Hyaluronan–CD44 interaction hampers migration of osteoclast-like cells by down-regulating MMP-9

Paola Spessotto,[1] Francesca Maria Rossi,[2] Massimo Degan,[2] Raffaele Di Francia,[2] Roberto Perris,[1,3] Alfonso Colombatti,[1,4,5] and Valter Gattei[2]

[1]Divisione di Oncologia Sperimentale 2, and [2]Nucleo di Ricerca Clinica e Laboratoristica in Ematologia, CRO-IRCCS, National Cancer Institute, 33081 Aviano, Italy
[3]Dipartimento di Biologia Evolutiva Funzionale, University of Parma, 43100 Parma, Italy
[4]Dipartimento di Scienze e Tecnologie Biomediche, and [5]MATI Center of Excellence, University of Udine, 33100 Udine, Italy

Osteoclast (OC) precursors migrate to putative sites of bone resorption to form functionally active, multinucleated cells. The preOC FLG 29.1 cells, known to be capable of irreversibly differentiating into multinucleated OC-like cells, displayed several features of primary OCs, including expression of specific integrins and the hyaluronan (HA) receptor CD44. OC-like FLG 29.1 cells adhered to and extensively migrated through membranes coated with fibronectin, vitronectin, and laminins, but, although strongly binding to HA, totally failed to move on this substrate. Moreover, soluble HA strongly inhibited OC-like FLG 29.1 cell migration on the permissive matrix substrates, and this behavior was dependent on its engagement with CD44, as it was fully restored by function-blocking anti-CD44 antibodies. HA did not modulate the cell–substrate binding affinity/avidity nor the expression levels of the corresponding integrins. MMP-9 was the major secreted metalloproteinase used by OC-like FLG 29.1 cells for migration, because this process was strongly inhibited by both TIMP-1 and GM6001, as well as by MMP-9–specific antisense oligonucleotides. After HA binding to CD44, a strong down-regulation of MMP-9 mRNA and protein was detected. These findings highlight a novel role of the HA–CD44 interaction in the context of OC-like cell motility, suggesting that it may act as a stop signal for bone-resorbing cells.

Introduction

Mononuclear osteoclast (OC)* precursors egress from the bloodstream and migrate to the bone surface where they form functionally active multinucleated bone-resorbing cells under the influence of several cytokines, including M-CSF and RANKL (Roodman, 1996; Lacey et al., 1998; Arai et al., 1999; Teitelbaum, 2000). Once attached to bone, OCs initiate the resorption process through the activation of a complex cascade of morphological and biochemical changes involving expression of adhesion molecules and secretion of proteolytic enzymes. In addition to cathepsin K (Inaoka et al., 1995), several lines of evidence indicate the critical role of matrix metalloproteinases (MMPs), and in particular, MMP-9 for OC migration and function. MMP-9 has been associated exclusively with OCs among the cells involved in bone formation and resorption in both human (Okada et al., 1995) and rabbit (Tezuka et al., 1994) tissues. In addition, it has proven to be indispensable for the migration of OCs through collagen both in periosteum and developing marrow cavity of primitive long bones (Blavier and Delaisse, 1995; Sato et al., 1998). Moreover, bone resorption was reduced in mice carrying a mutation in the type-I collagen site targeted by neutral collagenases (Zhao et al., 1999), and the natural tissue inhibitor of MMP activity, tissue inhibitor of metalloproteinase 1 (TIMP-1), expressed by OCs (Hill et al., 1994), inhibits bone resorption in organ cultures (Bord et al., 1999). Finally, the localization of membrane-type 1 MMP in invadopodia and lamellipodia of OCs suggested a role in OC invasion/migration phenomena for this membrane-bound protease (Sato et al., 1997).

Coordinated regulation and appropriate site-specific migration and bone adhesion of OCs toward different ECM substrates (such as collagens, fibronectin [FN], vitronectin [VN], and laminins [LNs]) in the spaces surrounding and

Address correspondence to Alfonso Colombatti, Divisione di Oncologia Sperimentale 2 CRO-IRCCS, National Cancer Institute, 33081 Aviano, Italy. Tel.: 39-0434-659-365. Fax: 39-0434-659-428.
E-mail: acolombatti@cro.it

*Abbreviations used in this paper: CAFCA, centrifugal assay for fluorescence-based cell adhesion; FN, fibronectin; HA, hyaluronan; LN, laminin; MMP, matrix metalloproteinase; OC, osteoclast; preOC, preosteoclast; TIMP-1, tissue inhibitor of metalloproteinase 1; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VN, vitronectin.

Key words: bone; cell migration; hyaluronic acid; metalloproteinase; osteoclast.

© The Rockefeller University Press, 0021-9525/2002/09/1133/12 $5.00
The Journal of Cell Biology, Volume 158, Number 6, September 16, 2002 1133–1144
http://www.jcb.org/cgi/doi/10.1083/jcb.200202120

