Human osteoclast-like cells selectively recognize laminin isoforms, an event that induces migration and activates Ca^{2+} mediated signals

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SUMMARY

Osteoclast precursors are chemotactically attracted to sites of bone resorption via migration pathways that include transendothelial crossing in blood capillaries. Transendothelial migration involves poorly understood interactions with basal lamina molecules, including laminins. To investigate osteoclast-laminin interactions, we used human osteoclast-like cell lines obtained from giant cell tumors of bone (GCT 23 and GCT 24). These cell lines are a well-characterized model for osteoclast functions, such as bone resorption and the behaviour of osteoclast precursors. Both GCT cell lines adhered to laminin-2 (merosin) coated wells in standard adhesion assays, but failed to adhere to laminin-1 (EHS-laminin). By light microscopy, GCT cells on laminin-2 were partially spread, with a motile morphology. None of the anti-integrin antibodies tested inhibited GCT cells adhesion to laminin-2. Peptides containing the integrin adhesion site RGD or the laminin adhesion sequence IKVAV did not inhibit GCT cell adhesion to laminin-2. By immunofluorescence, β_1 integrins were organized in focal adhesions. However, in the presence of monensin this reorganization of β_1 integrins was abolished, indicating that it was probably due to secretion of fibronectin by GCT cells subsequent to adhesion to laminin-2. GCT cells transmigrated through membranes coated with laminin-2, much more efficiently than through membranes coated with collagen. Migration was induced by osteocalcin, as a chemoattractant, in a dose-dependent manner. At low osteocalcin concentrations, transmigration was detectable on laminin-2 but not collagen. In cells loaded with fura-2, a sharp increase in intracellular Ca^{2+} was detected upon addition of soluble laminin-2, but not laminin-1, due to release from thapsi-gargin-dependent intracellular stores. In summary, osteoclasts may recognize laminin isoforms differentially. Initial adhesion to laminin-2 appears to be due to integrin-independent mechanisms. Such adhesion, though, may trigger secretion of fibronectin that could then support spreading and efficient chemotactic migration. These mechanisms may play an important role in facilitating chemotactic migration of osteoclast precursors toward the bone surface.

Key words: Osteoclast, Laminin, Adhesion, Migration

INTRODUCTION

Osteoclasts are highly motile cells specialized for bone resorption which originate in the bone marrow from the monocyte-macrophage cell lineage (Suda et al., 1992). They migrate to bone sites and during this process encounter different extracellular matrix proteins recognized by integrins or non integrin receptors. The chemotactically traced pathway that attracts osteoclasts to bone is poorly understood. We recently demonstrated chemotactic activity of osteocalcin for osteoclast-like cells, confirming that this protein may represent a component of bone matrix involved in the mechanism for attraction of the osteoclasts to the bone surface (Chenu et al., 1994). Bone matrix is very selectively resorbed when and where needed during skeletal morphogenesis and during bone remodeling in adult life. Osteoclast precursors must migrate from the bone marrow to their final resorption sites, in order to reach surfaces located as far away as the compact bone, where new haversian channels are formed. Because this migration may utilize blood vessels as a transport route, osteoclast precursors can encounter basal membranes during their transendothelial migration. The supramolecular structure of basal membranes is mainly formed by laminin, which can self aggregate and form a protein meshwork with type IV collagen (Yurchenco and Schittny, 1990; Yurchenco et al., 1992).

Laminins are heterotrimeric cross-shaped molecules (Timpl et al., 1979; Cooper et al., 1981; Tryggvason, 1993). A new nomenclature for laminins has been proposed (Burgerson et al., 1994), in which the previously designated A, B1 and B2 chains are renamed α, β and γ. The corresponding isoforms are numbered LN-1, LN-2, LN-3, LN-4, LN-5, LN-6, LN-7, and another two isoforms not yet completely characterized exist (Yurchenco and O'Rear, 1994). It is well known that interactions between cells and laminins are mediated by integrin or non-integrin cell surface receptors (Mercurio and Leslie, 1991). Among members of the β_1 integrin family α_1, α_2, α_3, α_6, and α_7 are known to recognize sites on the LN molecule (Hall et al., 1990; Ignatius et al., 1990; Languino et al., 1989;

Lotz et al., 1990; Gehlsen et al., 1989; Sonnenberg et al., 1990; Shaw et al., 1990; Kramer et al., 1989), $\alpha_6\beta_4$ also binds laminin, but its recognition site has not been identified. Some non-integrin proteins bind sequences or domains such as a binding site on the α_1 long arm of laminin-1, the IKVAV-containing peptide (Sephel et al., 1989; Weeks et al., 1994), and a sequence of nine amino acids in domain III of the β_1 chain, the pentapeptide YIGSR (Graf et al., 1987) responsible for cell attachment.

Laminin recognition promotes many biological effects, including adhesion, spreading, proliferation and differentiation (Martin and Timpl, 1987; Kleinman et al., 1985). In this study we investigated the interaction of osteoclasts with laminin in a model system of human tumor derived osteoclast cell lines (GCTs). These cell lines have previously been shown to faithfully reproduce many properties of primary osteoclasts, such as multinuclearity, calcitonin receptor, calcitonin inhibitable bone resorption, TRAP content (Grano et al., 1994a). We report that GCTs do not adhere or migrate on LN-1, while they adhere and migrate on LN-2 (merosin), but via non-integrin receptors.

