Osteocalcin Induces Chemotaxis, Secretion of Matrix Proteins, and Calcium-mediated Intracellular Signaling in Human Osteoclast-like Cells

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Abstract. Osteocalcin, also called Bone Gla Protein (BGP), is the most abundant of the non-collagenous proteins of bone produced by osteoblasts. It consists of a single chain of 46–50 amino acids, according to the species, and contains three vitamin K-dependent gamma-carboxyglutamic acid residues (GLA), involved in its binding to calcium and hydroxylapatite. Accumulating evidences suggest its involvement in bone remodeling, its physiological role, however, is still unclear. In this study the adhesion properties and the biological effects of osteocalcin on osteoclasts have been analyzed using as an experimental model, human osteoclast-like cells derived from giant cell tumors of bone (GCT). Osteocalcin promoted adhesion and spreading of these cells, triggering the release of bone sialoprotein (BSP), osteopontin (OPN) and fibronectin (FN), that in turn induced the clustering in focal adhesions of β_1 and β_3 integrin chains. Spreading was dependent upon the synthesis of these proteins. In fact, when the cells were incubated in the presence of monensin during the adhesion assay, they still adhered but spreading did not occur, focal adhesions disappeared and BSP, OPN, and FN were accumulated in intracellular granules. Furthermore osteocalcin induced chemotaxis in a dose-dependent manner. The action of BGP on osteoclasts was mediated by an intracellular calcium increase due to release from thapsigargin-sensitive stores. These results provide evidences that BGP exerts a role in the resorption process, inducing intracellular signaling, migration and adhesion, followed by synthesis and secretion of endogenous proteins.

OSTEOCALCIN, also called Bone Gla Protein (BGP),[1] is the most abundant of the non-collagenous proteins of bone produced by osteoblasts, and has several interesting features. It consists of a single chain of 46–50 amino acids, according to the species, and contains three vitamin K-dependent gamma-carboxyglutamic acid residues (GLA), involved in its binding to calcium and hydroxylapatite (19, 33). So far, BGP has been considered highly specific for bone, secreted by mature osteoblasts, and incorporated into the extracellular matrix. Recently, it has been demonstrated that the mouse genome contains an osteocalcin cluster composed of three genes (34, 13). The first two, named OG1 and OG2, are expressed only in bone, while the third one, the osteocalcin-related gene ORG, is transcribed in the kidney, where a protein similar to ostecalcin had already been described and called nephrocalcin (13). Despite its high degree of conservation between species and its specificity for bone tissue, the physiological role of osteocalcin is still not very clear. Accumulating evidences suggest that osteocalcin is involved in bone remodeling. A potential role of this protein in the induction of bone resorption has been suggested by in vitro experiments showing that it has chemotactic activity for a number of cells including monocytes which are known to be related to osteoclast precursor cells (25, 28, 29). Experimental rat models in which animals were maintained on high doses of warfarin in order to deplete their bone of osteocalcin, have shown impaired recruitment and differentiation of osteoclast-like cells by these bone implants (10, 14). Other experiments using this model have further suggested that osteocalcin might function as a matrix signal in the recruitment and differentiation of bone resorbing cells (15, 31).

Osteoclasts, the multinucleated giant cells present in bone, are the effectors of bone resorption (39, 41). The resorptive process involves proliferation of developing osteoclasts,

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1. *Abbreviations used in this paper*: BGP, Bone Gla Protein; BSP, bone sialoprotein; FN, fibronectin; GCT, giant cell tumors of bone; GLA, gamma-carboxyglutamic acid residues; MGP, matrix Gla protein; OPN, osteopontin; RT, room temperature.

migration to the site of resorption, adhesion, and polarization onto the bone matrix. When osteoclasts polarize, a sealing zone of close adhesion between their apical membrane and bone is formed, thereby isolating the ruffled border area where resorption takes place (21, 39, 41). Membrane receptors of the integrin family and RGD-containing bone matrix proteins are known to be involved in the adhesion of osteoclasts to the bone surface (35, 36, 37, 42), while the mechanism of osteoclast recruitment to the resorption sites remains very elusive.

In this paper, we present data indicating a specific relation between osteocalcin and osteoclasts. We show that osteocalcin induces chemotaxis, calcium-mediated intracellular signals, and secretion of matrix proteins by osteoclast-like cells, obtained from giant cell tumors of bone (GCTs), characterized for their osteoclast features (16).

Materials and Methods

Cell Cultures

Osteoclast-like cells were isolated from biopsy specimens of human GCTs, after a patient's informed consent at the Istituti Ortopedici Rizzoli as already described (16). Briefly, cells were mechanically dispersed with a Pasteur pipette, or, alternatively, released by a 30-min digestion with 2 mg/ml *Clostridium Histolyticum* neutral collagenase (Sigma Chem. Co., St. Louis, MO) in PBS (in mM: 138 NaCl, 2.7 KCl, 0.9 Na_2HPO_4, 1.4 KH_2PO_4, pH 7.3). Released cells were plated in 25 cm^2 Falcon flasks (Oxnard, CA) at a density of 10,000 cells/cm^2 and cultured in Iscove medium supplemented with 10% FCS (Gibco Limited, Uxbridge, UK), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 IU/ml mycostatin (Eurobio, Paris, France), at 37°C, in a water-saturated atmosphere with 5% CO_2. Cells were fed by medium replacement every 2–3 d.

The osteoclastic phenotype of cultured cells has been extensively studied and published elsewhere (16). Briefly, GCT cell morphology was observed by phase contrast microscopy and the number of nuclei per cell, as well as the number of multinucleated vs mononuclear cells, were evaluated. Binding of calcitonin on cells previously incubated for 3 h in the presence of 1 μCi/ml ^{125}I-human calcitonin (Amersham, UK) was investigated by autoradiographic technique. cAMP production in cells incubated in IBMX-buffer and treated for 10 min with 10^{-7} M calcitonin was measured using the Incstar Corporation (Stillwater, MN) radioimmunoassay. Bone resorption was biochemically detected incubating GCT cells for 48 h in the presence of [^{3}H]proline in vivo prelabeled bone particles, in control conditions or in the presence of 10^{-7} M calcitonin, as described by Blair et al. (3). Bone resorption was also morphologically evaluated according to Arnett and Dempster (1), by analysis of pits escavated by cells plated onto bone slices.

Proteins

Bovine carboxylated and decarboxylated osteocalcin, was purified and decarboxylated as described previously (26). Plasma fibronectin (FN) was from Sigma Chem. Co. The synthetic peptides GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) and GRGESP (Gly-Arg-Gly-Glu-Ser-Pro) peptides were purchased from Telios (La Jolla, CA).

