Interactions between the Bone Matrix Proteins Osteopontin and Bone Sialoprotein and the Osteoclast Integrin $\alpha_v\beta_3$ Potentiate Bone Resorption

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We have investigated the mechanism by which osteoclasts adhere to and resorb bone. We show that these cells express $\beta_1$ and $\beta_3$ integrins which are involved in attachment to purified bone matrix proteins. Binding to osteopontin and bone sialoprotein is mediated by $\alpha_v\beta_3$, while a $\beta_1$ integrin is responsible for attachment to fibronectin. Both the rapid attachment by osteoclasts to intact bone particles and their subsequent resorption are blocked by a monoclonal antibody directed to the $\alpha_v\beta_3$ complex but not by an antibody against $\beta_1$ integrins. Attachment of osteoclasts to bone is also inhibited with soluble osteopontin, Arg-Gly-Asp-containing peptides derived from both osteopontin and bone sialoprotein, or a monospecific polyclonal antibody against osteopontin. We conclude that both osteoclast adherence to bone and subsequent resorption of its matrix are dependent on interactions between the bone matrix proteins osteopontin and/or bone sialoprotein and the integrin $\alpha_v\beta_3$. Moreover, collagen, which constitutes 90% of its organic matrix, is minimally involved in binding of chicken osteoclasts to bone.

Bone resorption is a multistep process mediated by the osteoclast, a multinucleated cell of the monocyte/macrophage lineage (1, 2). Recent studies have established that if this cell is to degrade matrix it must develop a tightly sealed microenvironment at the osteoclast-matrix interface in which a steep proton gradient is established and where matrix-degrading enzymes are active (3).

Generation of such a highly defined compartment is believed to be mediated by interactions between specific matrix proteins and integrins, which are heterodimeric membrane receptors (4–6). Osteoclasts are known to express at least one such receptor, $\alpha_v\beta_3$ (7, 8) which recognizes a number of adhesive proteins including vitronectin, fibrinogen, thrombospondin, and von Willebrand's factor (9–11). Most importantly, an antibody to $\alpha_v\beta_3$ blocks formation of characteristic bone resorptive pits by human osteoclasts (12). The specific matrix proteins to which this or other integrins bind when initiating resorption are unknown, but immunoelectron microscopic studies of whole bone have shown that osteoclast $\alpha_v\beta_3$ and the matrix protein osteopontin colocalize (13), suggesting that their interaction may represent a mechanism by which the cell binds to bone.

Regardless of the molecule to which it attaches, $\alpha_v\beta_3$ recognizes a common tripeptide sequence, RGD (Arg-Gly-Asp), which has been shown to be crucial for specific binding (5, 9, 14) and which is present in the bone matrix proteins bone sialoprotein, osteopontin, thrombospondin, type 1 collagen, and fibronectin (15–17). The latter two proteins are potenti ally significant, since osteoclasts express $\beta_1$ integrins (8, 18), which are known to be involved in attachment to fibronectin and collagen (19), suggesting that such interactions may also play a role in the resorptive process.

In this report we investigate the identity of bone matrix proteins critical to resorption and the osteoclast integrins with which they interact. We show that while both $\beta_1$ and $\beta_3$ integrins are present on chicken osteoclasts, the cells bind to intact bone matrix via $\alpha_v\beta_3$, using osteopontin and bone sialoprotein for attachment. Osteoclasts do not attach significantly to the major matrix protein collagen, suggesting that, while abundant, it does not initiate the resorptive process. Most importantly, a polyclonal antibody to osteopontin inhibits attachment of osteoclasts to both the isolated protein and whole bone, providing the first direct evidence for the functional significance of such interactions.