MATERIALS AND METHODS

Cell cultures

GCT 23 and GCT 24 are osteoclast-like cell lines obtained from human giant cell tumors of bone (GCTs) and stabilized with passages. The osteoclastic phenotype of these lines has been extensively characterized (Grano et al., 1994a). Within these cells mulitplenucleated elements keep forming, deriving both from fusion and endomitosis. Both mononuclear and multinuclear cells are capable of calcitonin inhibitable bone resorption and are TRAP positive to various degrees. GCTs are maintained in culture in Iscove medium supplemented with 10% fetal bovine serum (FBS, Gibco Limited, Uxbridge, UK), 100 i.u./ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B and 50 i.u./ml mycostatin (Eurobio, Paris, France), at 37°C, in a water saturated atmosphere with 5% CO_2 and fed by medium replacement every 2-3 days. Cells used for the experiments were all from IX and XIV passages.

A bovine bone endothelial cell line, clone BBE-1, kindly provided by M. L. Brandi (Dipartimento di Fisiologia Clinica, Unita' di Endocrinologia, Università degli Studi di Firenze, Italy) was used in some experiments and cultured in Coon's modified Ham's F-12 medium containing 10% Nu-serum, 1% Ultraser-G and 200 mg/l galactose.

Proteins

Laminin from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma (LN-1), laminin from human placenta (LN-2), laminin fragments (2,091-2,108 from A chain (Cys-Ser-Arg-Ala-Arg-Lys-Gln-Ala-Ala-Ser-Ile-Lys-Val-Ala-Val-Ser-Ala-Asp-Arg) and 925-933 from B1 chain (Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg)), and plasma fibronectin (FN) were from Sigma Chemical Co (St Louis, MO). The synthetic peptides GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) and GRGESP (Gly-Arg-Gly-Glu-Ser-Pro) were purchased from Telios (La Jolla, CA).

Antibodies

Antibodies to integrin subunits were as follows: β_3 rabbit polyclonal serum against platelet GpIIIa (Dejana et al., 1988); β_1 monoclonal antibodies MAR4 (Pellegrini et al., 1992) kindly provided by Dr S. Ménard (Istituto Tumori, Milano, Italy) and BV7 by Dr G. Tarone (Dipartimento di Genetica, Biologia e Chimica Medica-Sezione di

Biologia-Università di Torino, Italy); α_3 monoclonal antibodies mAb F1 and MasF4a, kindly provided by Dr Zardi, PIB5 (Beckton Dickinson-San Jose Ca-USA) (Takada et al., 1988) or J145, kindly provided by Dr A. Albino USA; mAb IST-9 to the extra-domain (ED) specifically expressed by cell-assembled FN and not by plasma FN (Borsi et al., 1987; Carnemolla et al., 1987) were kindly provided by Dr L. Zardi (Istituto Scientifico per lo Studio e la Cura dei Tumori, Genova, Italy); mAb anti-α_v (69-6-5) (Lehmann et al., 1994), kindly provided by Dr Marvaldi (France). The localization of vinculin was assayed with mAb VIN (Dejana et al., 1988; Carnemolla et al., 1987) to chicken gizzard vinculin and cross-reacting with the mammalian form (Sigma, St Louis, MO).

Morphology

GCT cells were plated onto coverslips coated with 10 µg/ml of LN-2 and incubated in serum-free medium with or without monensin, and onto uncoated coverslips in the presence of 10% serum to evaluate cell morphology. After 1 or 6 hours incubation, cells were fixed with glutaraldehyde, pH 7.4, for 10 minutes, stained with 0.1% crystal violet, extensively washed in distilled water, and observed with a Zeiss Universal microscope. Images were recorded on Kodak T-Max 100 films and developed in a Kodak T-Max developer.

Adhesion

Adhesion assays were performed on 96-well microtiter plates coated overnight with 10 µg/ml LN-2 or LN-1 (diluted in 0.05 M Tris-HCl, pH 8.2, 0.3 M NaCl), at 4°C or with 10% FBS or 0.5% BSA, respectively, used as positive and negative control. Experiments were performed in serum-free medium, containing 0.5% BSA. After trypsinization cells were counted, diluted at a density of 2.5×10^5/ml, and 200 µl of GCT cell suspension, containing 50,000 cells, were placed in each microtiter well for 1 hour 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 minutes at room temperature (RT), followed by rinsing with PBS, air-dried and stained with 0.5% crystal violet for 15 minutes, followed by extensive rinsing. The dye was released from the cells by the addition of 0.1 M Na-citrate in 50% ethanol. The optical density of the released stain solution was read in a Titertek colorimeter at 540 nm. Similar experiments were also performed by plating the cells onto LN-2 or FN coated wells in the presence or in the absence of monensin in the medium. Some experiments were performed in the presence of 200 µg/ml of GRGDSP or GRGESP peptides or 50 µg/ml of 2,091-2,108 α fragment, containing the IKVAV sequence.

Results were expressed as percentage ± s.e.m. of the absorbance read in control samples.

Antibodies against β_1(BV7) (2 and 7 µg/ml), α_3 (PIB5 and J145) (from 1 to 20 µg/ml) and α_v (69-6-5) (5 and 10 µg/ml) were added during the adhesion assays onto LN-2 or FN at different concentrations in the presence or in the absence of monensin.

Results were expressed as percentage ± s.e.m. of the absorbance read in control samples represented by adhesion onto laminin.

Immunofluorescence

Coverslips were coated with 200 µl of laminin-2 (10 µg/ml) in 0.05 M Tris-HCl, pH 8.2, 0.3 M NaCl, for 15-20 hours at 4°C. Residual protein binding sites on coverslips were saturated by further incubation (30 minutes, RT) in a buffer containing 1% BSA (bovine serum albumin, fatty acid free; Sigma, St Louis, MO). Coverslips were then washed three times with Iscove medium plus 0.5% BSA.