Preparation and Coating of Glass Coverslips

Glass coverslips (12 mm in diam) were cleaned by immersion in nitric acid for 3 h, in water for 1 h, and in ethanol for another hour, and then dried and sterilized before use. The coverslips were coated with 200 μl of osteocalcin or FN (20 and 10 μg/ml, respectively) in PBS containing 1 mM $CaCl_2$, for 15–20 h at 4°C. Residual protein-binding sites on coverslips were saturated by further incubation (30 min, room temperature [RT]) in a buffer containing 1% BSA (fatty acid free, Sigma Chem. Co.). Coverslips were then washed three times with Iscove medium plus 0.5% BSA.

Antibodies

In this report, we used a set of antibodies recognizing the β_1 and β_3 integrin subunits, cellular fibronectin, bone sialoprotein (BSP), and osteopontin (OPN). β_1 antibodies were MAR4 (32), a mouse mAb for immunostaining, and a previously described goat anti-fibronectin receptor (8) used for adhesion assays; β_3 antibodies were rabbit polyclonal serum against platelet GpIIIa (11), mAb IST-9 to the extra domain (ED) specifically expressed by cell-assembled FN and not by plasma FN (4, 5) was kindly provided by Dr. L. Zardi (Istituto Scientifico per lo Studio e la Cura dei Tumori, Genova, Italy). BSP was immunolocalized by a polyclonal antibody raised against bovine BSP as described previously (7), by a polyclonal antibody, LF-6, raised against human BSP, as well as with LF83 and LF100 raised against synthetic sequences of human BSP; OPN was detected by a polyclonal antibody LF-7 raised against human OPN. LF6, LF7, LF83, and LF100 were all kindly provided by Dr. P. Gehron-Robey and by Dr. L. Fisher (National Institute of Dental Research, National Institutes of Health, Bethesda, MD). Antibodies raised against bovine osteocalcin were provided by Dr. C. Chenu. The localization of vinculin was assayed with mAb VIN 11-5 to chicken gizzard vinculin and cross-reacting with the mammalian form (Sigma Chem. Co.).

Adhesion and Spreading Assays

GCT cells were grown in 25 cm^2 flasks; at confluence, cells were trypsinized and resuspended in Iscove containing 0.5% BSA at a concentration of 20,000 cells/ml and 1 ml of cell suspension was seeded on each coated coverslip. Aliquots of cells were also resuspended in Iscove containing 10 μg/ml Monensin (Mon) (Sigma Chem. Co.), and seeded on coated coverslips.

After 6 h of incubation at 37°C, coverslip-attached cells were washed three times with $Ca^{2+}Mg^{2+}$ PBS and fixed in 3% paraformaldehyde, 2% sucrose in PBS pH 7.6 for 10 min at RT. After rinsing in PBS, cells were permeabilized to antibodies by soaking coverslips for 3 min at 0°C in Hepes-Triton X-100 buffer (20 mol/L Hepes, Ph 7.4, 300 mol/L sucrose; 50 mol/L NaCl; 3 mol/L $MgCl_2$; and 0.5% Triton X-100, from Sigma Chem. Co.). This procedure of fixation and permeabilization has been used for other studies of cytoskeleton and adhesion (12).

Adhesion assays were performed on 96-well microtiter plates coated overnight with 20 μg/ml of proteins (see above) and on uncoated wells. Experiments were performed in a serum-free medium, containing 0.5% BSA. Cells plated on uncoated wells in the presence of serum were used as positive controls. After trypsinization, cells were counted, diluted at a density of 2.5×10^5/ml, and 200 μl of GCT cell suspension were placed in each microtiter well for 6 h in a humidified atmosphere containing 95% air and 5% CO_2. Non-adhering cells were removed by gently washing the wells three times with PBS. Adherent cells were fixed with 3% paraformaldehyde for 20 min at RT, followed by rinsing with PBS, air-dried and stained with 0.5% crystal violet for 15 min, followed by extensive rinsing. The dye was released from the cells by addition of Na-citrate 0.1 M in 50% ethanol. The optical density of the released stain solution was read in a Titertek colorimeter at 540 nm. Results were expressed as percentage ± SE of the absorbance read in control samples. A group of experiments were performed for a shorter time (60 min) with or without 200 μg/ml GRGDSP or GRGESP peptides in the medium. To investigate the adhesion inhibitory effect of integrin antibodies, similar experiments were performed, with anti-β_1 and anti-β_3 antibodies added to the cell suspension at the time of plating at different dilutions, in the presence or absence of monensin. In the same conditions, the competitive effect of 20 μM osteocalcin in the culture medium during adhesion assay to osteocalcin-coated wells was evaluated.

Fluorescence Studies

Indirect immunofluorescence was used to show the topography of integrins and matrix molecules. The primary antibody was layered on fixed and permeabilized cells and incubated in a humidified chamber for 45 min at 37°C. After rinsing in PBS (pH 7.6), coverslips were incubated with the appropriate rhodamine-tagged secondary antibody (Dako-patts, Glostrup, Denmark) for 45 min at 37°C. After rinsing in PBS, coverslips were incubated with 25 μg/ml fluorescein-labeled phalloidin (F-PHD, from Sigma Chem. Co.) for 45 min at 37°C. Coverslips were then mounted in 20% Mowiol 4-88 (HOECHST AG, Frankfurt/Main, FGR). Observations were performed by epifluorescence in a Zeiss Universal microscope. Fluorescence images were recorded on Kodak T-Max 400 films and developed in a Kodak T-Max developer for 10 min at 20°C.

Measurement of Cytosolic Calcium Concentration

Cytosolic free calcium concentration in response to addition of BGP was

evaluated in single cells loaded with the intracellular CA^{2+} indicator fura-2 (Sigma Chem. Co.). Isolated cells seeded onto 24-mm diam round glass coverslips were loaded with 10 μM fura-2/AM in serum free, but otherwise complete, IMDM for 1 h at 37°C. Coverslips were washed three times and transferred to a Sykes Bellco open chamber (Bellco Biotechnology, Vineland, NJ) containing 1 ml Krebs-Ringer-Hepes buffer (KRH) (in mM: 125 NaCl, 5 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2 CaCl2, 265 Hepes, and 6 glucose). $[Ca^{2+}]_i$-dependent fluorescence was measured with a microfluorometer (Cleveland Bioinstrumentation, Cleveland, OH) connected with a Zeiss IM35 inverted microscope equipped with a Nikon CF X40 fluor objective. Recordings were performed at dual excitation wavelength (340 and 380 nm, bandwidth 0.5 nm) using an air turbine high-speed rotating wheel carrying the two excitation filters. Emission was collected by a photomultiplier carrying a 510-nm cutoff filter and analyzed by a demodulator. Emission from 340 and 380 nm and real-time 340–380 nm ratio were recorded by a Linseis L6514 recorder. At the end of each experiment, calibration was performed by adding 5 μM ionomycin followed by 7.5 mM EGTA to obtain Ca^{2+}-saturated and nominally Ca^{2+}-free fura-2 fluorescence, respectively. Thapsigargin (Sigma Chem. Co.) was used to deplete intracellular pools. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (18).

