bone calluses or tibia from 1-5-d-old rats. Resident macrophages were obtained from the peritoneal cavity of a rat, fixed as above, and used as tartrate inhibitable acid phosphatase positive cells.

Immunocytochemical and immunohistochemical studies. Slides of unstimulated and fMLP-stimulated PMNs and macrophages and 4-μm sections of the paraffin embedded calluses or tibia were immunostained using standard indirect avidin-biotin techniques (Hsu et al., 1981) (Vectastain ABC-Elite; Vector Labs., Inc. Burlingame, CA) with mouse monoclonal antibodies (IgG₁) that recognize: (*a*) rat monocyte/macrophage (ED-1, 1:500 dilution; Serotec, Oxford, England) (Dijkstra et al., 1985); (*b*) human neutrophil cytochrome b_{558} α- and β-subunits (449 and 48, respectively, 1:100 dilution) (Verhoeven et al., 1989); (*c*) muscle-specific actin (HHF-35, 1:1000 dilution; Enzo Biochem, Inc., New York) (Tsukada et al., 1987); and (*d*) rabbit antisera specific for human neutrophil p47-phox (p47-phox, 1:100 dilution) (Verhoeven et al., 1993). The samples were initially incubated in 1 ml of a solution of 50 ml of 3% H₂O₂ and 200 ml of methanol for 20 min at room temperature to inhibit endogenous peroxidase activity. The samples were next incubated in one ml of 5 or 10% nonimmune horse or goat serum at 23-25°C for 20 min, then incubated with antibody solution at 23-25°C for 90 min or at 4°C for 15 h. Biotinylated anti-mouse IgG (H + L) produced in horse (BA-2001; Vector Labs., Inc.) was used to detect the bound mouse monoclonal antibodies (23-25°C for 60 min), and biotinylated anti-rabbit IgG (H + L) produced in goat (BA-1000; Vector Lab.) was used to detect the bound p47-phox. This step was followed by incubation with avidin-biotin labeled-peroxidase, and finally 0.1 M Tris buffer, pH 7.6, containing 2.5 mM DAB and 0.02% H₂O₂ at 23-25°C for 2-4 min. Samples were counterstained with Harris hematoxylin.

DAB-Mn²⁺ histochemistry for the localization of superoxide. The DAB-Mn²⁺ histochemical procedure used in the present studies on tibia from 1-5-d-old rats was based on the method of Briggs et al. (1986). To specifically detect the production of superoxide by cells, freshly isolated and unfixed tibia were preincubated for 15-30 min in 0.1 M Hepes-NaOH buffer, pH 7.2, containing 5% sucrose with and without inhibitors, and then incubated in histochemical medium consisting of buffer, inhibitors when present, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) (1.0 mg/ml), plus 1.0 mM Mn²⁺ (MnCl₂·4 H₂O). The histochemical media were prepared just before use and filtered. It was necessary to readjust the pH of the medium after adding DAB with 0.1 N NaOH to return it to 7.2. The tibia were incubated in this medium at 37°C for 30 min and then washed in several changes of buffer at room temperature, fixed on ice in MIK fixative for 60 min and then embedded in paraffin. After being embedded in paraffin, sections of 6-μm thickness were stained with Harris hematoxylin. The principle of the DAB-Mn²⁺ histochemical method to localize the production of superoxide is based on the formation of visible brown deposits of DAB following its oxidation and the reactions are shown below: (*a*) generation of superoxide from molecular oxygen by NADPH-oxidase and (*b*) oxidation of Mn²⁺ to Mn³⁺ by superoxide, and the subsequent oxidation of DAB by Mn³⁺ (Patriarca et al., 1975; Curnutte et al., 1976; Kono et al., 1976).

1. O_2 — NADPH-oxidase → O_2^-
2. $2O_2^- + Mn^{2+}$-DAB → $H_2O_2 + Mn^{3+}$-DAB → oxidized DAB ↓ + Mn^{2+}
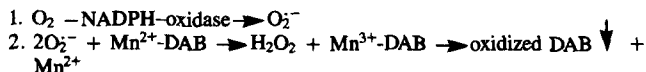
DAB histochemistry for cytochrome c oxidase activity. The DAB histochemical procedure for cytochrome c oxidase activity was based on the method of Seligman et al. (1968), and was performed as described above for DAB-Mn²⁺ histochemistry except no Mn²⁺ was added to the media.

