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Evidence for an Immunological and Functional Relationship Between Superoxide Dismutase and a High Molecular Weight Osteoclast Plasma Membrane Glycoprotein

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Abstract Large multinucleated osteoclasts are the major cells responsible for bone breakdown and have been reported to produce high levels of superoxides which may contribute to the process of bone resorption (Key et al.: *J Bone and Mineral Res* 4 [suppl. 1]:S206, 1989). Osteoclasts also possess high levels of superoxide dismutase, a protective enzyme capable of converting toxic superoxides to less toxic H_2O_2 (Fridovich: *J Biol Chem* 264:7761–7764, 1989). The amino acid sequence of manganese and/or iron superoxide dismutase has a conserved region which exhibits substantial homology with a fragment obtained from a high molecular weight osteoclast surface marker glycoprotein which is reactive with monoclonal antibody 121F. In this report, evidence is presented substantiating immunological, biochemical, and functional similarities between the osteoclast membrane antigen recognized by the 121F monoclonal antibody and superoxide dismutase. Western blot and immunoprecipitation studies show that a monospecific polyclonal antibody generated against immunoaffinity purified antigen is cross-reactive with superoxide dismutase. Both the antigen and a high molecular weight superoxide dismutase activity have been detected in osteoclast plasma membrane preparations. The levels of superoxide dismutase activity and the membrane antigen have been found to correlate in antigen depletion studies and in western blots probing osteoclasts and closely related marrow-derived giant cells. Moreover, regions of osteoclast superoxide dismutase activity identified by electrophoretic zymogram analysis have been shown by gel electrophoresis and western blots to contain the high molecular weight antigen, or complexes of the antigen with the 121F monoclonal antibody when these were premixed prior to nondenaturing electrophoresis. It is proposed that the osteoclast plasma membrane possesses a high molecular weight superoxide dismutase activity. Furthermore, it appears that this activity is associated with the osteoclast antigen recognized by the 121F monoclonal antibody.

Key words: bone resorption, osteoclast, superoxide dismutase

Osteoclasts are large multinucleated bone-resorbing cells which are related to cells of the mononuclear phagocyte lineage [1]. Osteoclasts on bone establish a tightly sealed extracellular lacuna, or external lysosome, into which protons and degradative enzymes are released to accomplish the removal of the organic and inorganic components of bone matrix [2]. These multinucleated cells have also been reported to produce and release significant levels of superoxides at the matrix-cell interface during active osteoclast-mediated resorption [3,4]. Key et al. [4] have hypothesized that these superoxides may be either directly involved in the resorption process, or else, generated as a by-product of resorptive activity. Furthermore, superoxide levels may be augmented by the high level of aerobic activity associated with the many mitochondria present in osteoclasts [2]. Oxygen-derived free radical influences on osteoclast-mediated bone resorption have been examined by Garrett et al. [5]. Although the generation of moderate levels of free radicals stimulated bone resorption, production of high levels conversely inhibited this activity. Therefore, intermediate levels of osteoclast-generated superoxide radicals may enhance the bone resorption process. However, overproduction of these reactive radicals diminishes bone resorption and can lead to potential cell damage,

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unless quickly neutralized by superoxide dismutase. Fortunately, osteoclasts possess high levels of superoxide dismutase [6]. The well-documented function of superoxide dismutase is to rapidly convert toxic superoxides to less toxic H_2O_2, which is then further cleaved by catalase into H_2O and O_2 [7,8].

During the course of studies revolving around the identification and characterization of an osteoclast surface protein, amino acid sequence data was obtained which suggested that there was a relationship between the osteoclast membrane component and superoxide dismutase. This osteoclast marker was originally identified by its reaction with one member, 121F, of a monoclonal antibody library raised against isolated chicken osteoclasts [9]. The antigen is strongly expressed on avian osteoclasts but not observed by immunohistochemical analysis on other cells within a variety of tissues, including bone [9]. Closely related marrow-derived giant cells do not demonstrate detectable levels of this 121F antibody-reactive antigen unless stimulated by exposure to osteoblast conditioned media prior to their fusion in vitro from marrow mononuclear precursors [10,11]. Induced giant cells display antigen levels intermediate between those of unstimulated giant cells and isolated mature osteoclasts. Webber et al. [12] have demonstrated a direct correlation between the level of the antigen recognized by the 121F monoclonal antibody and the presence of the characteristic osteoclast ruffled border on giant cells formed in response to resorbable substrates. Together, these observations suggest a direct relationship between the ability of multinucleated cells to carry out extracellular resorption and the expression of the plasma membrane glycoprotein recognized by the 121F monoclonal antibody.

Monoclonal antibody 121F has been used to identify and purify the osteoclast plasma membrane-associated glycoprotein. A 30 kDa hydroxylamine fragment derived from the 150 kDa disulfide reduced form of the purified antigen has been found to contain a region of significant amino acid sequence similarity to manganese/iron superoxide dismutase [13]. This finding prompted further investigations of the potential relationship between the osteoclast membrane glycoprotein and superoxide dismutase. Since the techniques currently employed to purify the osteoclast membrane antigen are not conducive to retaining superoxide dismutase activity, direct measurement of such antigen-associated activity was not possible and so, a series of correlative studies were performed. In addition to demonstrating significant immunological, biochemical, and functional similarities between the osteoclast membrane glycoprotein and both bacterial and human superoxide dismutase, the studies reported here describe the existence of a high molecular weight osteoclast plasma membrane-associated superoxide dismutase (SOD) activity. The prime candidate for the identity of this SOD appears to be the 121F MAb-reactive osteoclast membrane antigen.

MATERIALS AND METHODS

Cell Isolation and Culture

For each preparation, osteoclasts were isolated by a modification of the method cited by Oursler et al. [9,13] from the tibias and humeri of 15 white leghorn chick hatchlings maintained on a low calcium ($< 0.1\%$ calcium) diet (Purina) for a minimum of 28 days. The pelleted cells, obtained from the top layer and interface of the 35% Percoll gradient, were either used immediately or stored frozen at $-70°C$.

Marrow mononuclear cells were obtained from the flushed marrow cavities of 15 chicks for each preparation as described by Oursler et al. [9,13]. Ficoll-Paque (Pharmacia) separated marrow mononuclear cells were plated at 50×10^6 cells per 100 mm tissue culture dish (Falcon) in alpha-minimal essential medium (alpha-MEM), containing 10% fetal calf serum (FCS, GIBCO) and 1% antibiotic-antimycotic (GIBCO). Cultures were maintained at 37°C in a 95% air/5% CO_2 gas environment. Media was replaced every other day, and by the second to fourth day of culture, cells had begun to fuse to form marrow-derived multinucleated giant cells. For antigen induction, marrow cells were fed on day 3 and every other day thereafter with media containing 25% UMR 106 conditioned media [10,11]. Cells from day 12 cultures, which were typically more than 80% confluent with giant cells, were rinsed several times with warm Hanks' balanced salt solution, pH 7.4 (HBSS, GIBCO), scraped off dishes using a rubber policeman, centrifuged 5 min at 1,180*g*, and stored as a pellet at $-70°C$.