MATERIALS AND METHODS

Attachment Proteins—Chicken fibronectin was obtained commercially (Biomedical Technologies, Stoughton, MA), while rat tail collagen and rat albumin were purchased from Sigma. Purified type 1 chicken collagen was a generous gift from Dr. R. Mayne, Department of Cell Biology, University of Alabama, Birmingham, AL. Rat bone matrix protein fractions were separated by minor modifications of a published method (20), and the fractions were characterized by SDS-PAGE1 and staining with either Coomassie Blue or Stains-all (21). The procedure yielded samples highly enriched for osteocalcin, solubilized bone collagen, osteonectin, bone sialoprotein, and a mixture of proteoglycans. Rat osteopontin and a protein shown to be the rat homologue of human $\alpha_2\HS$-glycoprotein were purified by published methods (22, 23). Pure bone sialoprotein from the osteosarcoma cell line ROS 17/2.8 was purified as described and donated by Dr. Ron

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1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
were used to coat 96-well microtiter plates (Falcon, 1008). A 2-pl drop
were dissolved in PBS at between 0.6 and 200 pg/ml, and the solutions
visualized by autoradiography as described previously (9).

incubated at room temperature in 100% humidity for 3 h. The wells
were washed free of EDTA with PBS, resuspended at the appropriate

were lysed with a buffer containing 2% Renex detergent and

Bone Attachment—Attachment of bone particles to adherent cells
was determined according to methods developed in our laboratory
(27). Briefly, radiolabeled rat bone was obtained by a published
method (3), in which rate were injected with [3H]proline, and after

Chicken Osteoclasts Adhere Selectively to Bone Matrix Proteins—To
determine the nature of the adhesive ligands in bone, osteoclasts were
allowed to attach to microtiter wells coated with different matrix proteins.
The fractions tested represented the major proteins in bone matrix and
included all those with known chemotactic and/or attachment activity.

Miscellaneous Reagents—General reagents were purchased from
Hazelton Biologics, Lenexa, KS. "H-labeled proline was provided by
Amersham Corp., while [14C]NaI and [(35S]methionine/cysteine, as
TranSlabs, were from ICN, Irvine, CA. The monoclonal antibodies
LM 609, which bind to a functional domain of \( \alpha_{5} \beta_{3} \) (28) and CSAT,
which binds to chick \( \beta_{1} \) integrins (29, 30), were purified from ascites
or spent culture medium by Protein A-Sepharose chromatography.
The proteins were concentrated, dialyzed into PBS, sterile filtered,
and kept at 4 °C. Irrelevant, isotype-matched antibodies (IgG, for
LM 609, IgG4, for CSAT) were obtained from The Binding Site, La
Jolla, CA. A rabbit antiserum to rat osteopontin which is known to contain
small amounts of cross-reactivity to BAG, an acidic glycoprotein
found in bone (31), was purified of this contamination by passage
over an osteopontin affinity column. On subsequent analysis this
antiserum was monospecific for rat osteopontin (data not shown).

Statistical Methods—The results for cell binding are from repre-
sentative experiments and are presented as the mean and standard
deviation. Statistical significance was determined by the use of the
paired Student's t test.

RESULTS

Chicken Osteoclasts Express \( \alpha_{5} \beta_{3} \) and Multiple \( \beta_{1} \) Integrins—To
examine the repertoire of integrins expressed on chicken osteoclasts
were surface labeled with lactoperoxidase/121I, the lysate was immunoprecipitated with the monoclonal
antibodies CSAT (anti-\( \beta_{1} \)) and LM 609 (anti-\( \alpha_{5} \beta_{3} \)), and the resultant fractions were subjected to autoradiographic
analysis.
FIG. 1. Attachment of chicken osteoclasts to bone matrix proteins. Osteoclasts were allowed to attach for 30 min to various protein fractions in coated microtiter plates. Non-adherent cells were washed off and the attached multinucleated cells were counted after staining with toluidine blue. Significant binding was seen only in the case of rat osteopontin (rOPN), rat bone sialoprotein (rBSP), and chicken fibronectin (cFN). Fractions to which cells did not adhere included those enriched for rat osteocalcin (rOC), rat osteonectin (rON), mixed rat bone matrix proteoglycans (rFGs), soluble rat bone collagen (rCOL-s) as well as pure albumin (rALB), rat serum glycoprotein α2-HS (α2-HS), rat tail collagen (COL-1), or buffer alone (PBS).
Integrin-mediated Matrix Osteoclast Interactions