Desferal-Mn³⁺ complex preparation. This was prepared according to the method of Beyer and Fridovich (1989). In brief, deferroxamine mesylate (328 mg) was dissolved in 10 ml of dH₂O, then MnO₂ was added (56 mg). After stirring at 25°C for 2-4 h the unreacted MnO₂ was removed by centrifugation and the dark green solution was passed through a 0.2-μm syringe filter (Millipore Corporation, Bedford, MA) and lyophilized for storage (-20°C).

Results

Identification of Osteoclasts in Rat Tibia

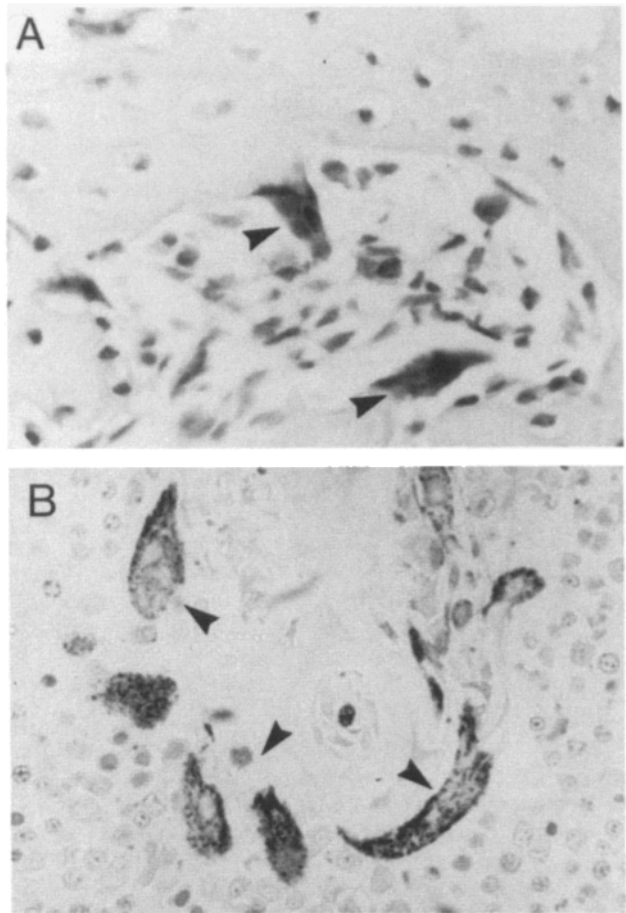Osteoclasts were identified in day 14 calluses from healing

Figure 1. Identification of osteoclasts in rat tibia using the tartrate-resistant acid phosphatase assay of Cole and Walters (1987). Day 14 calluses from healing tibial fractures of 28-35-d old rats were fixed overnight at 25°C and simultaneously decalcified in mss (50 ml of saturated zinc chloride in 0.6% NaCl, 6.0% glacial acetic acid, and 2.0% formalin), or fixed overnight at 4°C in MIK fixative (0.05% glutaraldehyde, 4.0% paraformaldehyde, and 15% saturated picric acid in 0.1 M Cac, pH 7.2) and then decalcified. Tibia from 1-5-d-old rats were fixed for one hour at 25°C in mss or on ice for 1 h in MIK fixative. Multinucleate, tartrate-resistant acid phosphatase positive osteoclasts were localized to bone surfaces in 4-μm paraffin sections of (*A*) day 14 calluses from healing tibial fractures of 28-35-d-old rats and (*B*) tibia removed from 1-5-d-old rats.

tibial fractures of 28-35-d-old rats as large, multinucleate cells (three or more nuclei), which appeared to be tightly adhered to bone surfaces, and contained tartrate-resistant acid phosphatase activity (Fig. 1 *A*). Similarly, tibia from 1-5-d-old rats contained numerous osteoclasts which were identified as multinucleate, tartrate-resistant acid phosphatase positive cells localized to the bone surfaces (Fig. 1 *B*).