Immunolocalization

Immunohistochemical localization of the antigen recognized by the 121F monoclonal antibody was carried out as previously described [9].

For electron microscopic immunocytochemistry, chicken bones were harvested from animals maintained on a low calcium diet for 4 weeks and prepared by the immunocytochemical localization method described by Brown and Farquhar [14], as modified by Baron et al. [15]. Briefly, bones were fixed in periodate-lysine-paraformaldehyde fixative (PLP) for 4 h at 4°C, incubated for 15 min in 50 mM NH_4Cl in phosphate buffered saline (PBS), then placed in blocking solution consisting of 1% bovine serum albumin (BSA) and 10% calf serum (GIBCO) in PBS for an additional 15 min. Following a 90 min incubation in 10% dimethyl sulfoxide in PBS, samples were frozen in liquid nitrogen, and 30 μm sections were cut on a cryostat. Sections were collected in PBS containing filtered 1% ovalbumin, fixed for two additional hours in PLP fixative, and incubated for 15 min with 50 mM NH_4Cl in PBS, then for 15 min in blocking solution. Sections were incubated overnight at room temperature in blocking solution with or without 121F antibody ascitic fluid (1:100 dilution in blocking solution). After rinsing 5 times for 15 min with blocking solution, sections were incubated for 2 h at room temperature with a 1:50 dilution in blocking solution of peroxidase conjugated biotinylated goat anti-mouse IgG (Vector Laboratories), rinsed 6 times for 15 min each with blocking solution, then further incubated and processed as described by Brown and Farquhar using 0.2% diaminobenzidine as the peroxidase substrate [14]. Samples were postfixed in 1% osmium tetroxide containing 1% potassium ferrocyanide in 0.1 M cacodylate buffer, pH 7.4. After rinsing samples in cacodylate buffer, sections were dehydrated in an alcohol series (50–100%) containing 0.5% uranyl acetate, embedded in Epon, and 600 μm sections were prepared and viewed on a Philips 300 electron microscope.

Membrane Association Assays

Triton X-114 (TX-114) (Sigma Chemical Co.) phase partitioning of integral membrane proteins from osteoclast extracts was carried out by the method of Malik and Low [16]. The fractions were fixed by addition of 1/100 volume of 25% aqueous glutaraldehyde (Sigma Chemical Co.), dried onto a 96-well poly-L-lysine precoated dish, and assayed for the antigen recognized by the 121F monoclonal antibody (MAb) using an enzyme-linked immunosorbent assay (ELISA), as described in Oursler et al. [9]. Results are presented as a percentage of the antigen in one fraction relative to the total antigen levels determined by the sum of the two fractions. In separate studies, high pH washing of osteoclast membranes was conducted to remove peripherally associated proteins by the method of Salas et al. [17]. The pH treated membranes were glutaraldehyde fixed as above, dried onto poly-L-lysine precoated 96-well ELISA dishes, and assayed for the antigen by ELISA [9,13]. In a third study to assess antigen distribution, isolated osteoclasts suspended in Hanks' balanced salt solution, pH 7.4 (HBSS, GIBCO) were dispensed into 96-well culture dishes, spun at 50*g* for 5 min, fixed by addition of a 3-fold excess of 1% paraformaldehyde in PBS for 15 minutes, and rapidly rinsed with −20°C methanol to permeabilize the cells. After rinsing with chilled PBS, the permeabilized cells were analyzed for the antigen by ELISA [9,13]. ELISA results were expressed as relative absorbance at 405 nm. For the methanol permeabilized cell experiment, sample protein content was determined [18], and the results were expressed as absorbance at 405 nm per mg protein. Indicated errors are standard deviations from the mean.

Plasma Membrane Isolation

Approximately 1×10^6 isolated avian osteoclasts (as determined by hemocytometer counting) were suspended in 2 ml of a 1.06 g/ml Percoll solution containing 0.25 M sucrose and 1 mM phenyl-methylsulfonyl fluoride (PMSF). Cells were homogenized by 12 strokes in a dounce homogenizer, the volume was raised to 12 ml with additional Percoll/sucrose/PMSF solution, and a self-generating Percoll gradient was formed by centrifugation at 40,000*g* for 60 min at 4°C. The resultant Percoll density gradient was determined using marker beads supplied by the manufacturer (LKB/Pharmacia). For comparison, chicken livers were also removed and 10 μg was processed as for osteoclasts. Osteoclast and liver plasma membrane Percoll density regions, as well as the density regions associated with mitochondria, were assayed for a mitochondrial membrane enzyme, rhodanese [19], to determine whether mitochondrial membranes were present in the plasma membrane fractions. Equivalent portions of each fraction were analyzed by ELISA for 121F MAb-reactive antigen. Each density fraction was also analyzed for superoxide dismutase activity using the zymogram method of Austen et al. [20].

Antigen-Affinity Purified Polyclonal Antibodies

Polyclonal antibodies directed against the antigen which is recognized by the 121F MAb were obtained by monthly subcutaneous injection of immunoaffinity purified antigen [13] into a New Zealand white rabbit. After seven injections of antigen, the animal was bled. Subsequent antibody was obtained by monthly boosts of antigen and bleeding. Monospecific polyclonal antibodies for the 121F MAb-reactive antigen were obtained by coupling the antigen to Sepharose CL-6B by the method outlined by Mishell and Shiigi [21], binding the polyclonal antibody to this affinity column, and eluting the antigen-specific polyclonal antibody.

Immunoprecipitation and Analysis

Superoxide dismutases (SODs) derived from *Barillas stearothermophilus, Escherichia coli,* and human mitochondria were purchased from Sigma Chemical Co. (St. Louis, MO). Purified SODs were iodinated using the Pierce Iodogen method [22] and rapidly desalted through minicolumns of BioGel P6DG (BioRad) to remove unincorporated label [23]. MAb 121F, antigen affinity-specific polyclonal antibody, and a less-specific macrophage and osteoclast-reactive monoclonal antibody, designated 29C, were separately used in the following immunoprecipitation schemes. One microgram of antibody and 300 ng of protein A (Sigma Chemical Co.) were incubated in PBS overnight at 4°C with 10 μg of each of the purchased SODs described above. Control samples received 1 μg of preimmune sera and 300 ng of protein A. After an overnight incubation at 4°C, the samples were microcentrifuged at 10,000*g* for 30 min, and the pellets were washed 3 times by resuspension in PBS and recentrifuged. Immunoprecipitates were counted on a Packard gamma counter. In separate, but related studies, these SODs were also analyzed by ELISA for 121F MAb binding [9]. Each SOD (6 μg) was diluted in 700 μl of PBS, 7 μl of 25% aqueous glutaraldehyde was added, and 100 μl was plated per well of a poly-L-lysine precoated ELISA dish, dried and analyzed.

SDS-PAGE and Western Blot Analysis of the Antigen in Superoxide Dismutase Activity Regions

Osteoclast SOD activity was localized after gel electrophoresis in 5% polyacrylamide gels run under non-denaturing conditions [24] and zymogram detection in the presence of 1.0 mM cyanide to eliminate Cu/Zn SOD activity [20]. In each experiment, ¼ of an isolated osteoclast preparation was used for each lane. A separate, but identical, electrophoresis lane was reserved at 4°C until the zymogram was developed, then the region corresponding to osteoclast SOD activity was excised from the reserved lane, split into two equal pieces, boiled in SDS-PAGE sample buffer and run on reducing SDS-PAGE [25]. An SDS-PAGE lane from half of the sample was analyzed by silver staining [26]. The remaining gel sample lane, and a companion lane containing purified antigen, were analyzed by western blot [13] using a 1:100 dilution of the monospecific polyclonal antibody.