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Fig. 2. Identification of several $\beta_1$ integrins and $\alpha_\beta_3$ in chicken osteoclasts. Cells were surface labeled with $^{125}$I, and the lysate was immunoprecipitated with the monoclonal antibodies CSAT (anti $\beta_1$) and LM 609 (anti $\alpha_\beta_3$). The precipitated material was analyzed under both non-reducing and reducing conditions. Lanes 1 and 2 in panel A and lane 2 in panel B represent the results of the non-reducing gels, with lanes 3 and 4 in panel A and lane 1 in panel B those of the reducing gels. Several $\beta_1$ integrins were present (panel A, lanes 2 and 4), with the expected sizes for the $\beta_1$ subunit (115 kDa non-reduced and 125 kDa reduced) and with several $\alpha$ subunits in the range 130-160 both reduced and non-reduced. These integrins were not characterized further. Bands corresponding to $\alpha_\beta_3$ were also present, with sizes of 95 and 110 kDa and 160 and 130 kDa for the non-reduced and reduced $\alpha$ and $\beta$ subunits, respectively (panel A, lanes 1 and 3). Cells were also labeled metabolically with $[^{35}]S$ methionine/cysteine, lysed, and the lysate purified by passage over a column containing bound GRGDSPK. The fraction eluting with a solution of GRGDSPK was immunoprecipitated with LM 609 and analyzed by SDS-PAGE (panel B). The material exhibited bands at 95 and 160 kDa (lane 2), which upon reduction migrated at 110 and 130 kDa (lane 1), characteristic of $\alpha_\beta_3$.

It had been shown that freshly isolated human monocytes fail to express the integrin $\alpha_\beta_3$, but elaborate the dimer while in culture (34). Therefore, we were concerned that all of our studies implicating this molecule as a critical mediator of bone resorption may be artifactual. However, our previous studies demonstrating that the monoclonal antibody LM 609 (which specifically recognizes $\alpha_\beta_3$) reacted with both generated and freshly isolated osteoclasts (25) suggest that this is not likely to be the case.

The ability of the cells to bind bone-derived proteins was examined using an assay in which only the center of microtiter plates was coated with individual proteins. This simple and rapid method permits the use of small amounts of rare protein fractions and each well acts as its own control, since under the described conditions osteoclasts were totally non-adherent to the surrounding uncoated plastic. Among the many known proteins in bone (16), osteoclasts bound significantly only to fibronectin, osteopontin, and bone sialoprotein. Interestingly, the latter two proteins were not derived from chicken bone, underscoring the homology of the RGD domains in osteopontin and bone sialoprotein in the chicken and a number of mammalian species (35-39).

Collagen comprises 90% of the organic matrix of bone and therefore would be a logical candidate to play an important role in osteoclast recognition, particularly as it contains both an RGD motif known to serve as a cell-binding site (40) and an RGD-independent binding domain (41). Moreover, human osteoclasts express the $\alpha_\beta_3$ integrin subunit (18) and hence must be presumed to have a collagen receptor. Thus, the failure of chicken osteoclasts to bind to either rat tail or bone collagen fragments, even in the presence of magnesium ions which have been shown to support attachment via the integrin $\alpha_\beta_3$ (42), is a critical finding of this study. This observation suggests that, in contrast to their human counterparts, chicken cells do not have this collagen receptor (the unavailability of an antibody precluded us from performing this experiment) or that the appropriate sequence in collagen is unavailable for binding. The significance of this finding is that while collagen is the overwhelmingly abundant bone protein (16), it appears to play, at best, a minimal role in the attachment of osteoclasts to matrix.