Freshly detached cells were resuspended in the same Iscove medium with or without 10 µg/ml monensin (Mon) (Sigma, St Louis, MO), and seeded onto coated coverslips.

After 1 or 6 hours of incubation at 37°C, coverslips were washed three times with Ca^{2+}, Mg^{2+} PBS and fixed in 3% paraformaldehyde, 2% sucrose in PBS, pH 7.6, for 10 minutes at RT. After rinsing in

PBS, cells were made permeable to antibodies by soaking coverslips for 3 minutes at 0°C in Hepes-Triton X-100 buffer (20 mmol/l Hepes, pH 7.4, 300 mmol/l sucrose, 50 mmol/l NaCl, 3 mmol/l MgCl$_2$ and 0.5% Triton X-100) from Sigma. This procedure of fixation and permeabilization has been described (Dejana et al., 1988).

For indirect immunofluorescence, primary antibody was layered on fixed and permeabilized cells and incubated in a humidified chamber for 45 minutes 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 minutes at 37°C. After rinsing in PBS, coverslips were incubated with 25 μg/ml fluorescein-labeled phalloidin (F-PHD, from Sigma, St Louis, MO) for 45 minutes at 37°C. Stained coverslips were then mounted in 20% Mowiol 4-88 (Hoechst AG, Frankfurten on Main, Germany). Observation were performed by epifluorescence in a Zeiss axioplan microscope. Fluorescence images were recorded on Kodak T-Max 400 films and developed in a Kodak T-Max developer for 10 minutes at 20°C.

Measurement of cytosolic free calcium concentration

Cytosolic free calcium concentrations ($[Ca2+]_i$) in response to addition of laminin-1 or laminin-2 at concentrations ranging from 10 to 70 μg/ml, were evaluated in single cells loaded with the intracellular Ca^{2+} indicator fura-2 (Sigma, Chemical Co., St Louis MO). GCT cells cultured onto 24 mm diameter round coverslips were loaded with 10 μM fura-2/AM in serum-free, but otherwise complete, IMDM for 1 hour 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) (125 mM NaCl, 5 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2 mM CaCl$_2$, 265 mM Hepes and 6 mM glucose). $[Ca^{2+}]_i$-dependent fluorescence was measured with a microfluorometer (Cleveland Bioinstrumentation, Cleveland, OH) connected to 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 the real-time 340-to-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 ethylene glycol-bis(β-aminoethyl ester)-*N,N,N′,N′*-tetracetic acid (EGTA) to obtain Ca^{2+}-saturated and nominally Ca^{2+}-free fura-2 fluorescence, respectively. Thapsigargin (Sigma, MO) was utilized to deplete intracellular pools and 3 mM EGTA to chelate extracellular calcium. $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. (1985).

Cell migration

Chemotactic assays were done as previously described (Dejana et al., 1985; Bussolino et al., 1989) with modified Boyden chamber technique. Polycarbonate filters (8 μm pore size polyvinyl-pyrrolidone-free; Nucleopore Corp., Pleasanton, CA) were coated with 10 μg/ml collagen or 10 μg/ml laminin-2. Osteocalcin, already demonstrated to be chemotactic for GCT cells, was seeded in the lower compartment of the chambers in the presence of 1% FCS at concentrations from 1 to 4 μM. Cells (1.5×10^5) suspended in medium containing 1% FCS, were seeded in the upper compartment. After 5 hours of incubation 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 through coated filters were fixed with 3% paraformaldehyde in PBS, stained in 0.5% crystal violet and lysed in 0.1 M sodium citrate to read the absorbance at 540 nM. Negative control was medium alone.

Control experiments were also performed to evaluate cell migration through porous filters coated with 10 μg/ml of LN-1, utilizing osteocalcin as chemoattractant. The experiments were performed in tripli-cate and cell migration activity was expressed as mean ± s.e.m. of the triplicates.

Statistical analysis

Quantitative data are expressed as average ± s.e.m. Statistical analysis was performed by Student's *t*-test.