Chemotaxis Assay

Chemotaxis activity of BGP was determined in vitro with the Boyden chamber technique (28). Briefly, the osteoclast-like cells (150,000 cells/500 μl) were seeded in the upper compartments of modified Boyden chambers (Costar Corp., Cambridge, MA) and osteocalcin at different concentrations (2×10^{-6} M to 6×10^{-6} M) was added in the lower compartments. Both the cells and osteocalcin were diluted to the desired concentration in IMDM 1% serum. The compartments were separated by polycarbonate membranes (Nucleopore, Costar Corp.) with pores of 8 μm in diameter. The chambers were incubated for 5 h at 37°C in a 5% CO_2/95% water-saturated atmosphere, cells attached to the upper side of the filter were mechanically removed while cells that had migrated to the lower side were fixed with 3% paraformaldehyde in cacodylate buffer, stained in 0.5% Crystal Violet and lysed in 0.1 N sodium citrate to read the absorbance at 540 nm. Negative control was medium alone. Controls with osteocalcin added in the upper compartment on the cell side of the membrane were also performed. Three chambers were used for each treatment and final activity was expressed as mean ± SEM of triplicates.

Statistic Analysis

Quantitative data are expressed as average ± SEM. Statistic analysis was performed by Student's *t* test.

Results

Cell Characterization

Human osteoclast-like cells obtained from giant cell tumors of bone were used in passages between VII and XI. The osteoclast phenotype of the cells used for the experiments has been extensively characterized and recently published elsewhere (16). They consist of homogeneous lines (GCT23, GCT24) derived from tumors in which the transformed cell was in the osteoclastic lineage. These cells keep dividing in vitro and polycarions derive both from fusion or endomytosis (16). The percentage of mononuclear vs multinuclear cells is inversely related to the division rate of the cell line (28 ± 2 multinuclear in GCT23, 72 ± 1 in GCT24). In all the experiments performed (16, 17, 30), differences in behavior between mononuclear and multinuclear cells were not found. TRAP activity was both histochemically and biochemically detected. Calcitonin receptors were present on the membrane of all the cells, independently from the number of nuclei and cAMP content was doubled by calcitonin treatment. Bone resorption was evident both by pit assay and by [^{3}H]proline-prelabeled bone particles degradation. Resorption was significantly reduced in the presence of calcitonin. These features have been extensively described and discussed in reference (16). The shape shown by the cells in culture is variable. Mononuclear cells are usually polygonal, elongated or macrophage-like, while multinuclear contain a variable number of nuclei (2-12) and present rounded edges, more similar to bona fide osteoclasts (Fig. 1). The possible presence of contaminating cells of different phenotype has been excluded by the absence of alkaline phosphatase–positive cells, absence of collagen fibrils in the culture as well as by the homogeneous integrin pattern in control conditions and when GCTs were plated on different substrata (17).

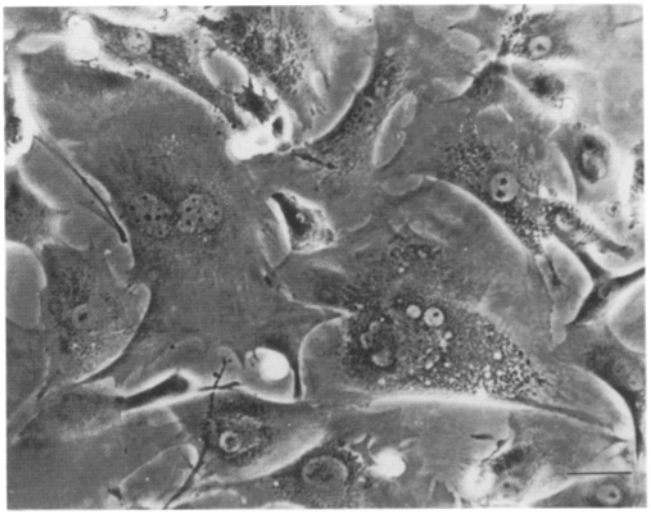

Figure 1. Phase contrast microscopy of cells from a giant cell tumor of bone (GCT24) in culture at the 8th passage. Multinuclear and mononuclear cells are present. Bar, 40 μm.

Adhesion of GCT Cells onto BGP

When GCT cells were plated onto 20 μg/ml BGP-coated cov-

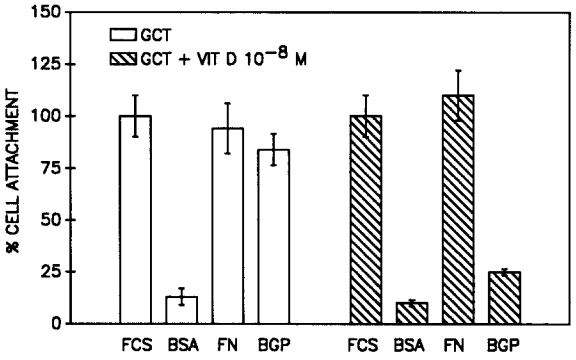

Figure 2. Adhesion onto osteocalcin of cells obtained from human giant cell tumors of bone. The adhesion assay was performed plating cells on wells coated with osteocalcin 20 μg/ml, FN 10 μg/ml or uncoated in the presence of serum or BSA as positive and negative controls, respectively. In some experiments, the GCT cells were maintained for three weeks in the presence of 10^{-8} M $1.25(OH)_2VitD_3$ before the assay. The number of attached cells is expressed as percentage of the adhesion obtained in the presence of serum. Results represent the mean ± SE of three experiments performed in triplicate. Ligand abbreviations: *FCS*, medium containing 10% FCS; *BSA*, bovine serum albumin; *FN*, fibronectin; *BGP*, osteocalcin.