Monoclonal antibodies to individual integrins decreased cell attachment to osteopontin, bone sialoprotein, and fibronectin in a highly specific manner. Inhibition of cell binding by LM 609, directed to $\alpha_\beta_3$ (28), is consistent with earlier reports on the ability of this receptor to mediate attachment to osteopontin and bone sialoprotein (43). On the other hand the monoclonal antibody CSAT, which binds to $\beta_1$ integrins (29), interfered with attachment only to fibronectin. While we have not identified which integrin is involved in such binding, our results suggest that it is likely to be the high affinity fibronectin receptor $\alpha_\beta_3$. The fact that the antibody to $\alpha_\beta_3$ only inhibited partially both binding and resorption (Figs. 5 and 6) suggests that other integrin- and/or non-integrin-mediated attachment mechanisms may be active. In support of these ideas it has been demonstrated in a number of systems that the processes of initial attachment and subsequent spreading are independent (44-47).

Matrix-integrin interactions are probably conformation-dependent. Thus, it has been shown that soluble and surface-attached forms of fibronectin have different affinities for the same receptor (48). Furthermore, studies on the interactions between $\alpha_\beta_3$ and synthetic peptides have also demonstrated marked conformation dependence (43). Since osteopontin and bone sialoprotein had been purified in the presence of strong chaotropic agents (20, 22), partial denaturation may have resulted, and the proteins may not have been in their native configuration. This latter reason and proven conformation dependence of fibronectin binding to its receptor raised the possibility that the results obtained with proteins coated on plastic were not representative of the in vivo situation. To address this issue, intact bone particles were incubated with
creased attachment by 50% revealed that interactions between these two matrix proteins also interfered with binding. These critical results containing peptides derived from osteopontin and bone sialoprotein also inhibited the process. Further support for such specificity was obtained in experiments where RGD-containing fragments of human osteopontin (OPN-f) and bone sialoprotein (BSP-f) and irrelevant antibodies (IgG1 and IgG2) all at 200 μg/ml, followed by the addition of a suspension of [3H]proline-labeled rat bone particles (100 μg, 3–5 × 10⁸ counts/minute). Thirty min later, non-adherent material was rinsed off, and the attached bone was determined as described under "Materials and Methods." The rapid binding of bone to cells was inhibited by blocking αβ₃, but not β₁, function. The soluble bone matrix protein and fragments also inhibited attachment. *p < 0.01.

A monospecific antibody to rat osteopontin inhibits binding of osteoclasts to both osteopontin and whole bone. Panel A, microtiter wells were coated with osteopontin, excess protein was washed off, and the plate dried overnight. A drop of preimmune or anti-osteopontin goat antiserum, diluted 1:50 in PBS, was added for 30 min, after which freshly lifted osteoclasts were added, allowed to bind for 30 min, and then rinsed off prior to fixing and staining. The antibody-containing area (marked with a horseradish peroxidase-labeled second antibody to goat IgG) was identified, and the number of bound cells was counted. The results represent the mean of two separate experiments. The specific antibody totally inhibits attachment of osteoclasts to osteopontin. Panel B, intact bone particles were preincubated with either PBS alone or the same sera described in panel A. After 30 min quadruplicate aliquots were added to adherent osteoclasts, while a fifth sample was processed directly to determine isotope content. Non-adherent bone was rinsed off and that which adhered was determined by dissolution and hydrolysis, as described previously. The results are expressed as the ratio of bone bound in the presence of immune and preimmune serum. (Bone incubated with non-immune serum bound about 70% as well to osteoclasts as did that incubated with PBS alone (not shown)). The results shown are the mean of four separate experiments with different preparations of osteopontin. The anti-osteopontin antibody decreased attachment by 50% (p < 0.05).