RESULTS

Adhesion onto LN

GCT 23 and GCT 24 are recently established cell lines well characterized for their osteoclastic phenotype (Grano et al., 1994a); they contain mono and multinuclear osteoclast-like cells that continuously divide in culture, giving rise to other mononuclear or multinuclear cells. Adhesion of GCTs to LN was quantitatively evaluated on cells plated onto Titertek wells coated with LN-1 and LN-2. Positive and negative controls were FCS and BSA, respectively. GCTs promptly recognized LN-2 with an adhesion percentage of 79±2 versus FCS, while adhesion to LN-1 was negligible, similar to the negative control (Fig. 1A). No adhesion was found at higher concentrations of LN-1 (10 to 80 μg/ml) (not shown). Bovine bone endothelial cells (BBE-1), utilized as controls, adhered onto LN-2 or onto LN-1 in a similar way (Fig. 1B). The morphological aspect of GCTs 23 and 24, seeded in serum-free medium onto LN-2 coated coverslips or in control conditions in the presence of serum was evaluated. Cells plated in the presence of serum onto uncoated coverslips were well spread,
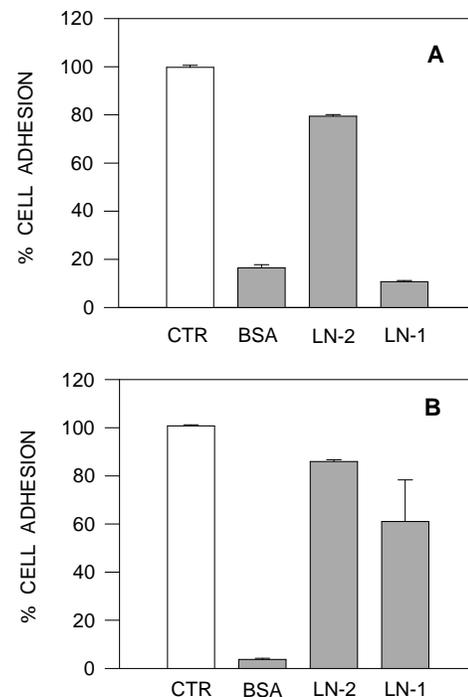
Fig. 1. Adhesion of GCT cells (A) and BBE cells (B) plated onto wells coated with LN-2, LN-1, 10% serum (CTR) or 5% BSA in Iscove medium. Cells were trypsinized, resuspended in serum-free medium and then seeded on coated wells for 60 minutes at 37°C. Attached cells were evaluated by Titertek technique. Results represent the mean ± s.e.m. of three experiments performed in triplicate. All values are expressed as percentage versus control.
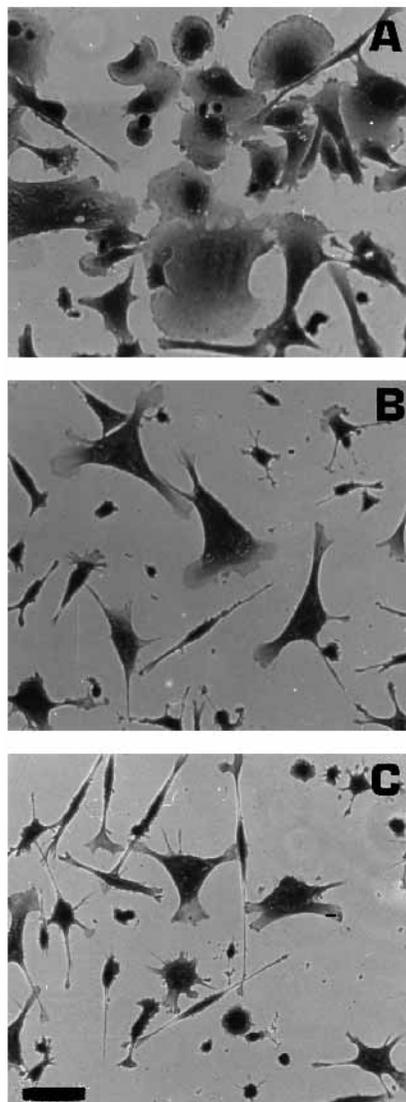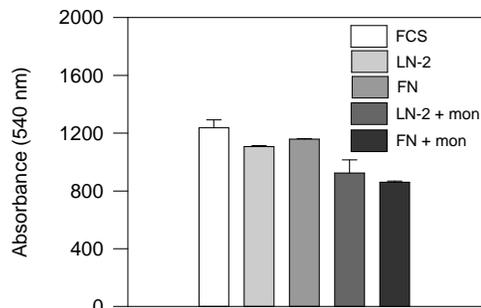
Fig. 2. Morphology of GCT cells plated onto uncoated coverslips in the presence of serum (A) or onto coverslips coated with LN-2 in serum-free medium without (B) or with (C) monensin. In control conditions cells are almost completely spread with a rounded shape (A), while on LN-2 cells appear elongated and motile (B,C). Bar, 25 μm.

Fig. 3. Adhesion of GCT cells plated onto wells coated with 10 μg/ml of LN-2 or FN was evaluated in the presence or in the absence of monensin in the medium. Results were expressed as absorbance values of cell adhesion versus cells plated onto FCS.

Table 1. Adhesion assay onto laminin or fibronectin coated wells in the presence of peptides containing adhesive sequences

Substrates	Control	GRGDSP % vs control	GRGESP % vs control	IKVAV containing peptide % vs control
LN-2	100±7.0	92±4.0	100±8.0	96±5.0
FN	100±2.0	40±2.0*	70±3.0**	108±5.0

Cell adhesion is expressed as percentage of adhesion to LN-2 or FN in control conditions. *$P<0.001$; **$P<0.05$.

displaying a mostly rounded appearance (Fig. 2A). In contrast, cells plated onto LN-2, with or without monensin in the medium, displayed large pseudopodia or membrane ruffling, suggesting a motile status (Fig. 2B,C).

GCT cells, upon recognition of and adhesion onto several extracellular matrix substrata, secrete fibronectin (Grano et al., 1994b). To prevent interference of such a secretion (FN) during adhesion assays, experiments were also performed in the presence of monensin. Although overall adhesion was ~20% lower, results were substantially unchanged (Fig. 3), and LN-2 recognition was not inhibited (Fig. 3).

Since we had already demonstrated that GCTs express several integrin chains (Grano et al., 1994b) involved in laminin recognition such as β_1, β_3, α_v, α_3, adhesion assays onto LN-2 in the presence of functional anti-β_1, anti-α_v and anti-α_3 antibodies were performed (Fig. 4). In the presence of anti-α_3 antibodies no inhibitory effect was found (data not shown), while the presence of anti-β_1 monoclonal antibody (BV7) tested at two different concentrations inhibited cell adhesion onto LN-2 of 40% with respect to controls (Fig. 4A). However, the presence of monensin anti-β_1 antibody, at

the same concentrations, failed to inhibit cell adhesion onto LN-2 (Fig. 4B).

These results suggest that $\alpha_3\beta_1$, or other β_1 containing integrins were not involved in GCT recognition and adhesion to LN-2, and that the 40% inhibition found with anti-β_1 was due to self-produced FN. Adhesion inhibition experiments with anti-α_v antibodies, known to be associated with β_3 in the osteoclasts (Grano et al., 1994b), with or without monensin in the medium showed that in both conditions adhesion to laminin was not modified (Fig. 4D-C). The role of GRGDSP and of its control GRGESP as well as of IKVAV-containing peptides, on osteoclast adhesion onto LN-2, was also evaluated. Neither GRGDSP nor GRGESP inhibited adhesion to LN-2, while GRGDSP partially inhibited adhesion onto FN. IKVAV-containing peptides had no effect (Table 1).