erslips and incubated in serum-free medium for 6 h 70–80% of the cells attached to BGP (Fig. 2). A similar adhesion was noted on coverslips coated with fibronectin but, while most of the cells spread onto fibronectin, spreading onto BGP was less pronounced. Absence of cell adhesion resulted in wells coated with BSA as a negative control. The positive control was FCS. When the GCTs were maintained continuously for three weeks in the presence of 10^{-8} M $1.25(OH)_2$ vitamin D_3, their capability of adhesion to BGP lost while they still adhered onto fibronectin. This could indicate that more differentiated osteoclasts may not recognize this protein, while less mature cells as they are in this tumor population, can recognize BGP. The cells adhered in the same way to decarboxylated BGP indicating that cell attachment was not related to electrostatic interactions between the cells and the highly negative charges of GLA residues contained in this protein. Furthermore the adhesion in both cases was not inhibited by the presence of RGD-containing peptides (data not shown). Cation effect on adhesion was also assayed plating the cells in the presence of EGTA (3 mM), but no changes were found (not shown). When adhesion experiments were performed in the presence of monensin, the cells attached, but spreading was inhibited (Fig. 3). The competitive presence of 20 μM osteocalcin in the culture medium produced a reduction of 35% from control adhesion values when monensin was also present, while no changes were found without monensin (not shown).

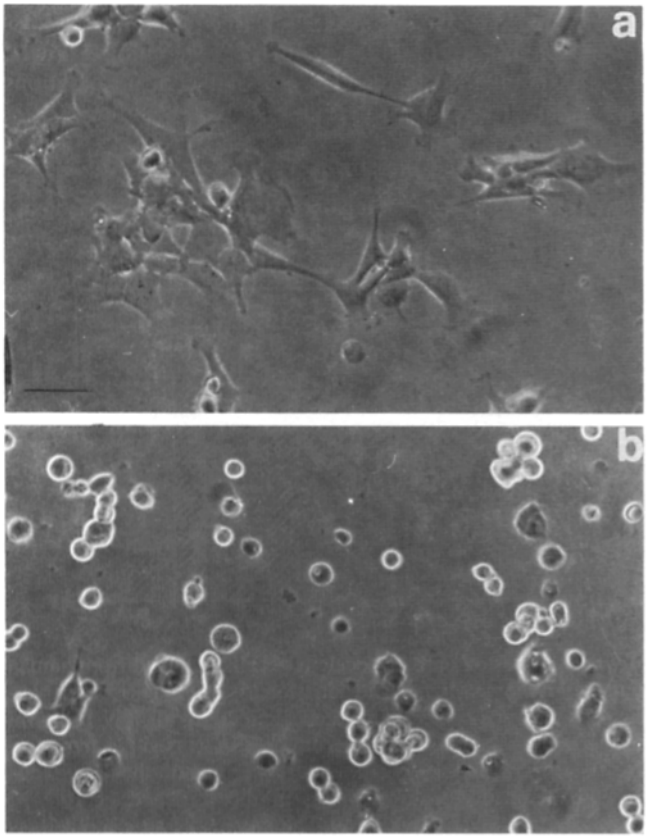

Figure 3. GCT cells plated onto osteocalcin in the absence of serum and fixed after 3 h. Cells are adherent and spread (*a*). In the presence of monensin (*b*) the cells maintain a rounded shape. Bar, 50 μm.

Cytoskeletal Organization and Integrin Expression of GCT Cells Plated onto BGP

Cytoskeletal organization and integrin expression of cells plated onto osteocalcin were analyzed by immunofluorescence using antibodies directed against cytoskeletal proteins and integrins. Actin microfilaments were detected organized in stress fibers at the edge of which vinculin was clustered in focal adhesions (Fig. 4, *a* and *b*). The presence of focal adhesions suggested the involvement of integrins, and, using monoclonal antibodies directed against the β_1 and β_3 integrin chains, we demonstrated a strong positive staining of the cells for these subunits. β_3 was found clustered in focal adhesions at the edge of the stress fibers (Fig. 4, *c* and *d*), while β_1 was organized both along stress fibers and in focal adhesions (Fig. 4, *e* and *f*). The presence of organized focal adhesions and integrins in osteoclasts adherent to osteocalcin was somehow unexpected, as this protein does not contain any known adhesive sequence. As a consequence we investigated if GCT cells were synthesizing proteins which could allow their spreading through interactions with integrin receptors. We searched for the presence of fibronectin, BSP, and osteopontin using antibodies directed against these proteins. Cellular fibronectin has been previously shown to interact in these cells with $\alpha_v\beta_3$ and $\alpha_5\beta_1$, while BSP and osteopontin bind to $\alpha_v\beta_3$ (6, 9, 17, 20, 27). We found that fibronectin was indeed secreted by the cells and organized in an extracellular network of filaments matching the stress fiber organization under the basal cell membrane (Fig. 5 *a*). BSP and osteopontin were also present but with a different pattern. BSP was detected inside the cells, distributed in granules crowded at the proximity of cell surfaces, suggesting a secretory activity (Fig. 5 *b*), while osteopontin was clearly extracellular with a granular distribution under the focal adhesion sites (Fig. 5, *c* and *d*). If cells were occasionally detached during the staining procedure, granules were still found on the coverslips also if the cell was lacking (Fig. 5, *e* and *f*). When the cells were incubated in the presence of 10 μg/ml monensin during the adhesion assay, their spreading on osteocalcin was not completed (Fig. 3), focal adhesions were absent and actin was mainly organized in a thin fibrillar network with only a few fibers (Fig. 6 *a*). β_1 and β_3 integrins were diffuse (Fig. 6, *c* and *d*), fibronectin, BSP, and osteopontin appeared distributed in intracellular granules (Fig. 6, *e* and *f*). These results suggest that complete spreading and integrin organization were dependent upon new protein synthesis during the adhesion process. Using antibodies against osteocalcin, a homogeneous staining of the coverslips could be detected and we could observe that after cell adhesion the protein was endocytosed, leaving a typical dark pattern in the sites where cells were or had been attached (not shown).