Expression of NADPH-Oxidase in Osteoclasts of Rat Tibia

To assess the expression of NADPH-oxidase by osteoclasts, immunohistochemical studies were performed on rat tibia using monoclonal antibodies (mAb) 449 and 48, and antisera specific for p47-phox. These antibodies recognize epitopes

Table I. Summary of Antibody Reactivities in 1–5-d-old Rat Tibia and 14-d Fracture Calluses of Rat Tibia

Mouse monoclonal antibodies subclass (IgG_1) and antiserum	Immunostained cells in 1–5-d-old rat tibia and 14 d fracture calluses
449 (α-subunit of cytochrome b_{558})	Osteoclasts and polymorphonuclear and some mononuclear leukocytes in bone marrow regions
48 (β subunit of cytochrome b_{558})	Osteoclasts and polymorphonuclear and some mononuclear leukocytes in bone marrow regions
p47-phox (cytosolic component of NADPH–oxidase)	Osteoclasts and polymorphonuclear and some mononuclear leukocytes in bone marrow regions
ED-1 (marker for monocytes/osteoclasts)	Osteoclasts attached to bone surfaces and monocytes in bone marrow regions
HHF-35 (muscle cell-specific actin)	Smooth muscle cells surrounding blood vessels

on the α (449) and β subunits (48) of cytochrome b_{558} (Verhoeven et al., 1989), and one of the cytosolic components, p47-phox, of NADPH–oxidase (Verhoeven et al., 1993). Table I summarizes the results of the immunohisto-chemical studies, and immunostaining for NADPH–oxidase in osteoclasts is shown in Fig. 2, *A-C*, and Fig. 3. Oxidized-DAB deposits of immunoperoxidase products were observed in osteoclasts attached to bone surfaces of calluses from healing tibial fractures and tibia from 1-5-d-old rats immunostained with mAbs 449 and 48 and anti-p47 antisera. In a number of adherent osteoclasts the cytochrome b_{558} subunits were localized to the ruffled border (Fig. 2, *A-C*; *arrowheads*).

Immunostaining with mAbs 449 and 48 and anti-p47 antisera was also observed in mature polymorphonuclear leukocytes and some mononuclear cells in bone marrow regions of tibia (Figs. 2 *C* and 3). Staining with mAb 449 and anti-p47 antisera was most intense in nonactivated polymorphonuclear leukocytes in the bone marrow regions of tibia, whereas, osteoclasts in tibia and healing fractures showed greater reactivity with mAb 48.

Results of several control antibodies are also presented in Table I. The presence of osteoclasts and preservation of antigens within cells of tibia fixed in mss or MIK fixative was confirmed using anti–ED-1 mAb. ED-1 antigen is an uncharacterized cytoplasmic molecule expressed at high level in osteoclasts (Sminia and Dijkstra, 1986), monocytes, and tissue macrophages (Dijkstra et al., 1985). Immunostaining was not observed in osteoclasts when a control mAb (HHF-35) of the same subclass IgG_1 was used, or when sections were incubated in the absence of mAb (data not shown).

Unstimulated and fMLP-stimulated peritoneal neutrophil and macrophage isolates from rats were prepared according to the procedure of Verhoeven et al. (1989) and used in immunocytochemical studies to confirm reactivity of mAbs 449 and 48, and anti-p47 antisera. As previously reported, immunostaining with mAbs 449 and 48 was observed in intracellular granules and plasma membranes of unstimulated phagocytic leukocytes (data not shown). Stimulation with 1 μM fMLP resulted in a more concentrated distribution of staining in the plasma membrane in response to both mAbs (Verhoeven et al., 1989). Staining with anti-p47 antisera was

more diffuse, and very little change in staining was observed when the cells were stimulated with fMLP.