Cytoskeletal organization and integrin expression

Cytoskeletal organization and integrin expression of cells plated and maintained for 1 hour or 6 hours (to allow complete spreading), onto coverslips coated with LN-2 or in control conditions in the presence of serum were analyzed by immunofluorescence. After 1 hour spreading was not complete in the majority of the cells; β_1, immmunostained with anti-β_1 mAb (MAR-4), was diffuse or, in a few cells, organized in focal adhesions (both on LN-2 or in the presence of serum) (not shown). After 6 hours GCTs plated onto LN-2 showed β_1 integrin chain clustered in focal adhesions along and at the end of stress-fibers (Fig. 5A), while this subunit appeared diffuse in cells plated in the presence of monensin (Fig. 5B). All the other integrins tested, such as α_v or α_5, gave a diffuse staining in both conditions (not shown). To confirm that the presence of β_1 in focal adhesions was caused by the secretion of cellular
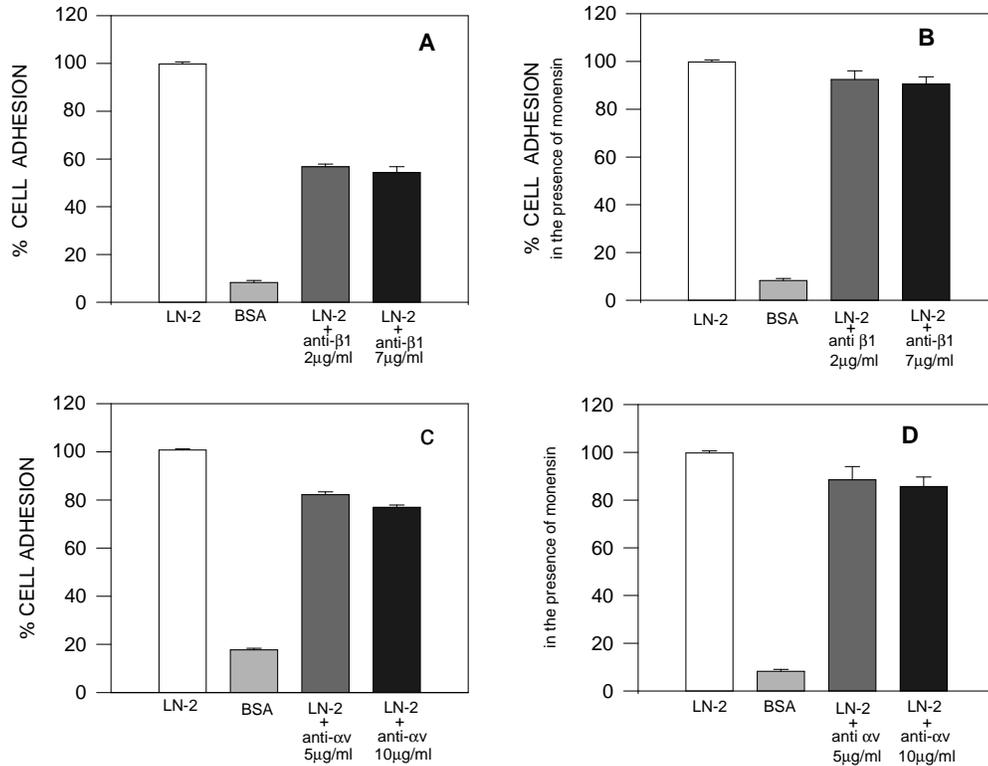
Fig. 4. GCT cells adhesion onto LN-2 in the presence or in the absence of anti-β_1 (BV7) or anti-α_v (69-6-5) antibodies was evaluated by Titertek technique. Mean % osteoclast adhesion is expressed compared to adhesion without antibodies (=100%). Anti-β_1 caused a 40% inhibition of cell adhesion (A), abolished in the presence of monensin (B). Anti-α_v failed to modify adhesion both in the absence (C) or in the presence (D) of monensin.

FN, osteoclast-like cells were immunostained with mAb against cellular FN (IST-9) and costained for F-actin with F-PHD both after 1 and 6 hours (Fig. 6). After 1 hour FN was already secreted and organized in fibrils underneath the ventral membrane of about 50% of the cells, while under the others it presented a granular, not yet organized, pattern (not shown). After 6 hours FN was organized in a fibrillar network corresponding to stress fibers (Fig. 6B); in the presence of monensin, however, F-actin organization (Fig. 6C) was similar to the control (Fig. 6A), while FN was retained in intracellular granules (Fig. 6D). These results indicated that β_1 clustering in focal adhesions was dependent upon FN secretion.

Cell migration

Because laminin is a protein encountered by preosteoclasts during their transendothelial migration and because morphological observations indicated a motile status for these cells on LN-2, the possibility that LN-2 could affect GCT motility was investigated. Cell migration through porous membranes toward the osteoclast chemoattractant osteocalcin (Chenu et al., 1994) was performed by coating membranes of modified Boyden chambers with LN-2 or collagen. Cell migration through both LN-2 and collagen coated membranes occurred in a dose-dependent manner, but at low chemoattractant concentration (1μM) cell migration was evident only through LN-2 coated membranes (Fig. 7). In similar experiments performed with LN-1 coated membranes, cell migration was not found, stressing that cell adhesion to this protein did not occur (data not shown).