Size Partitioning

Chicken osteoclasts and liver tissue were extracted in 0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, 0.05% CHAPS with 0.03% soybean trypsin inhibitor, 0.1 M epsilon-amino-N-caproic acid, 0.002 M PMSF, 0.4 trypsin inhibitor units/ml aprotinin, and 10 μg/ml each leupeptin, chymostatin, antipain, and pepstatin (all these reagents were obtained from Sigma Chemical Co.), pH 7.4 (CHAPS extraction solution). Extracts were centrifuged at 10,000*g* for 5 min at 4°C. Protein equivalent to 1.000 absorbance unit at 280 nm was used for each chicken osteoclast and liver sample. Extracts were separated by centrifugal ultrafiltration using an Amicon Centricon microconcentrator with a 100 kDa cut-off membrane. The flow-through from this membrane was centrifuged through an Amicon Centricon microconcentrator with a 10 kDa cut-off membrane. As an additional control, 1 μl (1 μg) human SOD (Sigma) dissolved in 1 ml of chicken serum was treated similarly. Residual volumes retained above the membranes were washed by the addition of two volumes of extraction solution midway through the centrifugation. Samples were then analyzed for antigen reactive with the 121F MAb by ELISA [9], and the results expressed as a percentage of the total activity. Alternatively, samples were electrophoresed on non-denaturing gels [24] and processed for zymogram analysis.

Antibody Localization in Zymogram

Cell extracts, each from 1×10^6 osteoclasts, were mixed with 121F MAb (1 μg MAb/extract sample) prior to loading on non-denaturing gels. Zymogram (SOD) analysis was carried out as

described earlier [20], and regions of the gel to be assayed for antibody content were excised, boiled in gel sample buffer, subjected to SDS-PAGE [25], and western blotted as outlined previously [13]. Blots were blocked for 1 h with 5% non-fat dry milk in PBS, probed with ^{125}I-labeled goat anti-mouse IgG (Organon Teknika-Cappel) in PBS at 4°C for 16 h, washed repeatedly with PBS containing 0.05% Tween 20 (Sigma Chemical Co.), and dried. The presence of the 121F MAb was indirectly detected by autoradiography.

Antigen Depletion Studies

Osteoclast CHAPS extracts from ½ of an isolated osteoclast cell preparation containing proteins larger than 100 kDa were obtained from centrifugal ultrafiltration as described above, then mixed with 5 ml of either 121F MAb or 29C MAb immunoaffinity matrices at 4°C for 4 h as described in Oursler et al. [13]. The unbound material from this step was again mixed for 4 h with an additional 5 ml batch of either the 121F MAb or 29C MAb immunoaffinity matrices. The antigen-depleted unbound fractions from these preparations were concentrated using a 100 kDa cut-off centrifugal ultrafiltration membrane, electrophoresed on non-denaturing gels, and analyzed by zymogram for SOD activity [20].

RESULTS

Antigen Membrane Localization

Prior to these studies, immunohistochemical and ELISA analyses of the osteoclast (OC) antigen recognized by monoclonal antibody (MAb) 121F indicated that the antigen was likely to be a plasma membrane protein [9,13]. In light of a possible relationship between superoxide dismutase (SOD) and this antigen which was based on shared amino acid sequence similarities, and the fact that SOD is not typically a membrane-associated enzyme, the association of the antigen with the osteoclast membrane was examined more closely by immunolocalization on sections of bone at both the light and electron microscopic levels (Fig. 1). Panels A (phase) and B (fluorescence) demonstrate that the antigen appeared to be restricted primarily to the plasma membrane of osteoclasts, with little intracellular staining. Furthermore, electron microscopic (EM) immunocytochemistry (panel C) also indicated that the antigen was localized on the osteoclast surface plasma membrane. In assessing antigen distribution by transmission EM in six separate trials, the majority of multinucleated osteoclasts displayed membrane-bound antigen. Some osteoclast samples did not, perhaps as a result of viewing sections cut from a region originally inaccessible to the immunocytochemical reagents. Examination of adjacent marrow regions which contained other cell types consistently failed to show any detectable antigen levels on mononuclear cell membranes, consistent with earlier findings [9].

Intracellular localization of the osteoclast antigen recognized by the 121F MAb was also investigated by ELISA analysis on intact (63.6 ± 19) or methanol-permeabilized (46.6 ± 5) osteoclasts and did not reveal an intracellular pool of the antigen. TX-114 detergent phase partitioning of integral membrane proteins, high pH membrane washing to release peripherally associated proteins, and subcellular membrane fractionation of osteoclasts were therefore employed to further explore whether, and in what fashion, the antigen was associated with the plasma membrane. The majority of the 121F MAb-reactive antigen monitored by ELISA remained membrane-bound after high pH washing of osteoclasts, which was consistent with the antigen being an integral membrane protein (Table I). In contrast, ELISA analysis of the 121F MAb-reactive antigen in the aqueous and TX-114 phases, from partitioning of either whole cell extracts or purified antigen, demonstrated that the antigen was preferentially associated with the aqueous phase (Table I). However, the failure of this antigen to partition with TX-114 during detergent phase separation is not inconsistent with a possible membrane association, since not all membrane proteins partition with TX-114 [27]. Lastly, ELISA analysis of density gradient subcellular fractionations of osteoclast cell homogenates gave the highest detection of 121F MAb-reactive antigen in the density region corresponding to the plasma membrane (Fig. 2). Therefore, by several independent measures, the osteoclast antigen was shown to be associated with the osteoclast plasma membrane.

Antigen and SOD Immunological Relationship

The high degree of amino acid homology between a portion of the osteoclast membrane antigen and SOD suggested that there might be shared immunological features between these proteins which could be detected using antibod-