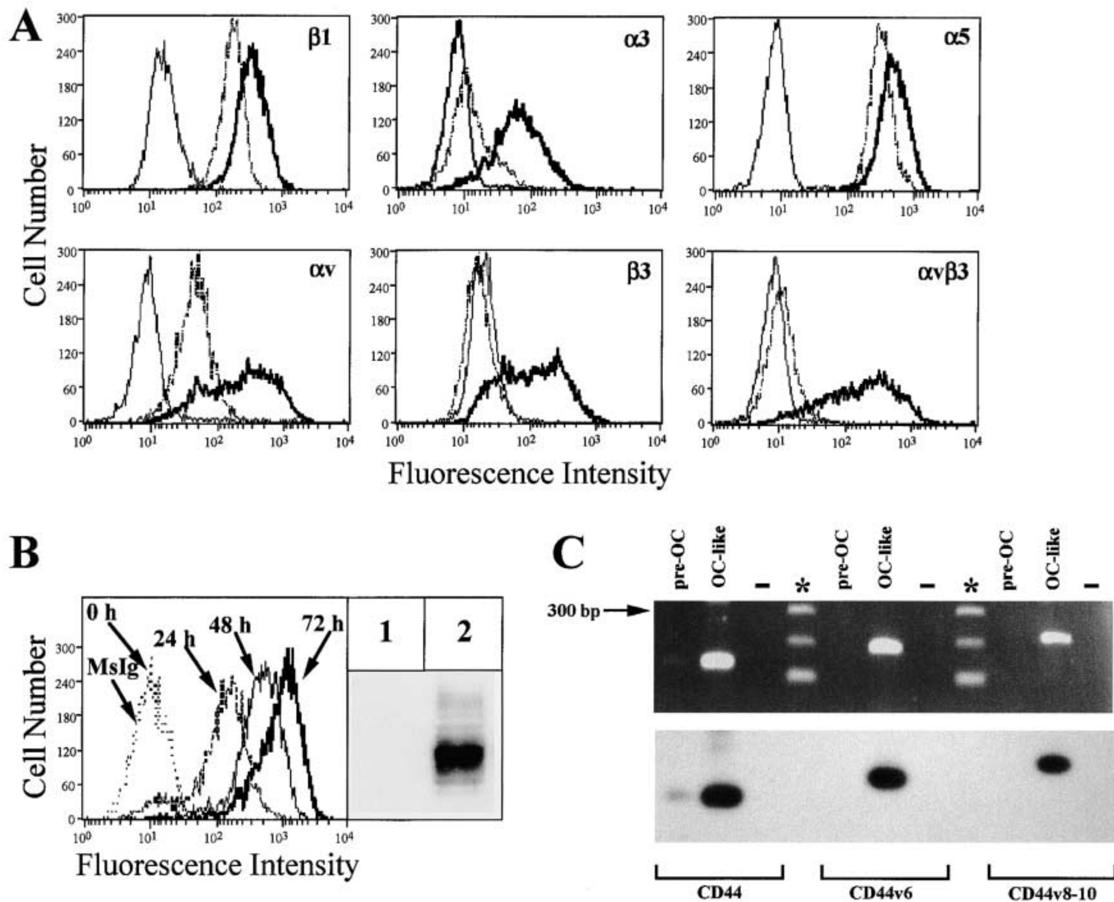

Figure 1. **Expression of adhesion receptors by preOC and OC-like FLG 29.1 cells.** (A) Cell surface expression of selected integrin receptors by pre-OC (dotted line) and OC-like (bold line) FLG.29.1 cells as assessed by flow cytometry. Binding of an unrelated isotype-matched control is reported as a thin line. Histograms indicate log fluorescence intensity (x axis) versus relative cell number (y axis). (B, left) Kinetics of cell surface expression of the CD44 receptor; the time in hours after induction is shown. MsIg indicates the binding of an isotype-matched unrelated antibody. (B, right) Immunoblotting of CD44. Lysates from preOC (lane 1) and OC-like (lane 2) cells were resolved on an 8% SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-CD44 pAbs. (C) RT-PCR analysis of the expression of CD44 alternatively spliced exons in OC-like FLG 29.1 cells. Amplified products were run on a 1.5% agarose gel (top), blotted onto nylon membranes, and hybridized with a probe chosen to recognize sequences within exon 5 (bottom). Negative control (−) was done by omitting cDNA. Asterisks indicate DNA markers.

within the bone matrix are mediated by various receptor systems, including integrins. Among the integrins involved in OC biology and motility, a fundamental role has been recognized for αvβ3, although bone resorption may also be partially inhibited by functional blocking of the α2β1 integrin (Duong et al., 2000). Another important class of OC adhesion receptors is represented by CD44 (Nakamura and Ozawa, 1996; Kania et al., 1997). CD44 is a cell surface, single-pass transmembrane part time proteoglycan expressed in a variety of cells of hemopoietic and nonhemopoietic origin (Aruffo, 1996; Naor et al., 1997; Borland et al., 1998; Lesley and Hyman, 1998; Rochman et al., 2000). The purported functions of CD44 are diverse, but by far the greatest interest in CD44 has been in relation to its function as a receptor for hyaluronan (HA). HA is a member of the glycosaminoglycan long nonbranching aminosugar polysaccharides that resides and exerts its function primarily within the extracellular space (Lee and Spicer, 2000), including those immediately surrounding bone matrices (Noonan et al., 1996). The roles of HA range from a purely structural function to regulation of cell motility and adhesion, as well as receptor-mediated changes in gene expression (Lee and Spicer, 2000). Experimental evidence indicates that binding of HA to CD44 can also be regulated through glycosylation and a variety of other posttranslational modifications (Bartolazzi et al., 1996). Furthermore, CD44 engagement can activate several downstream pathways through specific intermediates (Ilangumaran et al., 1999; Mangeat et al., 1999). The cellular and molecular mechanisms by which integrins contribute to cell migration processes have been extensively established (Holly et al., 2000); however, although the dependence of cell motility on CD44 has been demonstrated in several in vitro models (Thomas et al., 1993; Okada et al., 1996; Trochon et al., 1996; Ladeda et al., 1998; Okamoto et al., 1999; Kajita et al., 2001), the regulation of CD44-mediated motility has been less thoroughly investigated.