Chemotactic Activity of Osteocalcin Towards GCT Cells

A chemotactic role of osteocalcin has been already suggested in literature (25, 28, 29) but has never been tested directly on osteoclastic cells. We studied the chemotactic activity of osteocalcin using modified Boyden chamber technique. Fig. 7 shows the effects of various osteocalcin doses on migration of GCT cells in the presence of 1% FCS. A significant migration of the cells at concentrations varying from 2 μM to 6

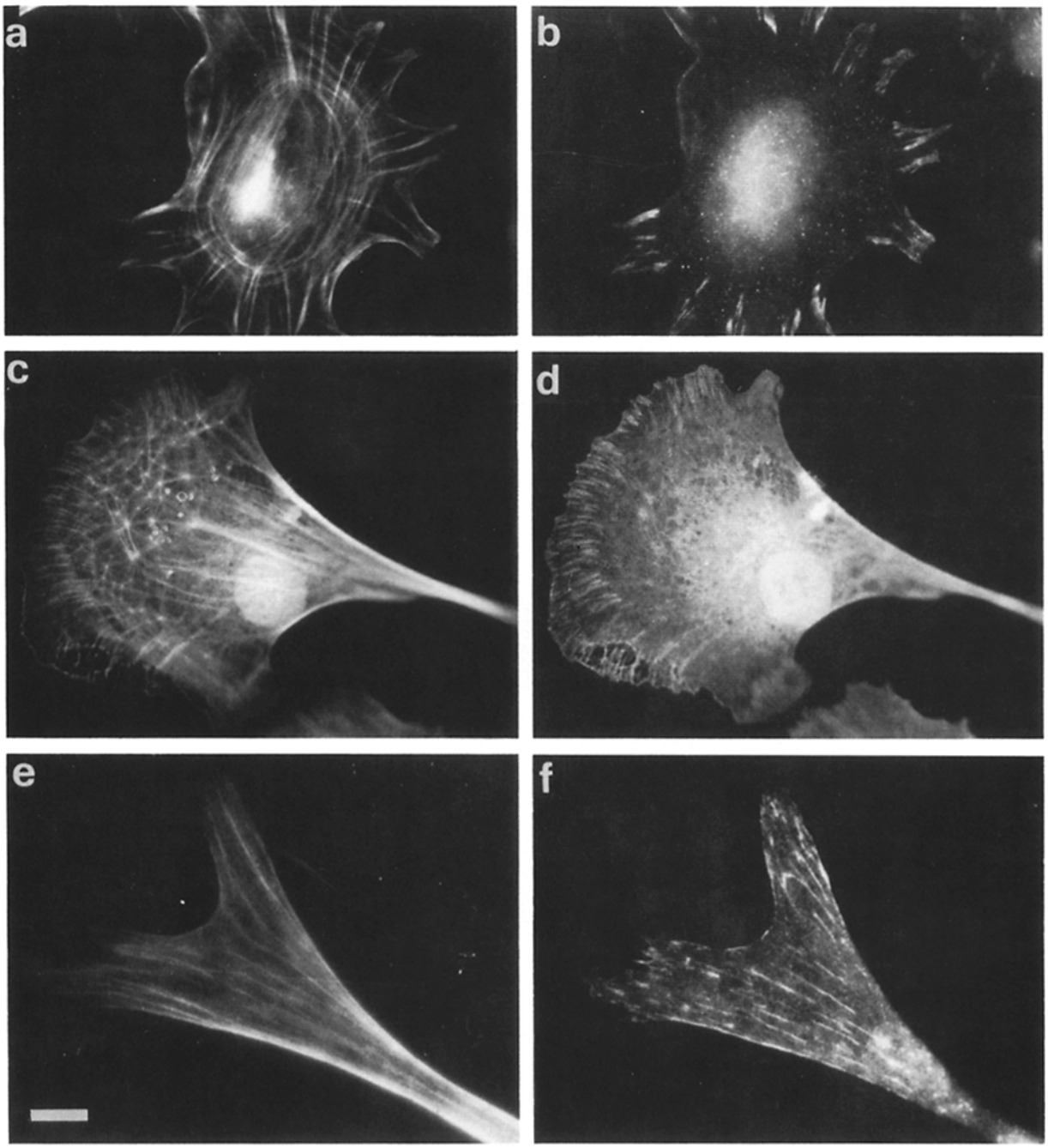

Figure 4. Actin microfilament distribution, vinculin localization, and integrin expression were studied by indirect immunofluorscence in osteoclast-like cells from giant cell tumors of bone cultured 6 h onto osteocalcin-coated coverslips. Actin (*a*, *c*, and *e*) was organized in stress fibers at the edge of which vinculin (*b*) was immunostained, localized in focal adhesions. β_3 integrin chain was immunostained, clustered in focal adhesions (*d*), while β_1 chain (*f*) was present both along stress fibers and in focal adhesions. Bar, 20 μm.

μM with a maximum effect already reached at 3 μM was found. The specificity of the response was established using medium alone supplemented with 1% of serum as a negative control. Experiments were also performed adding osteocalcin at the same concentrations on the cell side of the Boyden chambers and resulted in lack of migration (not shown). These results were reproducible in separate experiments.

Effects of Osteocalcin on $[Ca^{2+}]_i$

To understand the nature of the osteocalcin induced intracellular signals, we investigated the effect of osteocalcin addition on cytosolic free calcium concentrations. As shown in Fig. 8 *a*, $[Ca^{2+}]_i$ in basal conditions was 140 $\pm$ 19 nM, a value similar to what is observed in a number of cell types. Osteocalcin addition at a concentration of 10 μg/ml, induced an increase of $[Ca^{2+}]_i$ peaking at 283 $\pm$ 38 nM, which remained stable several minutes thereafter, followed by a return to a resting condition higher than the previous basal level. Such a response was observed in all the cells tested. Fig. 9 illustrates the dose response curve, testing addition