Effect of laminin on [Ca2+]i

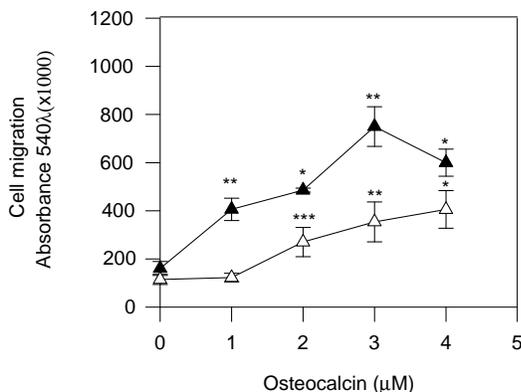Extracellular matrix proteins can activate a cascade of intra-
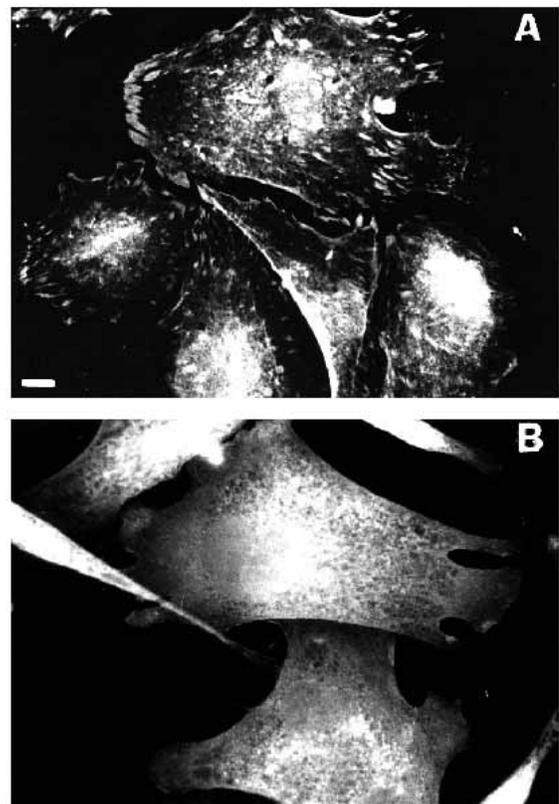
Fig. 5. GCT cells, cultured for 6 hours onto LN-2 coated coverslips, were immunostained with anti-β_1 mAb (MAR4). β_1 was clustered in focal adhesions (A) while in the presence of monensin appeared diffuse on the plasma membrane (B). Bar, 5 μm.
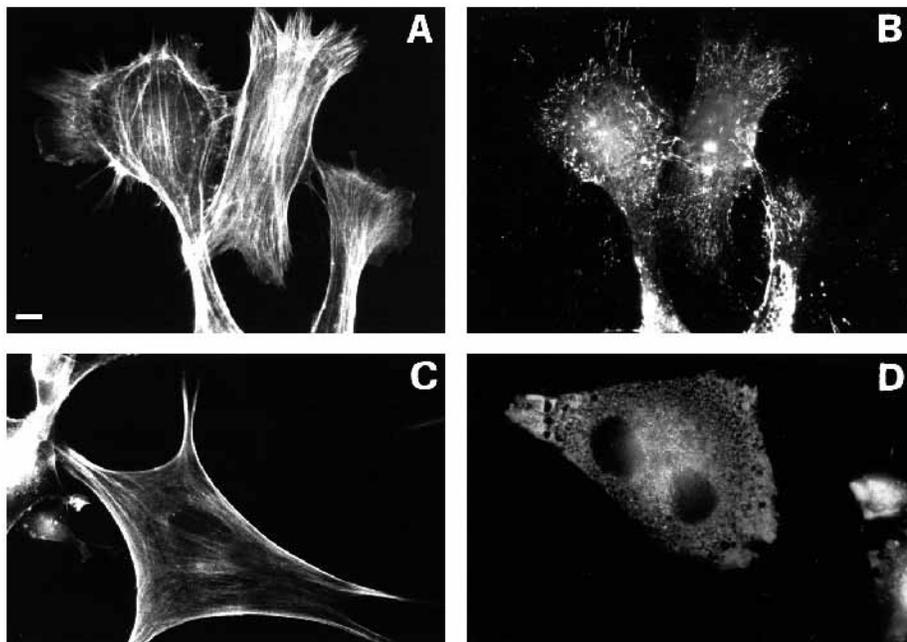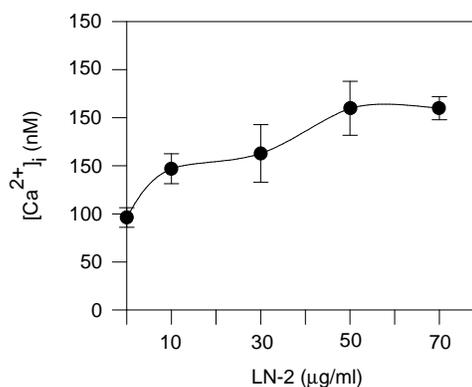
Fig. 6. GCT cells, cultured for 6 hours onto LN-2 coated coverslips, were immunostained with mAbto detect cellular FN (IST-9) and costained with F-PHD for F-actin. Actin microfilaments were organized in stress fibers (A,C), FN was secreted and organized in a fibrillar network corresponding to stress fibers (B) but in the presence of monensin was retained in intracellular granules (D). Bar, 25 μm.