The human continuous cell line (FLG 29.1) of bone marrow–derived preosteoclast (preOC) cells provides a valuable model to investigate specific aspects of OC biology (Gattei et al., 1992). FLG 29.1 preOC cells can be ir-

reversibly induced to differentiate into adherent, nondividing multinucleated OC-like cells displaying several features of primary OCs, including a specific immunophenotypic profile, tartrate-resistant acid phosphatase, and calcitonin and estrogens receptors, as well as the capability to degrade bone (Fiorelli et al., 1994, 1995; Gattei et al., 1996). Using these cells, we demonstrate a new role for the CD44-HA interaction in the regulation of cell migration. Engagement of CD44 by its specific ligand HA significantly impairs the migration of OC-like FLG 29.1 cells toward several ECM substrates by down-regulating the production of the locomotion-associated protease MMP-9. According to this in vitro model, the CD44-HA interaction may represent a novel motility stop signal that could also play a role in vivo to counterbalance the invasive activity exhibited by OC cells to gain access to and for anchorage at the bone surface.

Results

Integrin expression by OC-like FLG 29.1 cells

In agreement with data obtained on primary OCs (Kania et al., 1997), the acquisition by FLG 29.1 cells of the OC-like phenotype was associated with an up-regulation of the constitutively expressed β1, αv, and α5 integrin chains, and a de novo expression of αvβ3, β3, and α3 integrins (Fig. 1 A). Moreover, independently of the differentiation status, FLG 29.1 cells transcribed mRNA for both α8 and α9 integrin subunits (unpublished data), whereas they did not express selectins, β2 family integrins, β5, β7, α1, α2, α4, and α6 integrin subunits (unpublished data).

CD44 expression by OC-like FLG 29.1 cells

CD44, undetectable in preOC FLG 29.1 cells, was strikingly induced in a time-dependent manner upon OC-like differentiation (Fig. 1 B, left). Immunoblotting of cell lysates were in full accord with flow cytometry data and indicated no expression of CD44 in preOC cells and expression of the standard hemopoietic CD44 form in OC-like cells (Fig. 1 B, right). Only upon long exposures, very faint higher M_r bands were visible in OC-like cells, suggesting that variants including additional exons could be expressed at very low levels. Consistently, as analyzed by RT-PCR, only low amounts of standard CD44 mRNAs were detected in preOC cells. Transcripts of this isoform were increased in OC-like FLG 29.1 cells, along with those corresponding to CD44v6 and CD44v8–10 alternatively spliced variants (Fig. 1 C).

Cell adhesion to ECM substrates

PreOC FLG 29.1 cells bound in a cation-dependent manner to FN and to VN, but not to fibrinogen, type I collagen, and LNs (Fig. 2 A). Upon induction of differentiation, FLG 29.1 cells displayed an increased ability to attach to FN and VN, and acquired the capability to adhere to several LNs, mainly LN-8, and LN-10 isoforms (Fig. 2 A). As expected, adhesion of OC-like FLG 29.1 cells to FN, LN-8, and LN-10, but not VN, was completely abrogated by an anti-β1 integrin subunit-blocking mAb (Fig. 2 B). Similarly, anti-αvβ3 and anti-α3–specific mAbs significantly inhibited cell attachment to VN and LN-10, respectively (Fig. 2 B). OC-like FLG 29.1 cells also bound tenaciously and in a dose-dependent manner to surface-immobilized high M_r HA, reaching 50% cell binding at coating concentrations as low as 15–20 μg/ml HA (Fig. 3 A). HA binding to OC-like cells was entirely mediated by CD44 because staining with FITC-HA was fully abrogated by an excess of the function-blocking anti-CD44 mAb, BRIC235 (Fig. 3 B). Consistently, the same and other function-blocking anti-CD44 mAbs completely abolished adhesion of OC-like cells to immobilized HA, but not to FN substrates (Fig. 3, C and D).

HA engagement of CD44 inhibits migration of OC-like FLG 29.1 cells toward purified ECM molecules

Although preOC FLG 29.1 cells were totally unable to migrate toward all the various ECM substrates assayed (Fig. 4 A), differentiated OC-like FLG 29.1 cells migrated toward FN-, VN-, LN-8–, and LN-10–coated membranes. A significant number of migrated cells were detected as early as 6 h, indicating an enhanced locomotion activity on differentiation (Fig. 4 A, top). In agreement with the adhesive behavior and integrin expression pattern of OC-like FLG 29.1 cells, movement toward VN- and LN-10–coated membranes was