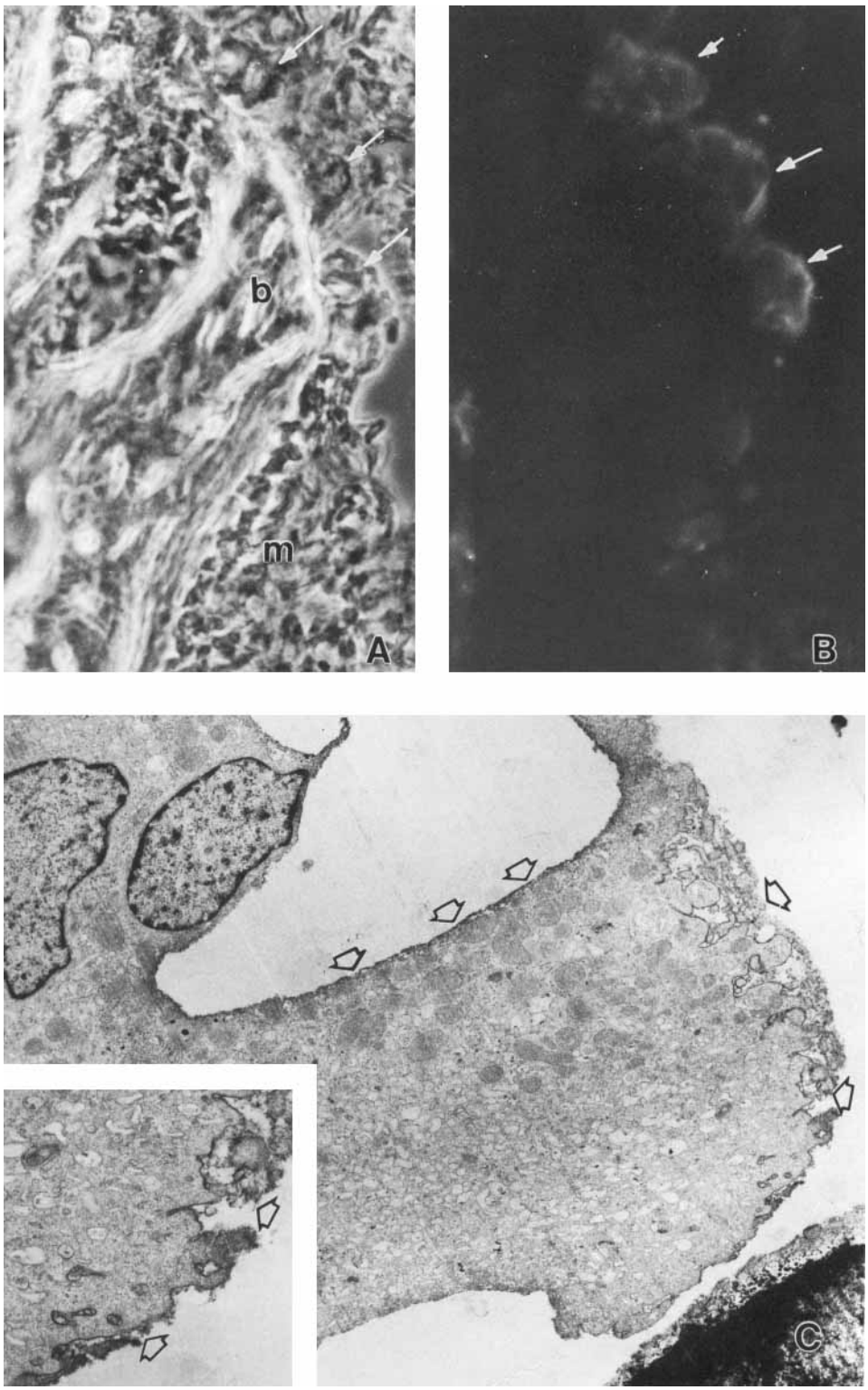


Fig. 1. Immunohistochemical localization of the 121F MAb-reactive antigen in bone. **A:** Phase micrograph of a frozen section of chick tibia from animals maintained 4 weeks on a low calcium diet. Osteoclasts (arrows) are observed associated with bone matrix (b). Marrow (m) surrounds the bone. ×1,240. **B:** Fluorescent micrograph of the field shown in A, illustrating that localization of the antigen reactive with 121F MAb is restricted to the osteoclast plasma membrane (arrows). ×1,240. **C:** EM-immunocytochemical 121F MAb localization on bone. Bone sections were prepared for peroxidase EM localization of the antigen as given in Materials and Methods. Note that the antigen is primarily observed on the multinucleated osteoclast plasma membrane (arrows). ×6,213. **Insert:** A higher magnification of the 121F MAb immunoreactive osteoclast plasma membrane. ×19,920.

TABLE I. 121F MAb-Reactive Antigen Association With the Osteoclast Membrane*

Assay	Sample	Aqueous Phase (%)	Detergent Phase (%)
TX-114 Detergent phase	Osteoclast	62	38
	Purified 121F MAb-reactive antigen	89	1
		Membrane released (%)	Membrane associated (%)
High pH Membrane wash	Osteoclast	11	89

*The proportion of 121F MAb-reactive antigen in the TX-114 aqueous or detergent phases and in the high pH membrane wash released or membrane-associated fractions was determined by ELISA as described in Materials and Methods. Equivalent proportions of each fraction were analyzed. The percentage of antigen in each fraction was determined from the ELISA absorbance at 405 nm for that fraction, compared with the total absorbance for both fractions.

ies. In order to obtain antibodies directed against a spectrum of epitopes on the antigen recognized by the 121F MAb, monospecific polyclonal antibodies were generated. Western blot analysis using this monospecific polyclonal antibody on electrophoresed whole cell extracts from marrow-derived giant cells (MAGCs) and avian osteoclasts (Fig. 3) supported the previously reported observation that 121F MAb-reactive antigen levels in MAGCs were below the levels detectable with this technique [9,13]. The monospecific polyclonal antibody bound the 150 kDa disulfide-reduced antigen from osteoclasts as well as a fainter 20 kDa band, unlike the 121F MAb, which uniquely recognized the reduced 150 kDa band.

The low molecular weight band detectable only with the monospecific polyclonal antibody migrated at a size expected for a typical SOD subunit. To examine this possibility, human and *E. coli* Mn SODs, as well as *Bacillus stearothermophilus* Fe SOD, were iodinated and immunoprecipitated with either the 121F MAb or the monospecific polyclonal antibody (Table II). Independently, each SOD was tested by ELISA for reactivity with the 121F MAb. Both the immunoprecipitation and ELISA analyses of these SODs demonstrated that the 121F MAb did not recognize any of the three SODs tested. Most notably, the *B. stearothermophilus* SOD, which is the species sharing the closest amino acid sequence similarity with the osteoclast antigen partial sequence determination [13], did not react with the 121F MAb. In contrast, immunoprecipitation with the monospecific polyclonal antibody, raised against immunoaffinity purified 121F MAb-reactive antigen, was capable of recognizing all three of these SOD species. When the polyclonal antibody immunoprecipitates were solubilized and analyzed by SDS-PAGE and autoradiography, bands migrating at a size corre-

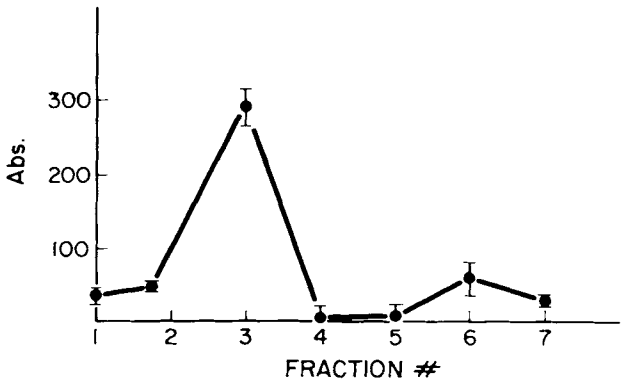


Fig. 2. Density gradient analysis for plasma membrane association of antigen. 121F MAb ELISA analysis of homogenized osteoclasts (1×10^6) which were fractionated on a Percoll density gradient as described in Materials and Methods. Equivalent aliquots of each fraction were fixed, plated on 96-well dishes and assayed for 121F MAb-reactive antigen. The relative ELISA absorbance at 405 nm of each fraction is plotted. A peak of detectable antigen is observed in the fraction corresponding to the plasma membrane buoyant density. Buoyant density (g/ml) of standard marker bead locations: Fraction 1 = lighter than 1.037; 2 = 1.037; 3 = between 1.037 and 1.049; 4 = between 1.049 and 1.054; 5 = between 1.054 and 1.067; 6 = between 1.067 and 1.081; 7 = heavier than 1.081.