cellular signals, including variation of intracellular calcium concentration $[Ca^{2+}]_i$ (Schwartz, 1992). In mammalian osteoclasts $[Ca^{2+}]_i$ is increased by a variety of extracellular signals, such as integrin occupancy (Shankar et al., 1993; Paniccia et al., 1993) or non integrin-mediated binding to bone matrix proteins such as osteocalcin (Chenu et al., 1994). The effect of LN-2 on $[Ca^{2+}]_i$ was measured in single GCTs by a fluorometric method, after loading with the Ca^{2+} sensitive probe fura-2. The $[Ca^{2+}]_i$ in basal conditions was 102±12 nM. The effect of laminin addition was evaluated at concentrations ranging from 10 to 70 μg/ml (Fig. 8), to find a dose-dependent intracellular calcium increase. The maximum response was reached at 50 μg/ml of LN-2; increasing the LN concentration beyond 50 μg/ml did not further modify intracellular calcium concentration. Upon the addition of 50

μg/ml of LN-2 an increase in $[Ca^{2+}]_i$, peaking at 210±26 ,followed by a sustained phase at peak values and a slow decrease reaching a plateau at 165±22 nM (Fig. 9a), was measured. To investigate whether the LN-2 induced $[Ca^{2+}]_i$ increase was dependent upon Ca^{2+} release from intracellular stores these were depleted by treatment with the Ca^{2+}-ATPase inhibitor, thapsigargin (Takemura et al., 1989). As expected, the treatment produced a transient increase in $[Ca^{2+}]_i$ (345±24 nM) slowly returning toward the baseline (149±24 nM). Under these conditions, addition of LN-2 failed to generate a further increase in $[Ca^{2+}]_i$ (Fig. 9b), indicating a role for intracellular stores in LN-2 induced $[Ca^{2+}]_i$ increase. To investigate if extracellular calcium was also involved, experiments were performed in a nominally calcium-free buffer. Under these conditions, LN-2 still induced the $[Ca^{2+}]_i$ increment (88±10 to 219±30 nM), but the peak was followed by an immediate return toward the


Fig. 7. GCT cells migration through porous membranes coated with LN-2 (▲) or collagen (△) toward increasing concentrations of osteocalcin, as chemoattractant, in modified Boyden Chambers. Cell migration occurs in a dose dependent manner through filters coated with both proteins, but remains always higher through LN-2 coated filters. *$P<0.001$ vs control; **$P<0.01$ vs control; ***$P<0.005$ vs control.

Fig. 8. Dose dependence of $[Ca^{2+}]_i$ increase elicited by laminin. Results represent the differences from average basal level after addition of increasing concentrations of laminin. Each values represents the mean of six determinations ± s.e.m.
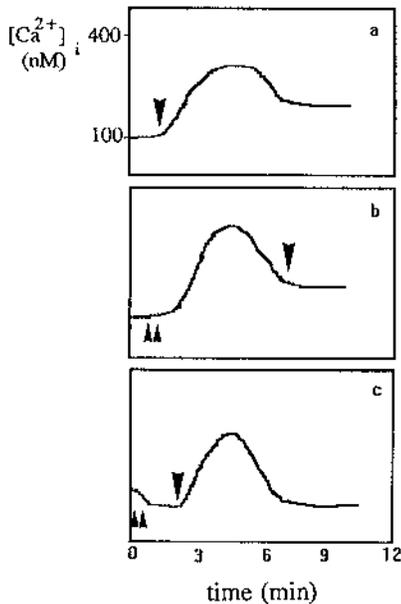
Fig. 9. The effect of LN-2 on intracellular calcium concentration was determined in fura-2 loaded single GCT cells. Cells were equilibrated in Krebs-Ringer-Hepes buffer (KRH) for 10 minutes at 37°C, and then 50 μg/ml of LN-2 was added (single arrowheads). $[Ca^{2+}]_i$-dependent fluorescence was measured with a microfluorometer. LN-2 produced an increase in cytosolic calcium, followed by a plateau which lasted several minutes (a). $[Ca^{2+}]_i$ elevation induced by LN-2 (single arrowheads) was abolished in cells pre incubated with 4 mM thapsigargin (double arrowheads) an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (b), while experiments performed in Ca^{2+}-free buffer by adding 3 mM EGTA (double arrowheads) showed no plateau phase (c).

baseline (109±19 nM) and the plateau was abolished (Fig. 9c), suggesting that the extracellular calcium rise was not involved in the $[Ca^{2+}]_i$ increase but, as already known, was responsible for the plateau phase due to store replenishment (Girard and Clapham, 1993).

Similar experiments were performed utilizing LN-1 at concentrations between 10 and 50 μg/ml, but no significant variations in intracellular calcium concentration were found.

DISCUSSION

Osteoclast precursors during skeletal morphogenesis and bone remodelling migrate from bone marrow areas through blood vessels to reach the bone sites that have to be resorbed. The presence of laminin in basement membranes suggests that osteoclasts would encounter this protein during some stages of this migration. The data presented in this paper demonstrate a highly specific interaction, apparently not mediated by integrins, between osteoclasts and LN-2. The same cells did not recognize LN-1. Other cells, published in the literature and confirmed by our control experiments as bovine bone endothelial cells (BBE-1), did not discriminate between the two laminin isoforms. Other authors recently reported that mature osteoclasts recognize different laminin isoforms only when they have been partially digested by proteases and expose otherwise cryptic fragments such as the RGD site in the α chain of LN-1 (Horton et al., 1994). To explain this apparent discrepancy two major differences have to be stressed. The first is related to species: we here report data obtained with human cells, while Horton et al. utilized rats. The second, probably more important, difference regards the cell model utilized: it has been obtained from a transformed osteoclast population

from giant cell tumor of bone and contains only osteoclast-like cells at various stages of differentiation. It has to be stressed, however, that they represent a homogenous population, all endowed with calcitonin receptors, TRAP positivity (more or less intense), and bone resorption capability. These cells in culture keep dividing and fusing, giving origin to other pre-osteoclasts. Eventually they become multinucleate and fully differentiated before degenerating. It has to be expected that osteoclasts interacting with laminin in vivo during transendothelial migration will be precursors and not completely differentiated, more similar to our model than to mature resorbing cells, as are isolated rat osteoclasts in vitro. It has also to be remembered that in our model we are dealing with transformed cells and there might be differences with non-transformed precursors in vivo. They are, in any case, the only available model of human osteoclasts in vitro.