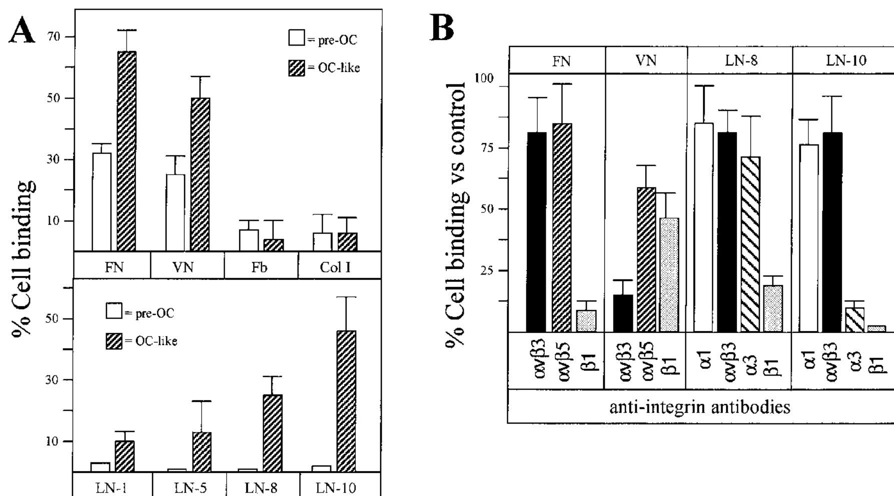

Figure 2. **Cell adhesion to ECM proteins.** (A, top) Cell adhesion to FN, VN, fibrinogen (Fb), and type I collagen (Col I). Substrate proteins were coated at 10 μg/ml and cell adhesion was performed in the presence of 1.0 mM Mg^{2+} and 1.0 mM Ca^{2+} or 10 μM Mn^{2+} for VN. The values reported represent the average of three experiments. (A, bottom) Cell adhesion to different LN isoforms coated at 10 μg/ml. Cell adhesion was performed as in A. (B) Inhibition of OC-like FLG 29.1 cell adhesion by integrin-specific antibodies. For the inhibition of cell adhesion, different antibodies were added at 5 μg/ml just before plating the cells. The following antibodies were used: FB12 (α1), P1F6 (αvβ5), LM 609 (αvβ3), P1B5 (α3), and 4B4 (β1).

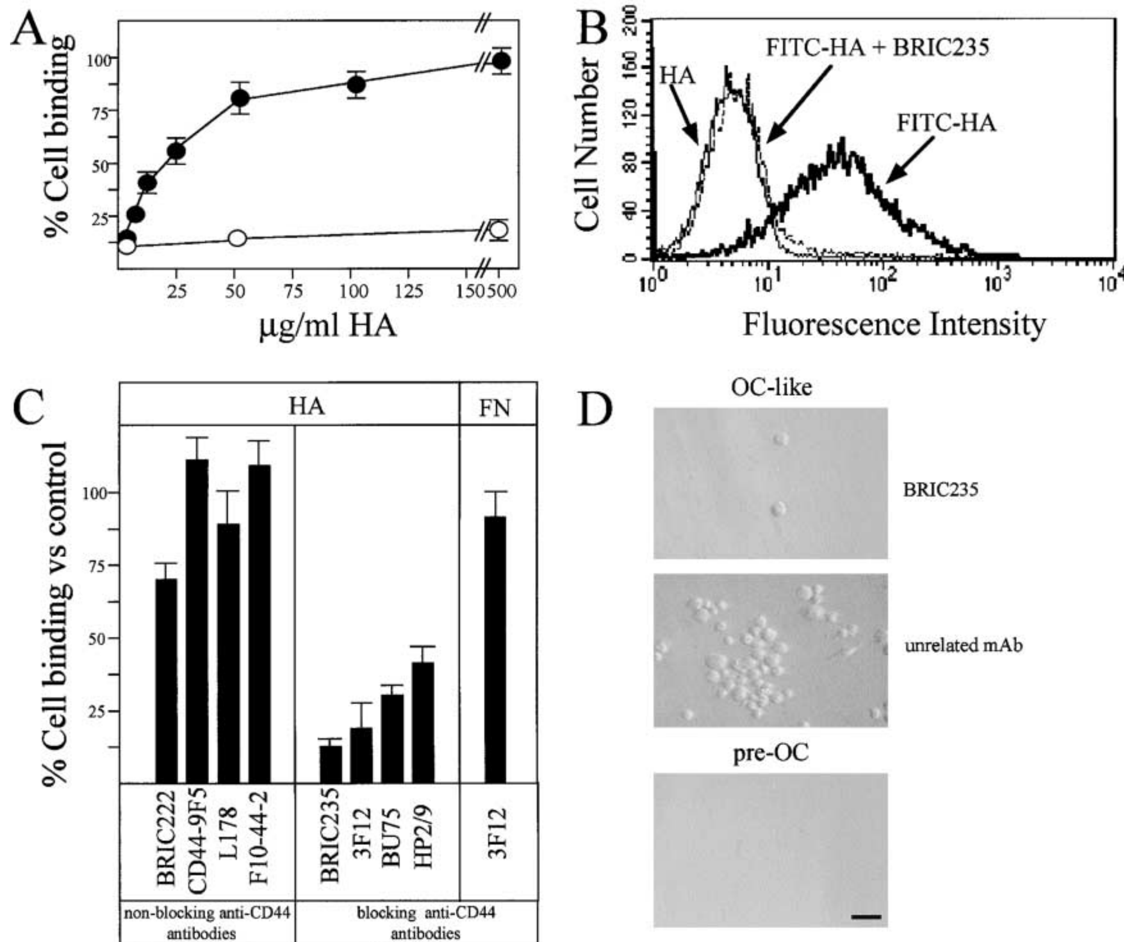

Figure 3. **Cell adhesion to HA.** (A) Dose–response of cell adhesion. HA was coated at different concentrations and the cell adhesion assay was performed with preOC (open circles) or OC-like (closed circles) FLG 29.1 cells in the presence of 1.0 mM Mg^{2+} and 1.0 mM Ca^{2+}. (B) The HA receptor on OC-like cells is CD44. Cells were probed with 0.8 μg/ml FITC-HA in the absence or presence of a CD44 function–blocking mAb (BRIC 235). (C) Inhibition of cell adhesion. For the quantitative evaluation of CD44-dependent inhibition of cell adhesion to HA coated at 0.5 mg/ml, the different function-blocking mAbs (5 μg/ml) were added just before plating the cells. Adhesion onto FN in the presence of an anti-CD44 function-blocking mAb is shown for comparison. (D) Adhesion to HA. Phase-contrast micrograph of adherent preOC or OC-like cells to HA-coated at 0.5 mg/ml in the absence or presence of BRIC 235. Bar, 100 μm.

mediated by the αvβ3 and α3β1 integrins, respectively, as indicated by inhibition of migration upon exposure to specific function-blocking mAbs (unpublished data). Similarly, migration toward FN was presumably largely mediated by the α5β1 integrin, because this movement was significantly hampered (>80%) by the addition of the anti-β1 4B4 mAb (unpublished data). In contrast, despite the significant expression of CD44 (Fig. 1 B) and the strong adhesion to HA (Fig. 3), OC-like FLG 29.1 cells completely failed to move toward HA-coated membranes at 6 and even at 20 h (Fig. 4 A). The motility response to HA was unaffected by any putative chemotactic stimulus provided by conditioned media from the C433 stromal cell line, known to contain several OC-specific differentiation-inducing factors (Gattei et al., 1996), or from NIH 3T3 fibroblasts (unpublished data).