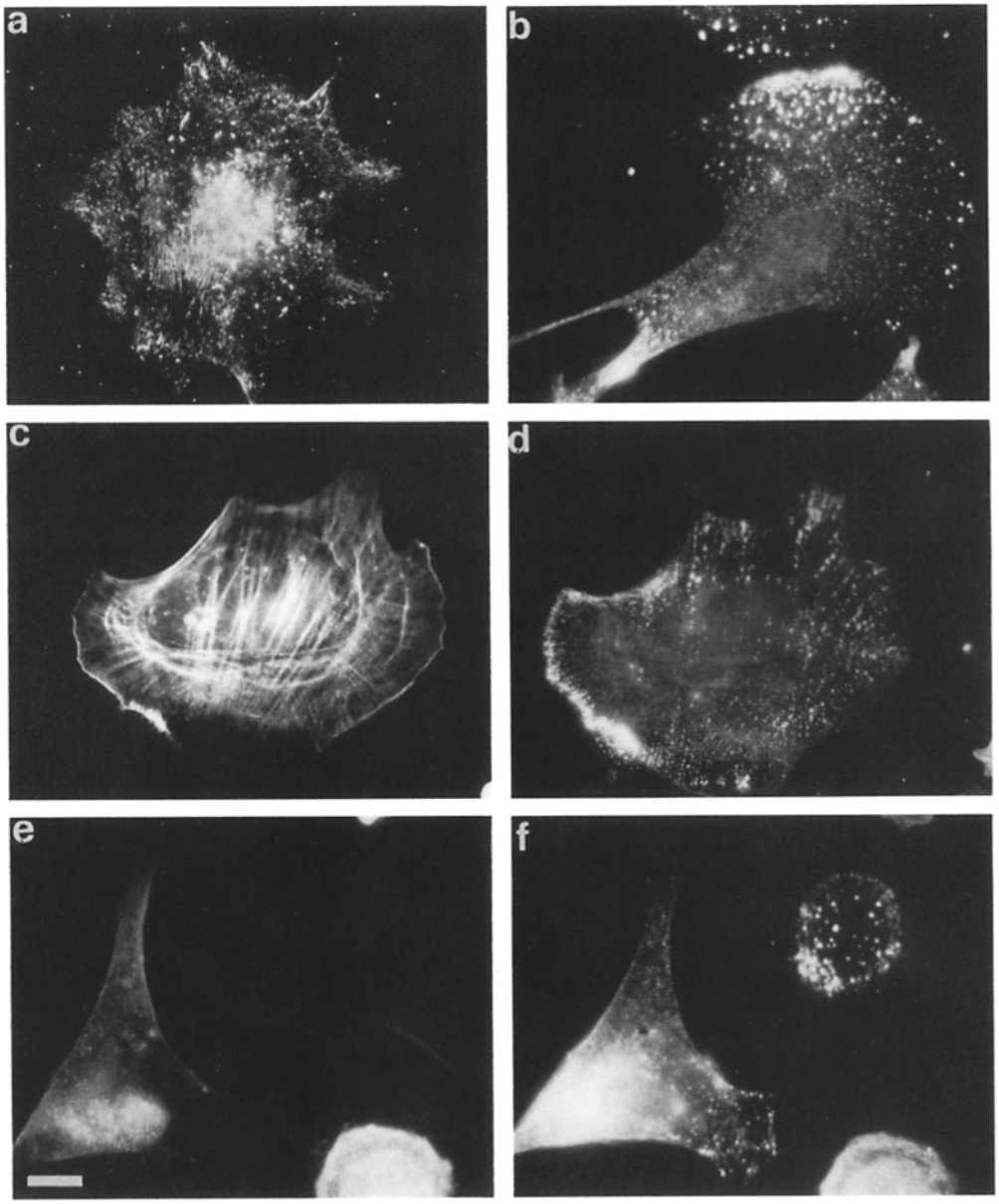

Figure 5. Secretion of matrix proteins was demonstrated for osteoclast-like cells from giant cell tumors of bone. Cellular fibronectin (*a*) was immunodetected by a specific antibody organized in an extracellular network under the basal membrane. BSP was localized in granules crowded at the proximity of the cell surface, suggesting a secretory pathway (*b*). Osteopontin was clearly extracellular with a granular distribution under stress fibers and focal adhesions (*c*, F-PHD; *d*, anti-osteopontin). If a cell had been occasionally detached during the staining procedure osteopontin granules were still present (*f*), while the corresponding cell was lacking in the same field stained for actin with F-PHD (*e*). Bar, 20 μm.

from 10 μg/ml to 100 μg/ml of BGP on $[Ca^{2+}]_i$ of the cells. The response was found to be dose dependent with a maximal increase already observed at a concentration of 20 μg/ml, which remained stable upon addition of 80 μg/ml of BGP and declined as BGP approached a concentration of 100 μg/ml. The increase of $[Ca^{2+}]_i$ induced by BGP could be either due to a Ca^{2+} influx through the plasma membrane or to a Ca^{2+} release from intracellular stores (38). To study the mechanisms by which BGP influenced $[Ca^{2+}]_i$, we used thapsigargin, a tumor promoter known to activate the release of Ca^{2+} from intracellular stores. After treatment with thapsigargin at 4 μM, BGP failed to modify $[Ca^{2+}]_i$ (Fig. 8 *b*), suggesting that the osteocalcin effect on calcium concentration was mainly due to release from intracellular stores. The role of extracellular calcium was investigated performing the experiments in a medium containing 3 mM EGTA. In these conditions, BGP increased $[Ca^{2+}]_i$, but calcium levels after a few minutes returned to the baseline indicating that the increase was dependent upon the release of calcium from intracellular stores, while the plateau phase was maintained by the entrance of extracellular calcium (Fig. 8 *c*).

Discussion

The results reported in this paper indicate that osteocalcin acts as a specific signal from the bone matrix for migration and adhesion of human osteoclasts and that intracellular calcium acts as a second messenger in this system.

Osteoclast-like cells, obtained from GCTs, used for these experiments represent a valid model as they present most of the characteristics of human osteoclasts, as multinucleation, tartrate resistant acid phosphatase activity, calcitonin receptors and ability of resorbing bone (16). They are at present