Superoxide Production by Osteoclasts In Situ in Freshly Isolated Tibia from 1-5-d-Old Rats

A specific histochemical method for the localization of superoxide production by cells (Briggs et al., 1986; Babbs et al., 1991a,b; Steinbeck et al., 1993) was used to determine if superoxide is actively being produced by osteoclasts in situ. Table II summarizes the results of the DAB-Mn²⁺ histochemical studies and several histochemical reactions are shown in Fig. 2, *D-F.* Osteoclasts within freshly isolated tibia incubated in DAB-Mn²⁺ histochemical medium contained oxidized deposits of DAB which were localized to the ruffled border and bone interfaces and to vesicles within these cells (Fig. 2 *D*). Greater than 95% of the osteoclasts contained oxidized deposits of DAB, and all of the cells were intensely stained as shown in Fig. 2 *D*. These results indicate that osteoclasts attached to bone surfaces are actively producing superoxide.

When excess SOD (400 U/ml) was added to deplete O_2^-, there was a dramatic decrease in the oxidation of DAB-Mn²⁺ by superoxide in most osteoclasts (Fig. 2 *E*, *arrows*). Decreases in oxidized DAB-Mn²⁺ deposits in osteoclasts more tightly attached to the bone surface (Fig. 2 *E*, *arrowheads*) was not complete. We did not observe a change in the oxidation of DAB-Mn²⁺ when desferal-Mn³⁺ (250–500 μM), a superoxide dismutase mimic, or desferal (500 μM) was added (Table II). However, a relatively short 15–30-min preincubation period with the various inhibitors before histochemical analyses was necessary in our studies to ensure that osteoclasts remained viable and actively producing superoxide. A short preincubation also eliminated the need to add an exogenous stimulator of bone resorption to stimulate superoxide production, and allowed us to measure the active production of superoxide in situ.