To investigate the effects of HA on OC-like FLG 29.1 cell migration in response to other ECM substrates, we hypothesized that HA may actively participate in some regulatory aspects of OC-like cell motility. Thus, soluble HA was added during a standard migration assay toward various ECM substrates (Fig. 4 B). In the presence of intact HA, the number of migrating cells on FN was found to be dose-dependently reduced at both 6 and 20 h (Fig. 4 B). This phenomenon was clearly dependent on binding of HA to CD44 because the addition of the anti-CD44–blocking mAb (BRIC235) fully restored the migratory behavior of OC-like cells toward FN (Fig. 4 B). A similar inhibitory effect of soluble HA on the migration of OC-like FLG 29.1 cells was also demonstrated when VN and LN-10 were used as substrates (unpublished data).

CD44 engagement does not affect adhesion of OC-like FLG 29.1 cells to ECM

Although mediated by a specific CD44-HA interaction, the inhibition of migration toward ECM substrates by soluble HA could depend on a mere steric blockade of the interaction between the ligand and its specific receptor. To address this issue, we investigated the relative adhesive strength of the cells to FN in the presence of HA. For this purpose, we performed centrifugal assay for fluorescence-based cell adhesion

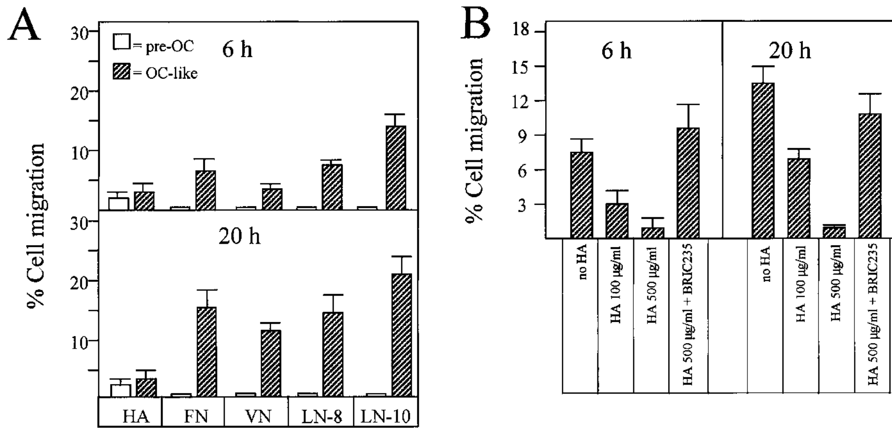

Figure 4. **Cell migration toward ECM substrates.** (A) Migration in response to various ECM substrates was evaluated at two time points (6 and 20 h). The filters were coated on the underside with 20 μg/ml of the various substrates. (B) Inhibition of cell migration toward FN by engagement of CD44 by soluble HA. Dose–response inhibition was evaluated at two time points in the presence of 100 or 500 μg/ml of soluble high molecular mass HA. The simultaneous presence of the BRIC235 mAb blocking the CD44-HA interaction reversed the HA-dependent inhibition. In both cases, the values shown represent the mean ± SEM of three experiments.

(CAFCA) assays in the presence of 0.5 or 1.0 mg/ml of HA, capitalizing on the fact that this assay can measure the strength of adhesion by varying the detachment forces (Spessotto et al., 2000, 2001). As shown in Fig. 5 A, soluble HA did not affect the attachment of OC-like FLG 29.1 cells to FN substrates, even when the maximal detachment force (400 *g*) was applied. Similar results were obtained using VN and LN-10 as adhesive substrates (unpublished data). This adhesion was mediated by the interaction between the FN substrate and its specific integrin receptor because the addition of a function-blocking anti-β1 mAb fully inhibited the adhesion of OC-like FLG 29.1 cells to FN, also in the presence of solu-