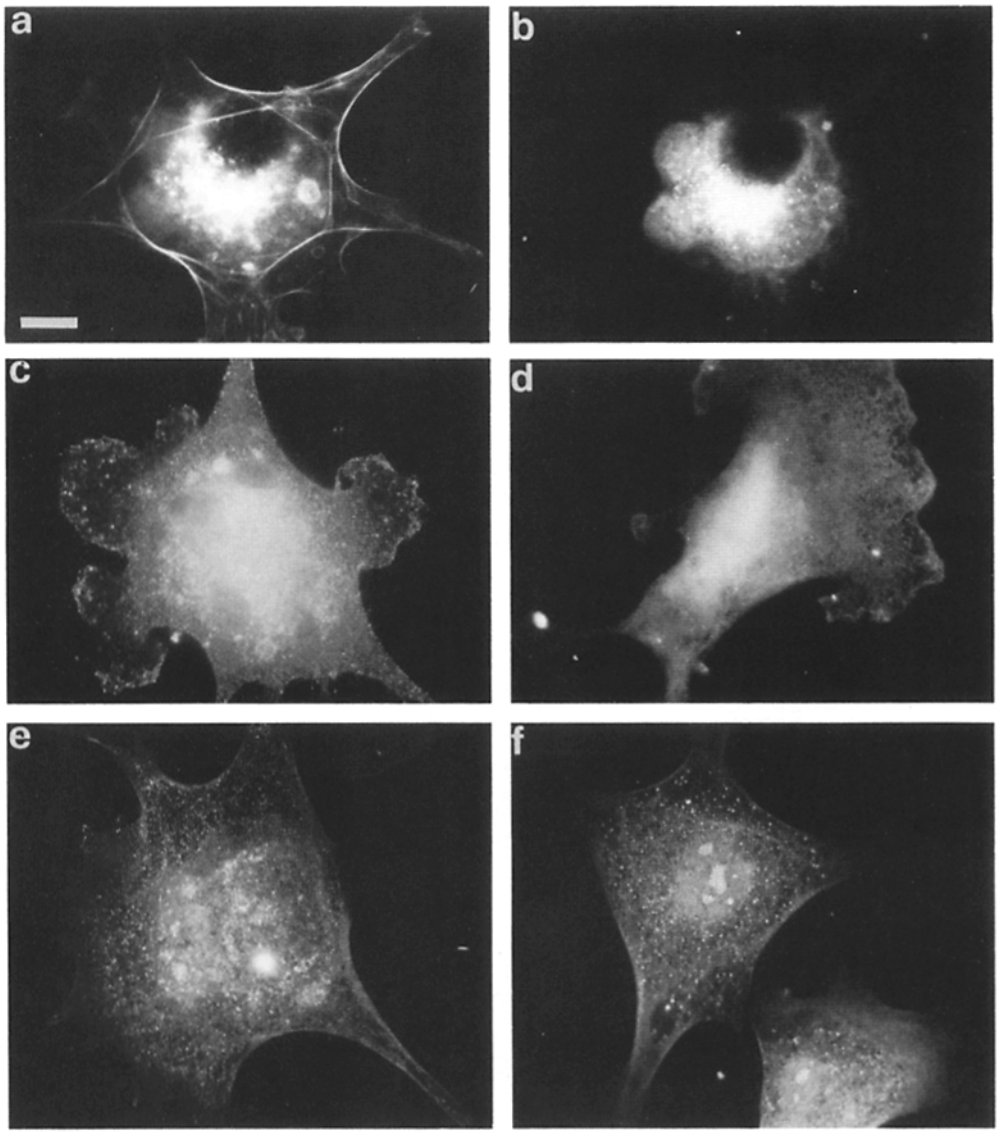

Figure 6. Osteoclast-like cells were plated onto osteocalcin in the presence of monensin. Focal adhesions were absent, and actin, detected with F-PHD, was diffused or organized in a few fibers (*a*). β_1 (*c*) and β_3 (*d*) integrins were diffused. Fibronectin (*b*), osteopontin (*e*), and BSP (*f*) were distributed in intracellular granules. Integrins and matrix proteins were detected by indirect immunofluorescence with specific antibodies. Bar, 20 μm.

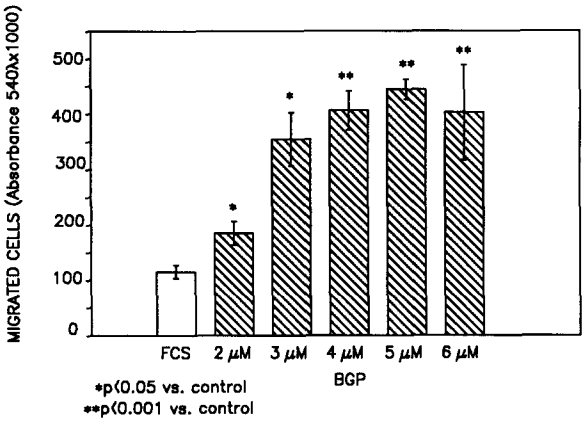

Figure 7. Chemotactic activity of osteocalcin toward osteoclast-like cells was measured by the modified Boyden chamber technique. Different concentrations (2–6 μM) of osteocalcin in Iscove medium containing 1% FCS were seeded in the lower compartment of the chambers, and 1.5 × 10^5 GCT cells, in the same medium, were seeded in the upper compartments. The chambers were incubated for 5 h at 37°C in a 5% CO_2/95% water-saturated atmosphere. Cells attached to the upper side of the filter were mechanically removed, while cells that had migrated to the lower side were fixed with 3% paraformaldehyde in cacodylate buffer, stained in 0.5% Crystal Violet and lysed in 0.1 N sodium citrate to read the absorbance at 540 nm. Negative control was medium alone. Three chambers were used for each treatment and final activity was expressed as mean ± SE of triplicates.

the only available model for human osteoclasts in vitro, nevertheless their transformed origin should be remembered in evaluating the obtained results.

Osteoclasts resorb bone by a complex process which implies their migration to the bone surface, adhesion to the matrix, followed by polarization and organization of sealing zone and ruffled border, allowing the resorption process to take place. Because of the impaired resorption of osteocalcin-deficient bone particles, osteocalcin has been speculated to be one of the putative signals arising from the bone matrix, affecting osteoclast recruitment and differentiation (10, 14, 15, 31). While osteocalcin was previously shown to

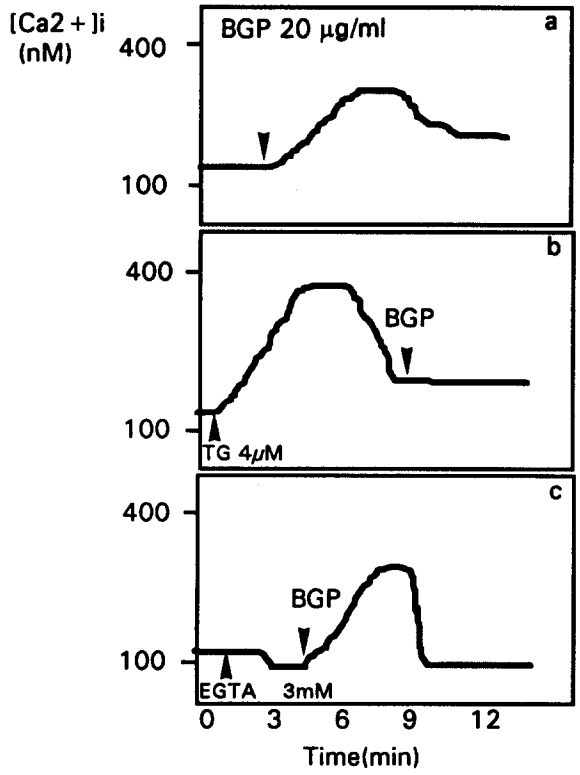

Figure 8. The effect of osteocalcin on intracellular calcium concentration was determined in fura-2 loaded single GCT cells. Cells were equilibrated in Krebs-Ringer-Hepes buffer (KRH) for 10 min at 37°C, and then 20 μg/ml of BGP was added (*arrows*). $[Ca^{2+}]_i$-dependent fluorescence was measured with a microfluorometer. Osteocalcin produced an increase in cytosolic calcium, followed by a plateau which lasted several minutes (*a*). $[Ca^{2+}]_i$ elevation induced by BGP was abolished in cells preincubated with 4 μM thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (*b*), while experiments performed in Ca^{2+}-free buffer by adding 3 mM EGTA (*c*) showed the absence of the plateau phase.

be chemotactic for a number of cell lines (25, 28, 29), this is the first study that demonstrates a chemotactic activity of osteocalcin towards osteoclast-like cells, confirming that it may represent a component of bone matrix involved in the mechanism for attraction of the osteoclasts to the bone surface. Osteocalcin was effective in increasing migration of the GCT cells at the same concentration range effective in the chemotaxis of monocytes in previous study (28, 29).