Oxidation of DAB-Mn²⁺ by superoxide was not inhibited by the addition of 0.15 mg/ml catalase which removes hydrogen peroxide and would block the formation of hydroxyl radicals, 10 mM 3-amino-1,2,4-triazole, an inhibitor of peroxidatic activity, or 1 mM KCN, a potent inhibitor of cytochrome c oxidase (Table II). KCN did not inhibit the oxi-
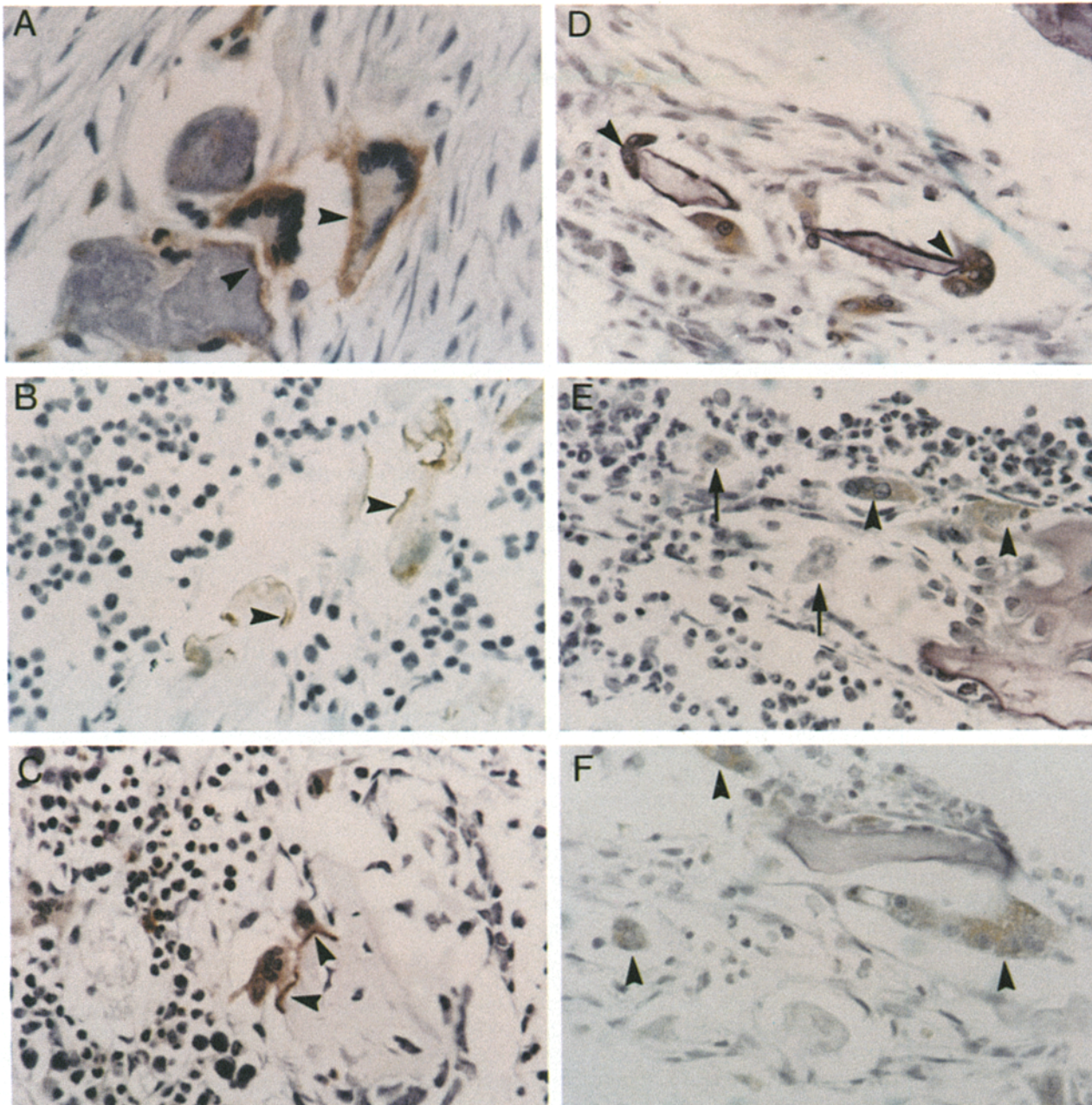
Figure 2. Immunohistochemical studies to determine the expression of cytochrome b_{558} of NADPH–oxidase in osteoclasts; performed on paraffin sections of calluses from healing tibial fractures of 28–35-d-old rats and tibia from 1–5-d-old rats. Sections of day 14 callus incubated with: (*A*) mAb 449, recognizing an epitope on the α-subunit of cytochrome b_{558} of NADPH–oxidase. Section of 1–5-d-old rat tibia incubated with: (*B*) mAb 48, recognizing an epitope on the β subunit of cytochrome b_{558} or (*C*) callus incubated with mAb 48. Using standard indirect avidin-biotin techniques, oxidized-DAB deposits of immunoperoxidase products were observed in osteoclasts attached to bone surfaces of day 14 calluses or 1–5-d-old rat tibia immunostained with mAbs 449 and 48. In a number of adherent osteoclasts these subunits were localized to the ruffled borders (*A–C, arrowheads*). Immunostaining with mAbs 449 and 48 was also observed in mature polymorphonuclear leukocytes and a few monocytes in bone marrow regions of tibia (*C*).

Freshly isolated tibia from 1–5-d-old rats were incubated in DAB-Mn^{2+} histochemical medium to establish the production of superoxide by osteoclasts in situ. Tibia halves were preincubated in 0.1 M Hepes–NaOH buffer with 5% sucrose, pH 7.2, containing 10 mM sodium azide, SOD, or KCN for 15–30 min at 25°C, then incubated for 30 min at 37°C in the following appropriate media: (*D*) DAB-Mn^{2+} medium containing sodium azide, an inhibitor of endogenous peroxidase or (*E*) DAB-Mn^{2+} medium plus SOD (400 U/ml) which was added to deplete extracellular O_2^-. Osteoclasts within tibia incubated in DAB-Mn^{2+} histochemical medium contained oxidized deposits of DAB, whereas, unstimulated leukocytes in the bone marrow regions did not contain deposits. Addition of SOD resulted in a dramatic, but variable, decrease (*E, arrowheads*) or inhibition (*E, arrows*) in the oxidation of DAB-Mn^{2+} by superoxide in many osteoclasts. In the absence of Mn^{2+}, osteoclasts also contained DAB deposits (*F, arrowheads*), but the formation of these deposits was completely inhibited by the addition of 1 mM KCN, a potent inhibitor of cytochrome c oxidase activity.
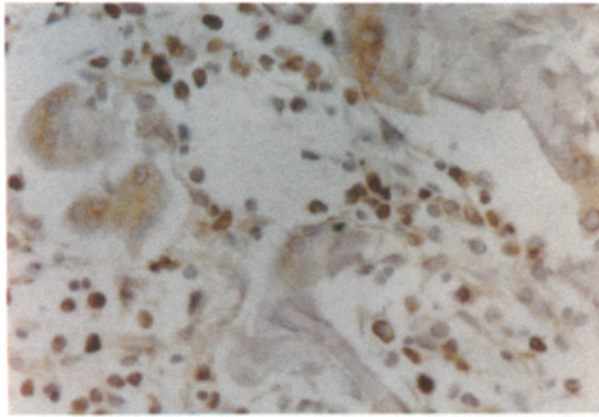
Figure 3. Immunohistochemical studies were performed on paraffin sections of 1-5-d-old rat tibia to determine the expression in osteoclasts of p47-phox, a cytosolic component of NADPH-oxidase. Section of 1-5-d-old rat tibia incubated with antiserum recognizing an epitope on p47-phox. Using standard indirect avidin-biotin techniques, oxidized-DAB deposits of immunoperoxidase products were observed in osteoclasts attached to bone surfaces of 1-5-d-old rat tibia. Immunostaining was also observed in mature polymorphonuclear leukocytes and a few monocytes in bone marrow regions of tibia.