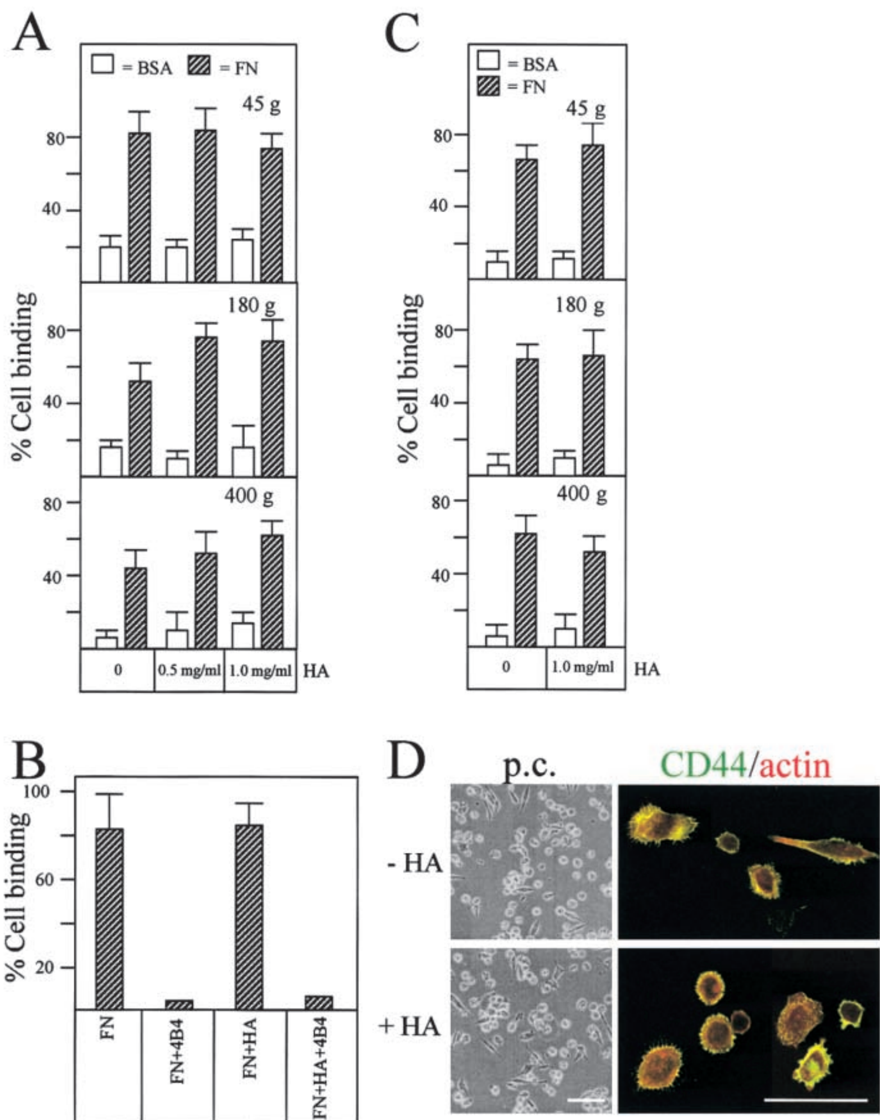

Figure 5. **Effect of HA on OC-like cell adhesion to FN.** (A) Assessment of the relative avidity of cell adhesion onto FN in the presence of HA by varying the centrifugal force (45 *g*, 180 *g*, and 400 *g*) applied to dislodge bound cells. Cells were allowed to adhere to FN coated at 5 μg/ml. (B) Adhesion to FN in the presence of HA or the 4B4 function-blocking anti-β1 mAb. (C) Assessment of the relative avidity of cell adhesion to FN of cells maintained for 24 h in the presence of soluble HA by varying the centrifugal force (45 *g*, 180 *g*, and 400 *g*) applied to dislodge bound cells. (D) Phase-contrast (p.c.) morphology and CD44/actin staining of adherent OC-like FLG 29.1 cells to FN-coated slides (20 μg/ml) at 20 μg/ml in the absence or presence of 1.0 mg/ml HA. Bars, 100 μm.

ble HA (Fig. 5 B). Another possibility could be that the addition of soluble HA could affect, via an inside-out mechanism mediated by the cytoplasmic domain of CD44, the affinity/avidity of integrin receptors for their cognate ligands. Thus, additional adhesion experiments were performed with OC-like FLG 29.1 cells grown in the presence of soluble HA for 24 h and then assayed with CAFCA at different detachment forces (45 *g*, 180 *g*, and 400 *g*) in the absence of HA. Again, no significant difference was noted when compared with control cells grown in the absence of HA (Fig. 5 C). Consistently, the presence of soluble HA affected neither the expression levels of $\alpha5\beta1$ and $\alpha v\beta1$ integrins on OC-like FLG 29.1, as evaluated by flow cytometry (unpublished data), nor the morphology of cells attached to FN, nor the even cell membrane distribution of CD44 molecules (Fig. 5 D).

MMP-9 is the major metalloproteinase involved in OC-like FLG 29.1 cell migration

MMPs represent indispensable factors for the regulation of specific OC functions, including migration to the bone surface (Blavier and Delaisse, 1995; Vu et al., 1998; Lee et al., 1999; Engsig et al., 2000). Accordingly, consistent levels of MMP-9 (M_r = 92 kD) were detected in conditioned media from fully differentiated OC-like FLG 29.1 cells (Fig. 6 A, lane 3), MMP-9 levels being further up-regulated by exposure to TNF-α (Fig. 6 A, lane 4), a strong inducer of MMP-9 expression (Zhang et al., 1998). On the contrary, although no MMP-9 was seen in preOC cell supernatants (Fig. 6 A, lane 1), a discrete MMP-9–related gelatinolytic activity was revealed on exposure of preOC cells to TNF-α (Fig. 6 A, lane 2). Other MMPs known to be expressed by OCs such as MMP-2 (M_r = 72 kD) and MMP-13 (M_r = 54/42 kD) were not expressed by FLG 29.1 cells both in zymograms (Fig. 6 A) and RT-PCR (unpublished data). By comparing MMP-9 levels in cell medium and cell lysates of OC-like cells, MMP-9 released in the supernatant represented ~90% of the whole detectable amount of activity both in the presence and absence of TNF-α (Fig. 6 B, lanes 1 and 2). However, in cell lysates, detectable amounts of active MMP-9 were demonstrable (Fig. 6 B, lane 3), which significantly increased on exposure to TNF-α (Fig. 6 D, lane 4).