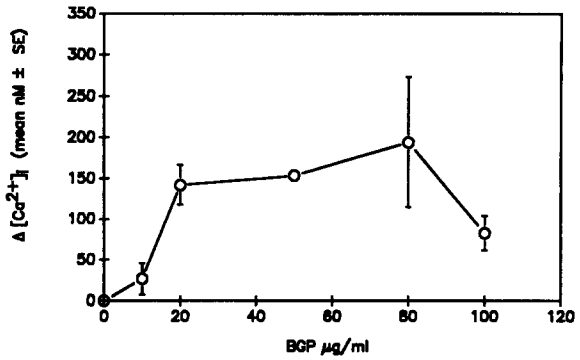

Figure 9. Dose dependence of $[Ca^{2+}]_i$ increase elicited by osteocalcin. Results represent the differences from average basal level after addition of growing concentration of osteocalcin. Each value represents the mean of six determinations ± SE.

In this study, we have shown that osteoclast-like cells attach and spread on coverslips coated with osteocalcin. Other authors (20) were not able to find similar results, their studies, however, differ in two respects. They used freshly isolated osteoclasts from different species, while our cells derived from human bone tumors. Moreover, our cells are not completely differentiated, as they still maintain mitotic activity. It is well possible that only less mature osteoclasts react chemotactically to osteocalcin. This is confirmed by the fact that treatment with vitamin D_3, a known differentiating agent, causes complete loss of osteocalcin recognizing capability. It is interesting to remark that this was the only detectable effect after vitamin D treatment, all other parameters of osteoclast differentiation and activity being unmodified.

This is not the first example of cell adhesion to a member of the vitamin K-dependent protein family. Previous cell adhesion to matrix Gla protein (MGP), has been already observed using different cell lines (24). Cell adherence to MGP nevertheless was inhibited by RGD-containing peptides and was requiring intact Gla residues. Our results show no difference in cell adhesion using decarboxylated osteocalcin, suggesting that the cell recognition site is not containing the Gla residues.

The presence of β_1 and β_3 integrins on GCT cells adhering to BGP suggests that full spreading, but not adhesion to osteocalcin, may depend upon new protein synthesis during the assay. Experiments using cycloheximide or monensin during the adhesion assay have shown that the number of GCT cells attached to BGP was not affected by the presence of inhibitors of protein synthesis and secretion. In addition, attachment of GCT cells to BGP was not dependent on RGD sequence as RGD-containing peptides did not inhibit cell adhesion. Nevertheless, monensin almost completely inhibited the spreading of cells on BGP and integrin clustering in focal adhesions was lacking under these conditions. These data suggest that spreading on BGP was dependent on proteins synthesized and secreted by GCT cells. Two adhesive proteins of the bone matrix, BSP and osteopontin, have been recently shown to be expressed by osteoclasts (2, 40), and GCT cells are known to release FN when adhering to various substrata (17). Our results demonstrated positive staining of GCT cells plated onto BGP for BSP, OPN, and FN using appropriate antibodies, indicating secretion of these proteins by osteoclast-like cells from giant cell tumors. This secretion in turn induced the recruitment in focal adhesions of β_1 and β_3 integrins. These results were confirmed by treatment of cells with monensin which induced arrest of the secretion pathway and concentration of BSP, OPN, and FN in intracellular granules. Specific recognition of and adhesion by GCT cells to osteocalcin seems therefore to occur in various steps. Attachment of the cells occurs via an RGD-independent mechanism, this step is followed by a generation of intracellular signals that ends via an unknown cascade in the nucleus, inducing synthesis and secretion of matrix proteins that finally allow spreading via an RGD-dependent mechanism, due to the release by the cells of RGD-containing proteins. We do not know if osteocalcin is recognized via an unknown integrin receptor, but it is unlikely, since the presence

of EGTA in the medium during the adhesion phase was without any effect and integrins require cations to be effective. Competition experiments with osteocalcin in the culture medium during the adhesion assays significantly reduced adhesion only in the presence of monensin. This finding is easily explained by the fact that osteocalcin induces synthesis and secretion of other proteins that affect osteoclast adhesion and spreading via integrin chains. It is possible that protein secretion, without monensin, is induced both in cells that encounter osteocalcin on the coated surface or in solution, and is followed by full spreading, while in the presence of monensin these proteins are not released and competitive inhibition can be found.

The signals generated in cells upon binding to osteocalcin have been studied. Changes in cytosolic Ca^{2+} are known to occur in cells upon recognition of some extracellular matrix proteins (22, 23, 27). We have shown that BGP stimulates an increase of $[Ca^{2+}]_i$ in GCT cells attached to glass coverslips. Similar specific increases of $[Ca^{2+}]_i$ have been demonstrated in GCT cells in response to OPN, BSP and related fragments (30), but while these RGD-containing proteins were inducing a prompt, transient response, the increase of $[Ca^{2+}]_i$ due to osteocalcin was less pronounced and lasting longer before return to the baseline. To establish the mechanism causing this increase in $[Ca^{2+}]_i$, we have tested whether the effect was depending upon Ca^{2+} influx through plasma membrane or upon release from thapsigargin-sensitive intracellular pools. As shown, BGP did not significantly affect the $[Ca^{2+}]_i$ after thapsigargin treatment. On the contrary $[Ca^{2+}]_i$ was also increased in the cells by BGP after addition of the Ca^{2+}-chelating agent EGTA, indicating the provenance of Ca^{2+} from intracellular stores.

In conclusion, this study provides evidences that osteocalcin, a specific protein of the bone matrix, exerts a role in the resorption process, including migration of osteoclasts toward the bone matrix, specific adhesion and intracellular signaling, followed by synthesis and secretion of proteins as osteopontin and BSP. These bone matrix proteins, recognized via $\alpha_v\beta_3$, have been suggested as putative inducers of cell polarization and bone resorption. It is possible that their secretion plays a role in osteoclast functions, contributing to osteoclast "homing" in the bone matrix, when they reach the site where chemotactic stimula were originated.

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