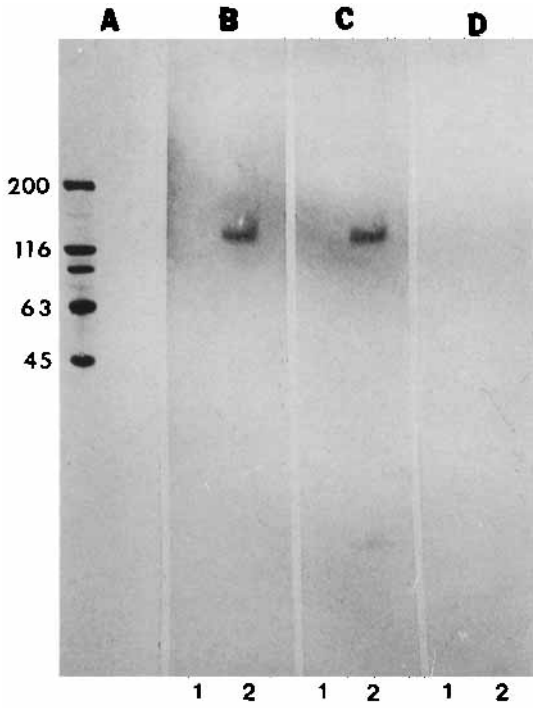


Fig. 3. Western blot analysis of osteoclasts and giant cells with monoclonal and polyclonal antibodies. Western blots (B–D) of 3×10^6 marrow-derived giant cells **(lane 1)** and 3×10^6 osteoclasts **(lane 2)**. **A:** Molecular weight markers (BioRad) stained with Coomassie blue: myosin (200 kDa), beta-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa); **B:** extracts probed with 121F MAb, then alkaline phosphatase conjugated goat anti-mouse secondary antibody; **C:** extracts probed with monospecific polyclonal antibody against the antigen, then secondary antibody as in B; **D:** extracts probed only with the alkaline phosphatase goat anti-mouse secondary antibody.

sponding to that for each purified SOD were observed (data not shown). Therefore, there is a significant immunological similarity between portions of the 121F MAb-reactive osteoclast membrane glycoprotein and the SOD protein family, complementing the homology initially reported on the basis of amino acid sequence similarity [13]. However, certain regions of the membrane glycoprotein, including the epitope for binding the 121F MAb, may be uniquely specific to this protein.

SOD Activity Levels Correlate With the Antigen

Although it has been known for some time that osteoclasts contain significant SOD activity [6], there have been few reports further characterizing osteoclast SOD. Due to the sequence homology and immunological cross-reactivity of SOD and the osteoclast antigen recognized by the 121F MAb, investigations were initiated to explore whether the antigen itself possessed SOD activity. A direct demonstration of SOD activity associated with the purified 121F MAb-reactive osteoclast antigen has not yet been possible. Presumably, any antigen SOD activity would be destroyed by the urea and/or EDTA exposure in the process of purifying the antigen, since SOD activity is difficult to maintain even under the best of circumstances [7,8,28]. Preliminary attempts to reconstitute an SOD activity by dialysis of the purified antigen in the presence of manganese have not been successful. Therefore, indirect methods were required to establish whether the osteoclast antigen possessed SOD activity. Although 121F MAb-reactive antigen cannot be detected by immunohistochemistry or in western blots of MAGCs, very low levels of this antigen are measurable in MAGCs by ELISA [10,11]. For MAGCs which have formed in the presence of osteoblast conditioned media, these antigen levels are induced to an intermediate level between that for uninduced MAGCs and osteoclasts [11]. To determine whether SOD activity levels and 121F MAb-reactive antigen levels varied in concert, SOD activities were assessed in comparable samples (based on equal protein content) of osteoclasts (high levels of

TABLE II. Immunoanalysis of Purified Superoxide Dismutases*

	Immunoprecipitation				
Species	MAb 121F	MAb 29C	PAb 121F	Preimmune serum	ELISA: MAb 121F
B. stearothermophilus	1,010	1,584	25,207	1,879	0.098 ± .006
E. coli	3,552	2,089	40,731	1,033	0.082 ± .005
Human	2,500	1,181	31,341	1,571	0.056 ± .007

*Commercially available SODs were iodinated and equivalent portions (cpms) were immunoprecipitated with either the monoclonal (MAb) or polyclonal antibodies (PAb) as designated above and described in Materials and Methods. Separately, ELISA analysis for 121F MAb-reactive antigen was performed for equivalent portions of SODs using the 121F MAb as outlined in Materials and Methods. Absorbance at 405 nm was measured for triplicate wells and the mean and standard deviation are recorded above.

Fig. 4. SOD zymogram analysis. Non-denaturing gel electrophoresis and SOD zymogram analysis of equivalent protein (2 mg) of osteoclasts (**lane a**), marrow-derived giant cells (**lane b**), and marrow-derived giant cells which have formed in the presence of osteoblast-conditioned media (**lane c**). Levels of the 121F MAb-reactive antigen vary in a similar fashion to the SOD zymogram activities with values from a typical ELISA being 39.26 ± 1.12 for osteoclasts, 1.69 ± 0.09 for unstimulated marrow-derived giant cells, and 4.09 ± 0.50 for osteoblast-conditioned media stimulated marrow-derived giant cells.

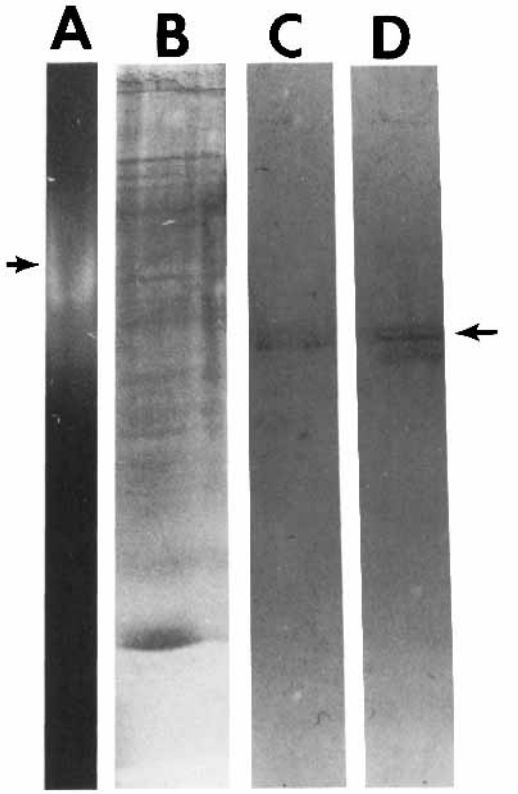


Fig. 5. Demonstration of antigen in SOD activity region. **A:** SOD zymogram analysis of extracts from 1×10^5 osteoclasts. **B:** SDS-PAGE and silver staining of SOD activity region from A. **C:** Western blot of sample identical to B, probed with the 121F monospecific polyclonal antibody, then alkaline phosphatase goat anti-mouse secondary antibody. **D:** Western blot of immunoaffinity purified 121F MAb-reactive antigen probed as in C. Arrow indicates the 150 kDa disulfide-reduced 121F MAb-reactive antigen.

antigen), MAGCs (low levels of antigen), and MAGCs stimulated by osteoblast conditioned media (mid-range levels of antigen). Figure 4 compares zymogram SOD levels amongst equivalent protein samples of these cell types. The SOD activities were found to be directly proportional to the 121F MAb-reactive antigen levels measured by ELISA for these cells. These findings were consistent with the osteoclast antigen having an SOD activity.