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Fig. 4. A monospecific antibody to rat osteopontin inhibits binding of osteoclasts to both osteopontin and whole bone. Panel A, microtiter wells were coated with osteopontin, excess protein was washed off, and the plate dried overnight. A drop of preimmune or anti-osteopontin goat antiserum, diluted 1:50 in PBS, was added for 30 min, after which freshly lifted osteoclasts were added, allowed to bind for 30 min, and then rinsed off prior to fixing and staining. The antibody-containing area (marked with a horseradish peroxidase-labeled second antibody to goat IgG) was identified, and the number of bound cells was counted. The results represent the mean of two separate experiments. The specific antibody totally inhibits attachment of osteoclasts to osteopontin. Panel B, intact bone particles were preincubated with either PBS alone or the same sera described in panel A. After 30 min quadruplicate aliquots were added to adherent osteoclasts, while a fifth sample was processed directly to determine isotope content. Non-adherent bone was rinsed off and that which adhered was determined by dissolution and hydrolysis, as described previously. The results are expressed as the ratio of bone bound in the presence of immune and preimmune serum. (Bone incubated with non-immune serum bound about 70% as well to osteoclasts as did that incubated with PBS alone (not shown)). The results shown are the mean of four separate experiments with different preparations of osteopontin. The anti-osteopontin antibody decreased attachment by 50% (p < 0.05).
Fig. 6. Bone resorption is blocked by pretreatment of osteoclasts with osteopontin, bone sialoprotein, and the antibody LM 609, but not CSAT. The experiment described in Fig. 4 was repeated, except that incubation was extended to 24 h. At this time the supernatant was assayed for the presence of [3H]hydroxyproline/proline. In the absence of any additions, about 10–15% of the isotope was released (not shown), and this amount was decreased by treatment of the cells with LM 609 and both intact rat osteopontin and RGD-containing fragments of the proteins osteopontin and bone sialoprotein, but not by CSAT. Repeated additions of LM 609 (6 hourly, 50 µg/ml) increased the degree of inhibition. *p < 0.05; **p < 0.01.

We have shown directly that interference in the interactions between the integrin αβ3 and osteopontin. Conversely, they suggest that αvβ3-mediated attachment of cells to fibronectin, while occurring in vitro, is not demonstrable and hence may have limited significance in vivo.

As an extension of these studies the bone particles were left in contact with the osteoclasts for 24 h to monitor resorption in the presence of various antibodies to integrins. Paralleling the bone adhesion experiments, both selected RGD-containing peptides and LM 609, but neither CSAT nor the isotype-matched controls, inhibited this process. When LM 609 was added at 6 hourly intervals there was an increase in inhibition, suggesting that the cells metabolize the antibody.

A recent report on the inhibition of osteoclast function in both mammalian and avian osteoclasts by the snake venom echistatin (50) has partially corroborated our results. These authors showed that this RGD-containing protein inhibited both bone resorption by and response of the cells to calcitonin in a manner suggesting that an αv integrin was implicated. However, they failed to identify clearly the integrin involved in these events, a question of growing importance since it is now known that, in addition to its classical interaction with the β3 subunit, αv can also associate with either β1, β3, β5, or β8 (51–57).

We have now shown by both immunoprecipitation and blocking of adhesion with an antibody which recognizes specifically αβ3 that this integrin is present and involved directly in these events. At this time we cannot exclude the presence and function of other integrins involving the αv chain and studies are in progress to address this issue.

In summary, we have demonstrated that chicken osteoclasts express the integrin αβ3 which mediate attachment of the cells to specific bone matrix proteins. Binding to fibronectin is mediated by an unidentified integrin, possibly α5β1, and can be demonstrated only when the protein is coated on plastic. In contrast αβ3 mediates attachment to osteopontin and bone sialoprotein both on plastic-coated plates and in, for the former molecule, in intact bone. While the predominant event in the dissolution of the organic matrix is the proteolysis by an acidic collagenase of the major matrix component collagen (3), this same protein (collagen) appears to play no role in the attachment of avian osteoclasts to bone or in subsequent matrix resorption, despite the presence of an RGD sequence. Thus, these studies have demonstrated separate roles for different matrix proteins found in bone. Most significantly, we have shown directly that interference in the interactions between the integrin αβ3 and a protein in the context of whole bone led to decreased ability of osteoclasts to bind to and resorb this tissue. This result suggests an approach for the design of drugs which will be efficacious in the treatment of a wide range of clinically important bone diseases.

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