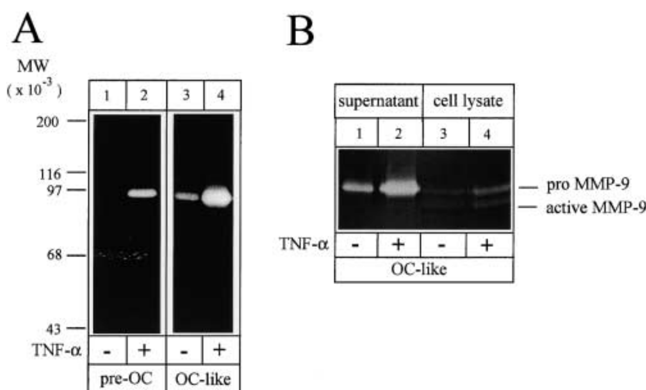

Figure 6. **Expression of MMP-9.** (A) Pro–MMP-9 expression in the supernatant of preOC or OC-like cells (5×10^5/lane) in the absence or presence of TNF-α (10 ng/ml). Expression was evaluated with zymography. (B) Pro–MMP-9 and active MMP-9 in supernatants and cell lysates of OC-like cells in the absence or presence of TNF-α. Only the relevant part of the gel is shown.

To evaluate whether MMP-9 may be critical for the motility of OC-like FLG 29.1 cells, migration assays were performed in the presence or absence of an excess of MMP inhibitors. As shown in Fig. 7 A, the addition of the natural inhibitor of MMPs, TIMP-1, as well as of the synthetic inhibitor of MMP activity, GM6001 (Leppert et al., 1995), almost fully abrogated cell migration toward FN-coated membranes. To formally demonstrate that MMP-9 was involved in migration of OC-like FLG 29.1 cells, we tested whether the inhibition of MMP-9 expression by antisense treatment could affect cell migration toward FN. On exposure to specific antisense oligonucleotides, the expression of MMP-9 mRNA, as investigated by real-time quantitative PCR, was dose-dependently reduced with a nearly full inhibition at 10 μM (Fig. 7 B). Accordingly, a consistent inhibition of cell migration was attained, reaching 75% reduction compared with control, when 10 μM of antisense oligonucleotide was added (Fig. 7 C). Expression of surface CD44 in OC-like cells exposed to antisense treatment, as evaluated by flow cytometry, was totally unaffected (unpublished data), thus excluding the possibility that the inhibitory effects on migration could result from a mere down-regulation of CD44. These experiments concordantly suggested that motility of OC-like FLG 29.1 cells was under the influence of MMPs, likely MMP-9.

HA engagement of CD44 affects MMP-9 expression

OC-like FLG 29.1 cells grown for 24 h onto ECM substrates such as FN or VN did not change the levels of MMP-9 detected in their supernatants when compared with cells grown on tissue culture plastic (Fig. 8 A, lanes 1, 7, and 9). On the other hand, in the presence of soluble HA, a strong and dose-dependent reduction of MMP-9 expression was observed (Fig. 8 A, lanes 2–6), reaching 85% in the presence of 1.0 mg/ml HA. Such inhibitory effect was maintained or even increased when cells were cultured on FN (Fig. 8 A, lane 8) or VN (Fig. 8 A, lane 10) substrates (~50% reduction at 0.1 mg/ml HA). Consistently, immunoblotting of TCA-precipitated supernatants from OC-like cells grown in the presence of 1.0 mg/ml HA demonstrated a 70% reduction of MMP-9, as compared with control samples (Fig. 8 B). The HA-dependent reduction of MMP-9 production in supernatants was confirmed in cytoplasms of OC-like cells also when the expression of MMP-9 was increased upon TNF-α stimulation (Fig. 8 C). In accordance with these findings, significantly lower levels of MMP-9 transcripts were detected by real-time PCR in OC-like FLG 29.1 cells grown onto different substrates in the presence of soluble HA (Fig. 8 D). This down-regulation was rescued by treatment with the function-blocking anti-CD44 mAb BRIC235 (Fig. 8 D, lanes 3 and 6). Taken together, these data concordantly suggest that the CD44-HA interaction was directly responsible for the inhibition of the MMP-9 synthesis. Conversely, engagement of CD44 with HA did not result in any change in the expression levels of the standard hemopoietic CD44 form both in OC-like cells (Fig. 8 E, lanes 2 and 3) and in OC-like cells exposed to TNF-α (Fig. 8 E, lanes 5 and 6). Consistently, no cleaved extracellular CD44 could be detected in the corresponding cell supernatants (unpublished data).