Several integrin receptors recognize LN, including integrins of the β_1 subfamily, e.g. $\alpha_3\beta_1$ (Wayner and Carter, 1987; Wayner et al., 1988; Takada et al., 1988; Gehlsen et al., 1988, 1989). Moreover, antibodies against the β_1 integrin subunit were shown to inhibit strongly interactions of many cells with LN-2 (Horwitz et al., 1985; Bozyczko and Horwitz, 1986; Hall et al., 1987; Tomaselli et al., 1987, 1988; Kramer et al., 1989). We investigated the role of $\alpha_3\beta_1$ in GCT cells LN-2 recognition utilizing functional antibodies against α_3 or β_1 subunits (Wayner and Carter, 1987; Carter et al., 1991). In the presence of anti-α_3 antibody cell adhesion onto LN-2 was not modified, indicating that this subunit was not involved in LN-2 recognition. A reduction in the percentage of cells adherent to LN-2 was detected in the presence of anti-β_1 antibodies, suggesting that this chain could be involved in LN-2 recognition, probably linked to a different α chain. However, in the presence of monensin, the number of cells adherent to laminin-2 with or without anti-β_1 antibodies was the same, suggesting that the inhibition observed in the absence of monensin could be due to protein secretion. Previously (Chenu et al., 1994; Grano et al., 1994b) we demonstrated that adhesion to many substrates such as osteopontin, osteocalcin and others specifically recognized by osteoclast-like cells could induce FN production and secretion, followed by clustering of β_1 integrin receptors in focal adhesions. Therefore, the inhibition of cell adhesion to LN-2 by anti-β_1 antibodies could be dependent on interference with the adhesion-dependent secretion of fibronectin. In fact, we demonstrated, by immunofluorescence, the production of endogenous fibronectin, followed by the relocation of β_1 in focal adhesions. In the presence of monensin FN was retained in intracellular granules and β_1 was diffuse on the plasma membrane. Thus, it is possible that GCTs recognize LN-2 and that this recognition triggers the non-specific secretion of fibronectin, followed by β_1 integrin clustering in focal adhesions. If anti-β_1 antibodies are present in the medium during this process, they can interfere with this clustering and as a consequence with the adhesion process while, in the presence of monensin, we can observe only the first step of laminin recognition not followed by protein secretion.

Other known LN-receptors are the heterodimers $\alpha_6\beta_4$ and $\alpha_6\beta_1$ (Lotz et al., 1990), but we previously demonstrated that GCT cells do not express α_6 or β_4 subunits (Grano et al., 1994b), ruling out these integrins. Finally we investigated the role of $\alpha_v\beta_3$, since the involvement of β_3 had been suggested by other authors (Horton et al., 1994), at least if proteolized

RGD-containing fragments were present in the LN-2 used in the experiments. The presence of anti-α_v antibodies during the adhesion assay, with or without monensin, did not modify LN-2 recognition, excluding any possible role for $\alpha_v\beta_3$ in LN recognition in our experiments. Control experiments were also performed in the presence of both GRGDSP and GRGESP as well as in the presence of peptides containing another sequence, IKVAV, indicated as responsible for LN recognition (Weeks et al., 1994). GCTs were not affected by the presence of these peptides during the adhesion assays (Table 1).

Osteoclast morphology may change according to functional status. GCT cells plated onto LN-2 appeared only partially spread, compared to the controls, showing a highly motile phenotype (Fig. 2). Because interactions with laminin may occur during motile phases such as transendothelial migration, we assayed if laminins could facilitate osteoclast migration. We previously demonstrated that osteocalcin, one of the most abundant non-collagenous proteins of bone, is highly chemotactic toward osteoclasts (Chenu et al., 1994). Utilizing this protein as chemoattractant, we compared migration through laminin or collagen-coated membranes and demonstrated significantly higher cell migration through LN-2 at any concentration tested. Migration through laminin already occurred at doses not effective for collagen. Therefore, laminin recognition facilitates osteoclast precursor migration from blood vessel to bone.

Interactions between cells and extracellular matrix proteins can activate a cascade of intracellular signals, involving many cytoplasmic transducers including intracellular calcium (Jaconi et al., 1991; Juliano and Haskill, 1993). We determined that LN induces an increase in $[Ca^{2+}]_i$ in GCT. Specific increases in $[Ca^{2+}]_i$ have been demonstrated in these cells in response to osteopontin, bonesialoprotein and related fragments (Paniccia et al., 1993). These RGD-containing proteins induce a prompt, transient response. In contrast the increase in $[Ca^{2+}]_i$ due to LN-2 was less pronounced and longer lasting. Thapsigargin and EGTA treatment clearly indicated that Ca^{2+} was released from intracellular stores.

In conclusion, this study provides firm evidence that human osteoclast-like cells discriminate between laminin isoforms. Taken together, our results support a model whereby initial interactions with LN-2 induce Ca^{2+}-mediated intracellular signals and appear to be due to integrin-independent mechanism. Such adhesion may trigger secretion of fibronectin, which could then support spreading and efficient chemotactic migration. These mechanisms may play a role in facilitating chemotactic migration of osteoclast precursors toward the bone surface.

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