The relationship between osteoclast SOD activity and the 121F MAb-reactive antigen was pursued further by analyzing the osteoclast electrophoretic SOD regions for the presence of the antigen. SOD zymogram activity bands from non-denaturing electrophoresis of osteoclast extracts were excised, and analyzed by SDS-PAGE and western blotting. Figure 5 illustrates the protein profile and 121F MAb-reactive species present in the SOD zymogram activity region. SDS-PAGE of the excised SOD activity region (lane A) revealed a broad spectrum of proteins (lane B). Western blot analysis of an identical sample (lane C), analyzed in parallel with a sample of the purified antigen (lane D), revealed that a single disulfide-reduced protein of 150 kDa was obtained from the SOD activity region which was reactive with the 121F monospecific polyclonal antibody. No band was apparent in the 20 kDa region, although western blots of osteoclast extracts probed with the 121F monospecific polyclonal antibody typically demonstrate a faint band in the 20 kDa region (Fig. 3). It is possible that the level of the 20 kDa species in this particular sample may have been below the level of detection. Therefore, despite the many protein species co-migrating in the SOD zymogram activity region, the discovery of only one 121F MAb-reactive protein having the molecular weight attributed to the osteoclast membrane antigen indicated that this membrane glycoprotein was contained within the SOD activity gel region.

In addition, further evidence was obtained by demonstrating that 121F MAb-reactive antigen

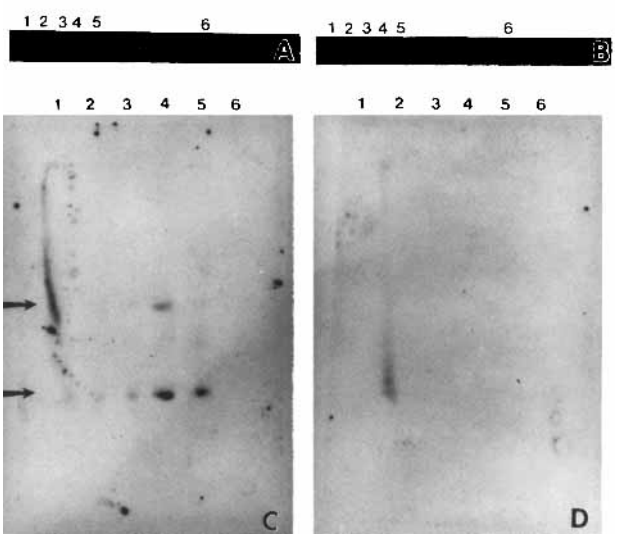


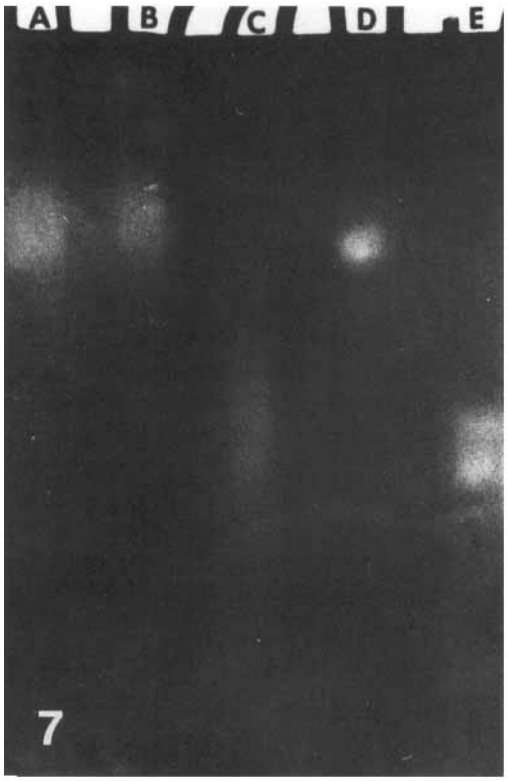
Fig. 6. Demonstration of 121F MAb-reactive antigen in SOD activity region. **A:** SOD zymogram analysis of osteoclast extracts pre-mixed with the 121F MAb. **B:** Zymogram analysis of osteoclast extracts in the absence of the 121F MAb. The bottoms of the non-denaturing gels in panels A and B are on the left. Numbers indicate gel regions excised for analysis by reducing SDS-PAGE and western blotting, as shown in C and D. **C:** Western blot of numbered regions indicated in A. **D:** Western blot of regions indicated in B. Blots were probed with iodinated goat anti-mouse IgG and autoradiographed. Arrows point to heavy chains (upper) and light chains (lower) derived from reduced IgG.

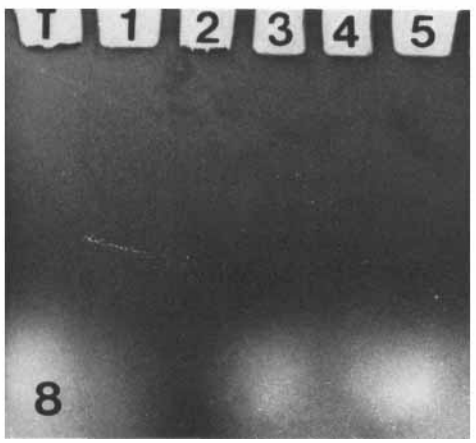
was present in zymogram SOD activity regions of non-denaturing gels (Fig. 6). Osteoclast extracts were pre-mixed with the 121F MAb, separated on non-denaturing gels, and the SOD activity regions were excised. These were subjected to reducing SDS-PAGE analysis, followed by western blotting. When probed with secondary antibodies to detect the mouse 121F MAb, the SOD activity region of osteoclast extracts pre-mixed with the 121F MAb displayed immunoreactive species at gel migrations expected for light chain and heavy chain IgG. Thus, complexes of 121F MAb and antigen co-localize with SOD activity on non-denaturing gels. The SOD activity region must therefore contain the 121F MAb-reactive antigen. None of the gel regions lacking SOD activity from the pre-mixed osteoclast extract and 121F MAb sample yielded IgG bands. Similarly, SOD regions from osteoclast extracts not pre-mixed with the 121F MAb did not contain any apparent mouse IgG.