dation of DAB-Mn^{2+} by superoxide, but variably reduced the amount of DAB deposit formation observed in many osteoclasts by eliminating the contribution of cytochrome c oxidase in DAB deposit formation in these cells (e.g., see Fig. 2 F and section below). These findings are consistent with the production of superoxide, and the subsequent oxidation of DAB by Mn^{3+}.

Unstimulated mature polymorphonuclear leukocytes in the bone marrow regions of freshly isolated tibia did not contain DAB deposits (Fig. 2, D-F). Treatment of the tibia with 4β-phorbol-12-myristate-13-acetate (150 ng/ml), a known activator of NADPH-oxidase in phagocytic leukocytes, resulted in DAB deposit formation in leukocytes and a small increase in deposit formation in some osteoclasts (data not shown). This was not unexpected since production of superoxide by cells containing the NADPH-oxidase requires

cell activation and assembly of cytochrome b_{558} with cytosolic components to form a functional NADPH-oxidase system (for review see Segal and Abo, 1993).

Cytochrome c Oxidase Activity as an Indicator of Active Mitochondrial Respiration

DAB without Mn^{2+} was originally used as a histochemical marker for the localization of peroxidase (Graham and Karnovsky, 1966), and then later for the localization of cytochrome c oxidase activity (Seligman et al., 1968). Table II summarizes the results of the DAB histochemical studies on freshly isolated tibia, and Fig. 2 F shows the amount of DAB deposits observed in osteoclasts. Under conditions of no Mn^{2+}, formation of oxidized deposits of DAB in these cells was completely inhibited by the addition of 1 mM KCN. No change in DAB deposit formation in osteoclasts was observed when 10 mM 3-amino-1,2,4-triazole or 10 mM sodium azide was added to the histochemical media. These compounds inhibit to varying degrees endogenous peroxidatic activity. Combined, the inhibitor studies strongly suggest that cytochrome c oxidase activity and not peroxidatic activity is responsible for the observed oxidation of DAB in the absence of Mn^{2+}.

Discussion

This study is the first to identify the enzyme involved in the production of superoxide by osteoclasts as NADPH-oxidase, and demonstrated the production of superoxide by osteoclasts in situ. NADPH-oxidase is a well known superoxide-generating enzyme expressed by phagocytic leukocytes. As in phagocytic leukocytes, the cytochrome b_{558} components in osteoclasts were found in both intracellular vesicles and plasma membranes (Verhoeven et al., 1989). In osteoclasts attached to bone surfaces in tibia from 1-5-d-old rats and in calluses from healing tibial fractures, these subunits were concentrated at the ruffled border regions.