High Molecular Weight SOD Activity

As a first prerequisite, size partitioning of osteoclast extracts should demonstrate a large molecular weight SOD activity if the membrane antigen itself contained SOD activity. The osteoclast antigen recognized by the 121F MAb migrates in non-reducing SDS-PAGE as a molecule well above 200 kDa. The largest previously reported SOD is a eukaryotic mitochondrial tetrameric SOD of 80 kDa [29]. To determine whether osteoclasts did, indeed, contain an SOD activity which was larger than 100 kDa in size, osteoclast CHAPS extracts were fractionated by centrifugation in Amicon Centricon microconcentrators to separate proteins greater than 100 kDa from those between 10 kDa and 100 kDa. As a control, human erythrocyte SOD was suspended in serum and fractionated in a similar manner, to assess whether an 80 kDa SOD species would be anomalously retained by the 100 kDa cut-off membrane during centrifugation in the presence of other protein species. These samples were then analyzed by non-denaturing gel electrophoresis and zymogram reaction (Fig. 7). SOD activity from osteoclasts was found both in the protein fraction larger than 100 kDa and in the fraction containing proteins between 10 kDa and 100 kDa (lanes B and D). In contrast, human erythrocyte SOD activity was observed primarily in the protein fraction partitioning between 10 kDa and 100 kDa (lane E), with significantly less activity present in the larger than 100 kDa fraction (lane C). Although apparent protein size determined by SDS-PAGE does not always correlate with native globular protein size in solution, SDS-PAGE analyses of these fractions demonstrated that there was a general enrichment of larger protein species in the greater than 100 kDa fraction and a complementary enrichment for

smaller protein species in the 10 kDa to 100 kDa samples (data not shown). When the osteoclast protein fractions retained by the 100 kDa and the 10 kDa cut-off membranes were analyzed separately by ELISA for the antigen recognized by MAb 121F, 90% (0.182 ± 0.003) of the antigen was found in the material retained by the 100 kDa cut-off membrane and only 10% (0.021 ± 0.003) was associated with material which had passed through the 100 kDa membrane but was retained on the 10 kDa cut-off membrane. The small amount of antigen which had passed through the 100 kDa membrane may represent partially degraded or partially synthesized antigen. Centrifugual size partitioning therefore established that, in addition to a low molecular weight SOD activity, there was an SOD activity derived from osteoclasts which behaved in solution, like the osteoclast membrane antigen, as a species larger than 100 kDa.





Osteoclast Membrane SOD Activity

If the high molecular weight SOD activity is linked to the 121F MAb-reactive membrane antigen, then the activity should be found associated with the osteoclast plasma membrane. The antigen recognized by the 121F MAb was shown to localize with the membrane fraction during density gradient centrifugation (Fig. 2). When each fraction from the Percoll density gradient centrifugation of osteoclast extracts was analyzed for SOD activity, only the plasma membrane and mitochondrial fractions revealed SOD activity (Fig. 8). SOD zymogram analysis of similarly prepared control liver extract membrane fractions demonstrated no measurable membrane-associated SOD activity (data not shown). Plasma membrane and mitochondrial density regions from both osteoclasts and liver were each assayed for the mitochondrial membrane enzyme, rhodanese [19], to determine whether mitochondrial proteins might be present in the plasma membrane fractions (Table III). In both osteoclast and liver extract preparations, rhodanese activity was predominantly found in the mitochondrial density fraction with

Fig. 7. Size partitioning of SOD activities. Osteoclast extracts (from 1×10^6 cells) or purified human erythrocyte SOD (5 mg) were fractionated by molecular weight exclusion membrane centrifugation. **A:** Unfractionated osteoclast extract. **B:** Osteoclast extract retained by 100 kDa cut-off membrane. **C:** Human SOD retained by 100 kDa cut-off membrane. **D:** Osteoclast extract retained by 10 kDa cut-off membrane (after non-retention by 100 kDa membrane). **E:** Human SOD retained by 10 kDa cut-off membrane (after non-retention by 100 kDa membrane). Equivalent portions of each sample were run on non-denaturing gels, then analyzed for SOD activity by zymogram.

Fig. 8. SOD zymogram analysis of density gradient fractions. Osteoclasts (1×10^5) were homogenized and fractionated by Percoll density centrifugation. Fractions were concentrated using 10 kDa cut-off membrane centrifugation, then analyzed on non-denaturing gels by SOD zymogram. **T:** Unfractionated osteoclast extract. Buoyant density (g/ml) was determined by marker bead locations: **lane 1** = lighter than 1.037; **lane 2** = 1.037; **lane 3** = between 1.037 and 1.049; **lane 4** = between 1.049 and 1.067; **lane 5** = denser than 1.067.

TABLE III. Mitochondrial Rhodanese Assay*

Chicken cell type	Buoyant density	Rhodanese activity
Liver	1.04	0.107
Liver	1.08	1.630
Osteoclasts	1.04	0.106
Osteoclasts	1.08	1.963

*Cell extracts obtained from 35% Percoll purified osteoclasts (1×10^6 cells) or chicken liver (10 μg) were subjected to Percoll density gradient separation (see Materials and Methods) to prepare plasma membrane (1.04 g/ml buoyant density) and mitochondrial (1.08 g/ml buoyant density) fractions. The plasma membrane and mitochondrial fractions were analyzed for the mitochondrial enzyme rhodanese as described by Sorba [19]. Rhodanese activity values represent absorbance at 460 nm.

little, if any, rhodanese activity detectable in the plasma membrane density fractions. Therefore, the SOD activity observed in the osteoclast plasma membrane fraction was unlikely to be the result of contaminating mitochondrial membranes.

Antigen Depletion Studies

Although the presence of membrane SOD activity strongly correlated with the 121F MAb-reactive antigen, a more convincing alliance between the two could be demonstrated if selective removal of the antigen consequently led to a loss of SOD activity. To investigate such a connection, osteoclast extracts retained by the Amicon 100 kDa molecular weight cut-off membrane were mixed with either a 121F MAb immunoaffinity matrix or a similarly prepared matrix coupled with an unrelated anti-osteoclast monoclonal antibody, 29C, to deplete the high molecular weight extract fractions of the respective antigens recognized by these antibodies. Extracts depleted of one or the other of these antigens were then subjected to non-denaturing gel electrophoresis and zymogram analysis (Fig. 9). Whereas SOD activity was still clearly retained in the 29C MAb-depleted high molecular weight osteoclast extracts, there was significantly diminished activity remaining in the 121F MAb-depleted high molecular weight osteoclast extracts. Presumably, complete removal of the 121F MAb-reactive antigen would eliminate all such high molecular weight membrane SOD activity. The depleted antigens could not be directly examined by zymogram analysis since non-denaturing gel electrophoresis does not dissociate the antigens from the immunoaffinity