It was perhaps not surprising that NADPH-oxidase is expressed in osteoclasts, since a proposed myelomonocytic precursor is common to both phagocytes and osteoclasts (Schneider and Relfson, 1988; Hagennaars et al., 1989), and functional similarities between these cells have been reported (Khan et al., 1978; Teitelbaum et al., 1979). How-

Table II. Summary of DAB-Mn^{2+} and DAB Histochemistry of 1-5-d-old Rat Tibia

DAB-Mn^{2+} or DAB histochemical media ± various inhibitors*	Observable DAB-deposits in cells of 1-5-d-old rat tibia
DAB [2.5 mM] + Mn^{2+} [1 mM]	Strongly positive osteoclasts (4$^+$)
+ SOD [400 U/ml]	Significant decrease, but variable (0-2$^+$)
+ catalase [0.15 mg/ml]	No effect
+ KCN [1 mM]	Some reduction due to loss of cytochrome c oxidase activity (3-4$^+$)
+ 3-amino-1,2,4-triazole [10 mM]	No effect
+ desferal [500 M]	No effect
+ desferal-Mn^{3+} [250 μM or 500 μM]	No effect
DAB only [2.5 mM]	Positive osteoclasts (2$^+$)
+ KCN [1 mM]	Complete inhibition
+ 3-amino-1,2,4-triazole [10 mM]	No effect

* Freshly isolated tibia were preincubated in 0.1 M Hepes–NaOH buffer with 5% sucrose, pH 7.2 containing 10 mM sodium azide ± SOD, 3-amino-1,2,4-triazole, desferal, or desferal-Mn^{3+} for 15–30 min at room temperature. Other samples were preincubated in histochemical buffer without sodium azide containing catalase or KCN. Tibia were then incubated for 30 min at 37°C in the various histochemical media listed above.

ever, differentiation of phagocytes and osteoclasts proceeds along divergent paths and the phenotypes and enzyme contents of these cells are known to differ (reviewed in Gay, 1992).

Cytochrome b_{558} has two subunits, a 22–23-kD protein (α subunit) and a 75–90-kD glycoprotein (β subunit) (Parkos et al., 1987; Segal, 1987). Two monoclonal antibodies raised against these subunits (Verhoeven et al., 1989) were used in the present immunohistochemical studies to establish the presence of NADPH–oxidase in osteoclasts, mAb 48 which recognizes an epitope of the β subunit, and mAb 449 which recognizes an epitope on the cytoplasmic surface of the α subunit. These antibodies are specific for cytochrome b_{558} and do not cross-react with other known heme-binding proteins, such as the subunits of cytochrome c oxidase or cytochrome P-450 (Verhoeven et al., 1989). In addition to mAbs 449 and 48, an antisera specific for p47-phox, one of the cytosolic components of the NADPH–oxidase, demonstrated the expression of this component of NADPH–oxidase in osteoclasts.

In addition to identifying an enzyme in osteoclasts capable of producing superoxide, we used freshly isolated tibia from 1-5-d-old rats to establish that superoxide is being produced by osteoclasts during the process of bone resorption. DAB-Mn^{2+} histochemistry, which specifically detects the production of superoxide and not other ROS (Briggs et al., 1986; Steinbeck et al., 1993), was used to localize superoxide production to osteoclasts in freshly isolated tibia. Under these conditions, oxidation of DAB-Mn^{2+} by superoxide was visible as oxidized deposits of DAB, strongly suggesting that osteoclasts in situ are already stimulated and actively producing superoxide when attached to bone surfaces.

In contrast to our approach, others have primarily investigated the production of superoxide by osteoclasts in tibial or calvarial cultures in response to exogenous stimulators of bone resorption (Garrett et al., 1990; Key et al., 1990; Ries et al., 1992). In addition, most of these studies relied on the use of NBT as a histochemical marker for superoxide production. Superoxide is not the only reactive agent that can reduce NBT to formazan (for review see Stoward et al., 1991), and the usefulness of this method to detect superoxide relies on the use of inhibitors to deplete superoxide. These inhibitors include SOD, an enzyme that catalyzes the conversion of superoxide to hydrogen peroxide and ground state molecular oxygen (McCord and Fridovich, 1969), and more recently, desferal-Mn^{3+}, a low-molecular weight superoxide dismutase mimic originally produced by Beyer and Fridovich (1989).

SOD has been reported to be both effective (Garrett et al., 1990) or marginally effective in inhibiting the reduction of NBT to formazan by osteoclasts in bone cultures (Ries et al., 1992). This discrepancy may be due to residual cytochrome c oxidase activity, suggested by the variable reduction in DAB-Mn^{2+} oxidation by KCN, or the fact that SOD inhibits the extracellular reduction of NBT by isolated rat osteoclasts, but the high molecular mass (40 kD) of SOD largely prevents its uptake by cells and, in particular, its diffusion into the space between the osteoclast and bone surface. In the present study, addition of excess SOD (400 U/ml) to deplete superoxide resulted in a significant decrease in the oxidation of DAB-Mn^{2+}, but in agreement with Ries et al. (1992) SOD was least effective in osteoclasts which appeared to be tightly adhered to bone surfaces.

Under the conditions used by Ries et al. (1992), involvement of O_2^- or one of its metabolites in the reduction of NBT was strongly supported by addition of desferal-Mn^{3+}. In their study, desferal-Mn^{3+} was added to the NBT histochemical media and to tibia that were maintained in culture for 48 h before histochemical testing. The long-term incubation with desferal-Mn^{3+} apparently allowed for its diffusion into the spaces between adherent osteoclasts and the bone surface, inhibiting the reduction of NBT by superoxide. We did not observe a change in oxidation of DAB-Mn^{2+} when desferal-Mn^{3+} was added to freshly isolated tibia. The ineffectiveness of desferal-Mn^{3+} in our studies could be due to either the use of desferal-Mn^{3+} to deplete superoxide in conjunction with a manganese-based detection system, and/or the relatively short period of preincubation with the various inhibitors before histochemical analyses. The relatively short incubation, as previously mentioned, was necessary in our studies to ensure that osteoclasts remained viable, allowing us to determine if osteoclasts were actively producing superoxide in situ.

The activity of cytochrome c oxidase was also investigated, since osteoclasts contain a large number of mitochondria and the activity of cytochrome c oxidase in osteoclasts has been reported to increase when these cells are actively resorbing bone (Noda et al., 1985; Yamamoto et al., 1991). To localize cytochrome c oxidase activity, the original DAB method without Mn^{2+} was used (Seligman et al., 1968). Multinucleated osteoclasts within the tibia contained intracellular deposits of oxidized DAB in the absence of Mn^{2+}. This oxidation, but not the oxidation of DAB-Mn^{2+} by superoxide, was inhibited by addition of 1 mM KCN. Oxidation of DAB by cytochrome c in the cytochrome c oxidase pathway involves a direct electron transfer and is not dependent upon the production of superoxide or other ROS, and KCN inhibits the oxidation of DAB by blocking the final electron transfer from cytochrome a_3 to oxygen, thus preventing further electron transfer through cytochrome c. Due to the relatively high activity of the mitochondria, it is not unlikely that in the presence of SOD some of the residual oxidized DAB can be contributed to cytochrome c oxidase.

DAB without Mn^{2+} was originally used as a histochemical marker for the localization of peroxidase activity (Graham and Karnovsky, 1966). The involvement of a peroxidase in the formation of DAB deposits in our study seems unlikely, since the addition of known inhibitors of peroxidatic activity to the histochemical media, 10 mM sodium azide or 3-amino-1,2,4-triazole was without effect, and an extensive study by Zheng et al. (1991) has indicated that rat osteoclasts, unlike phagocytic cells, do not contain endogenous peroxidatic activity.

In summary, this study detected the expression of an enzyme in osteoclasts capable of generating superoxide. Our findings indicate that osteoclasts express cytochrome b_{558}, a unique cytochrome that is part of the NADPH–oxidase electron transport system, and that cytochrome b_{558} is localized to the ruffled borders in these cells. In addition, osteoclasts expressed p47-phox, a cytosolic component of NADPH–oxidase, and produced superoxide in situ when attached to bone surfaces. The presence and location of the NADPH–oxidase components and the production of superoxide by osteoclasts attached to bone surfaces strongly support the involvement of NADPH–oxidase and ROS in the process of bone resorption by osteoclasts.

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