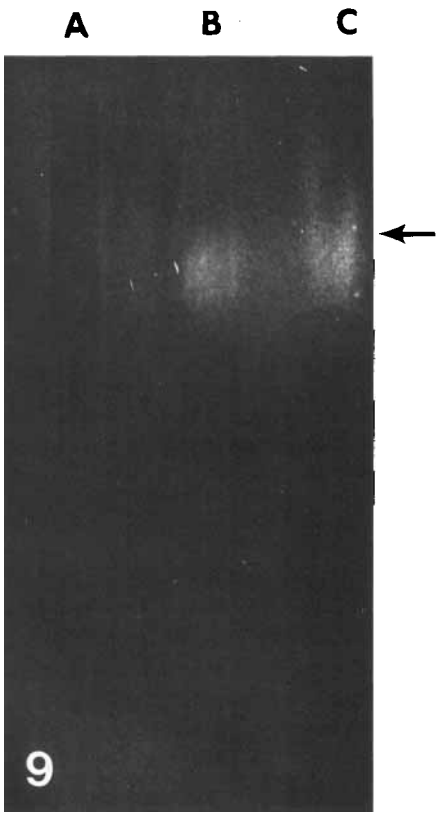


Fig. 9. Antigen depletion studies. Extracts from 5×10^5 osteoclasts were divided into 3 equal parts and concentrated with 100 kDa cut-off membranes. Two portions were depleted for either the 121F MAb-reactive antigen or the 29C MAb-reactive antigen as described in Materials and Methods. Samples were analyzed by nondenaturing electrophoresis and SOD zymogram analysis. **Lane A:** SOD activity levels were reduced in 121F MAb-depleted osteoclast extract. **Lane B:** SOD levels remained high in extracts exposed to 29C MAb. **Lane C:** SOD levels in untreated osteoclast extracts.

matrices and so, the antigens do not migrate into the native gel (data not shown). As reported by Oursler et al. [13], the antigens can be readily dissociated from the immunoaffinity matrices using a 0–8 M urea gradient, however, urea exposure abolishes SOD activity. When the above immunoaffinity matrices employed to deplete the 121F MAb and 29C MAb-reactive antigens were eluted with urea, the expected 150 kDa and 110 kDa antigen bands were obtained on reducing SDS-PAGE analysis, respectively (data not shown). These experiments have been performed using either 121F MAb ascitic fluid or Sephadex G-200 purified antibody for immunoaffinity matrix preparation and have yielded identical results (data not shown).

DISCUSSION

Aerobic metabolic activity generates potentially toxic levels of superoxide radicals. Cellular

defenses against superoxide radical damage have taken many forms, the most ubiquitous protective mechanism being the production of a family of enzymes known as the superoxide dismutases (SODs) [reviewed in 7]. There are two genetically distinct families of SODs: the cytosolic copper/zinc (Cu/Zn) SODs and the bacterial and mitochondrial manganese/iron (Mn/Fe) SODs. Most Cu/Zn SODs are homodimeric unglycosylated cytosolic polypeptides, however, a tetrameric glycosylated form has been identified in mammalian extracellular fluid [30]. Prokaryotic Fe SODs are also unglycosylated homodimers, whereas eukaryotic mitochondrial matrix Mn SOD is an unglycosylated homotetramer. There have been no reports describing a plasma membrane-associated Mn/Fe SOD, although a chloroplast membrane-associated form has been identified [28]. Thus, a membrane association for SOD is not completely unprecedented. The facultative aerobic bacterium *Nocardia asteroides* contains a cell wall-bound SOD [31]. This pathogen is resistant to superoxides produced during macrophage and neutrophil respiratory burst activity, presumably through its cell wall SOD which protects and enables it to grow within macrophages.

Osteoclasts, the major cell type responsible for bone resorption, are large multinucleated cells which establish a tightly sealed extracellular lacuna on the bone target surface, into which protons and a myriad of degradative enzymes are pumped [2]. It has been known for some time that osteoclasts contain high levels of SOD activity [6]. Recently, reports have described superoxide generation localized at the bone-cell interface during bone resorption [4]. The large number of mitochondria existing within osteoclasts further suggests a high level of aerobic activity. It is likely that the combination of these cellular activities generates large amounts of superoxide radicals which could potentially damage these cells unless neutralized by SOD.

A previous report described the unexpected amino acid sequence similarity between the Mn/Fe SOD family and a 30 kDa hydroxylamine fragment generated from the 150 kDa (disulfide-reduced) osteoclast membrane glycoprotein recognized by the 121F MAb [13]. Since this sequence represents only a small fraction of the osteoclast membrane protein, we have sought to obtain additional immunological and functional information with respect to the potential role of this protein in osteoclast activity and its possible relationship to SOD. Membrane-associated SOD activity is relatively uncommon; therefore, it was important to first establish conclusively whether the 121F MAb-reactive osteoclast antigen was localized to the plasma membrane. Several lines of evidence, including immunocytochemical staining, cell permeabilization assays, peripheral membrane protein stripping, and membrane fractionation studies confirmed that the antigen recognized by the 121F MAb is a plasma membrane protein. Furthermore, significant immunological similarities between portions of the 121F MAb-reactive osteoclast membrane glycoprotein and the SOD protein family were discovered using monospecific polyclonal antibodies generated against the purified antigen. Such shared immunological features complement the homology previously based on common amino acid sequence alone. Other regions of the membrane glycoprotein, including the epitope for binding the 121F MAb, appear to be uniquely specific to this osteoclast protein.

Due to the sequence homology and immunological cross-reactivity of SOD and the 121F MAb-reactive osteoclast antigen, we investigated whether the antigen itself possessed SOD activity. Since it was not possible to directly examine SOD activity in the purified antigen, an indirect approach was taken in a series of experiments to correlate the antigen with SOD activity. The studies reported here describe a high molecular weight SOD activity which is associated with the osteoclast plasma membrane. SOD activity varied in accordance with antigen levels when osteoclasts were compared with closely related stimulated or unstimulated marrow-derived giant cells. When the 121F MAb-reactive antigen was selectively withdrawn from high molecular weight osteoclast extracts, a corresponding drop in SOD activity was obtained. In addition, SOD activity regions on zymograms were shown to contain the 150 kDa (reduced) membrane antigen which was reactive with the 121F MAb.

Taken together, the evidence suggests that the 121F MAb-reactive antigen, a high molecular weight osteoclast plasma membrane glycoprotein, may exhibit an SOD-like activity. Although the acidic microenvironment generated by osteoclasts at the bone surface might contribute to the conversion of superoxides to progressively more reactive perhydroxyl radicals, a non-enzymatic mechanism may not be as rapid and efficient as needed to protect the cell [32,33]. In addition, the abundant membrane anion transporters in osteoclasts [34] could potentially al-

low externally generated superoxide radicals to gain quick access to intracellular targets if such radicals were not rapidly neutralized by a plasma membrane-associated SOD. Normal bone resorption may involve oxygen-derived free radicals which are generated at the osteoclast-bone interface as resorption occurs [4,5]. It is therefore plausible to hypothesize that osteoclasts may have evolved a specialized defensive mechanism localized to the plasma membrane which ensures that extracellular dissolution of bone matrix does not endanger its own cell integrity. The results presented in this report demonstrate that osteoclasts possess a high molecular weight membrane-bound SOD activity. The prime candidate for the identity of this SOD appears to be the 121F MAb-reactive plasma membrane antigen, based on amino acid sequence similarities, immunological cross-relatedness and direct correlations between antigen and SOD activity levels. If so, this might explain why there is a close association observed between this osteoclast membrane antigen and extracellular degradative activity [12].

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