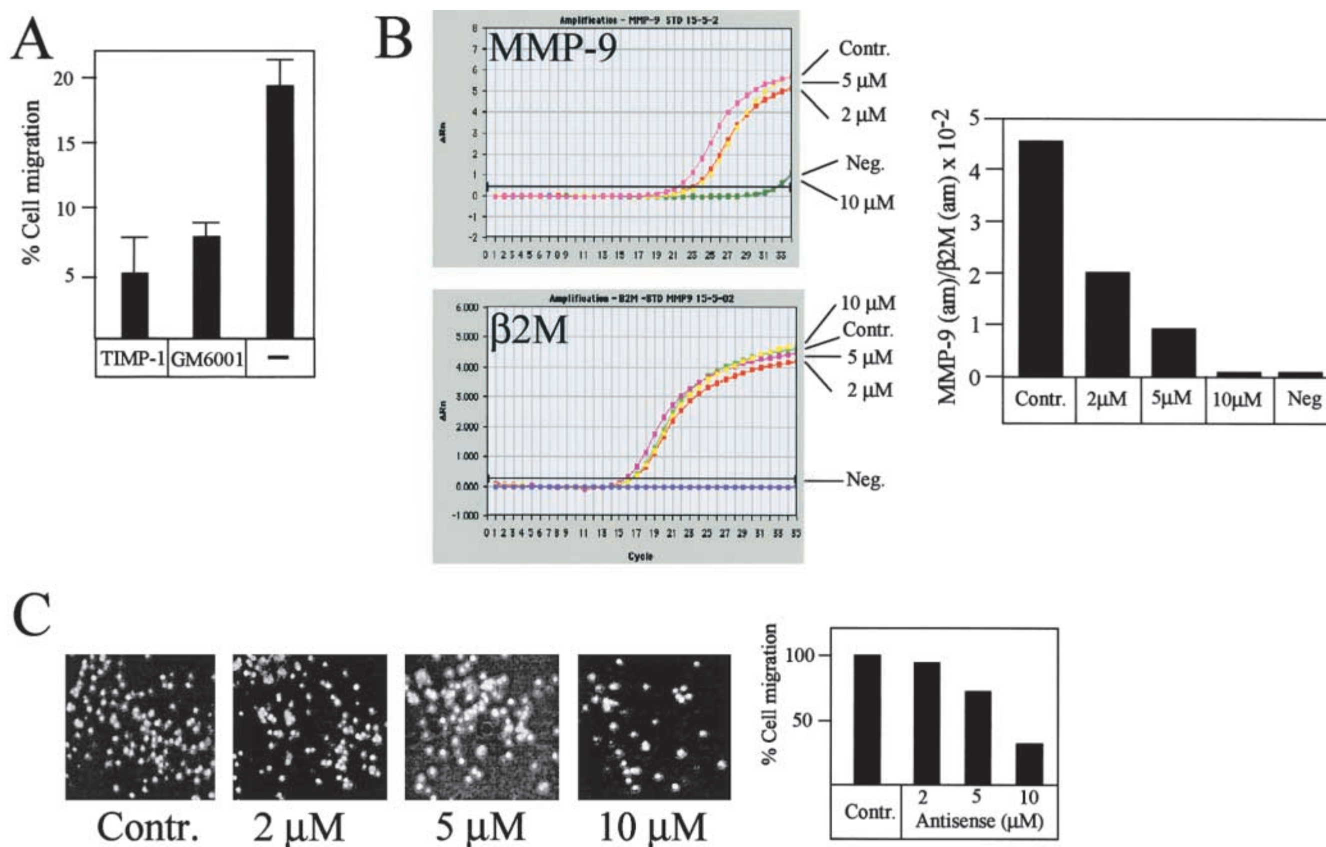

Figure 7. **Inhibition of MMP-9 and effects on cell migration.** (A) Inhibition of migration toward FN by TIMP-1 and GM6001. 10 μg/ml TIMP-1 and 4 μg/ml GM6001 were added to the cultures, and the number of cells that migrated toward FN-coated Transwells at 24 h was evaluated. (B) Quantitation of MMP-9 mRNA in OC-like cells treated with MMP-9 antisense oligonucleotides. The cDNA prepared from OC-like cells treated with MMP-9–specific antisense oligonucleotides or control scrambled oligonucleotide (Contr.) was subjected to quantitative real-time PCR with primers specific for β2M and MMP-9. The left panel shows a graphic representation of one experiment where the x axis indicates the number of PCR cycles (Cycle) and the y axis (Rn) indicates the normalized reporter signal, i.e., the ratio between the fluorescence signals of the reporter dye and of the passive reference dye. The right panel shows the levels of MMP-9, expressed as MMP-9/β2M ratios (am, attomoles) as obtained in two different experiments. (C) Inhibition of migration by MMP-9 antisense oligonucleotides. Representative fields of migrated cells at 24 h are shown on the left panel, and the quantitative data of migration, as obtained in two experiments, are shown on the right panel.

Discussion

The FLG 29.1 cell line had originally been proposed as a valid in vitro model of OC-like differentiation (Gattei et al., 1992). Since then, additional experimental evidence has been accumulated to strengthen the close relationship between normal OC precursors and the OC-like FLG 29.1 cells, further validating the use of this cell system for investigating OCs differentiation/maturation and their functional activity (Gattei et al., 1992, 1996; Fiorelli et al., 1994, 1995). In the present study, by taking advantage of the FLG 29.1 cell system, we demonstrate a novel MMP-9–dependent mechanism of inhibition of cell migration along specific ECM substrates, which is conferred by engagement of HA to CD44, one of the major adhesion receptors expressed by OCs (Aruffo, 1996; Nakamura and Ozawa, 1996).

Differentiation of FLG 29.1 cells into OC-like elements involved up-regulation of β1, α5, and αv integrin chains and de novo induction of αvβ3, β3, and α3 integrins. Consequently, adhesion of differentiated OC-like FLG 29.1 cells to specific ECM molecules, such as FN, VN, LN-8, and LN-10 were strongly up-regulated. Similarly, differentiation of FLG 29.1 cells was accompanied by a striking induction of CD44 expression, which conferred the capability to strongly adhere also to HA substrates. In accordance with their integrins profile, OC-like FLG 29.1 cells were able to migrate toward membranes coated with FN, VN, and LNs, but completely failed to move toward HA substrates. Given the high expression of CD44 molecules and the strong adhesion of OC-like cells to HA, the lack of migration toward HA was surprising. Moreover, soluble HA also strongly inhibited cell migration of OC-like FLG 29.1 cells toward other ECM substrates, such as FN, VN, and LN-10. This phenomenon was clearly dependent on the engagement of CD44 because the addition of specific anti-CD44 mAbs that blocked the interaction with HA fully restored the motility.

CD44 is expressed in many migratory and metastatic cells (Borland et al., 1998), and has been reported to provide motility and locomotion on HA-coated substrates in vitro (Thomas et al., 1993; Okada et al., 1996; Trochon et al., 1996; Ladeda et al., 1998; Okamoto et al., 1999; Oliferenko et al., 2000; Kajita et al., 2001; Sohara et al., 2001). The present findings describe a novel and apparently opposite function for the CD44-HA pair, and raise the question of the putative mechanism(s) by which the CD44-HA in-

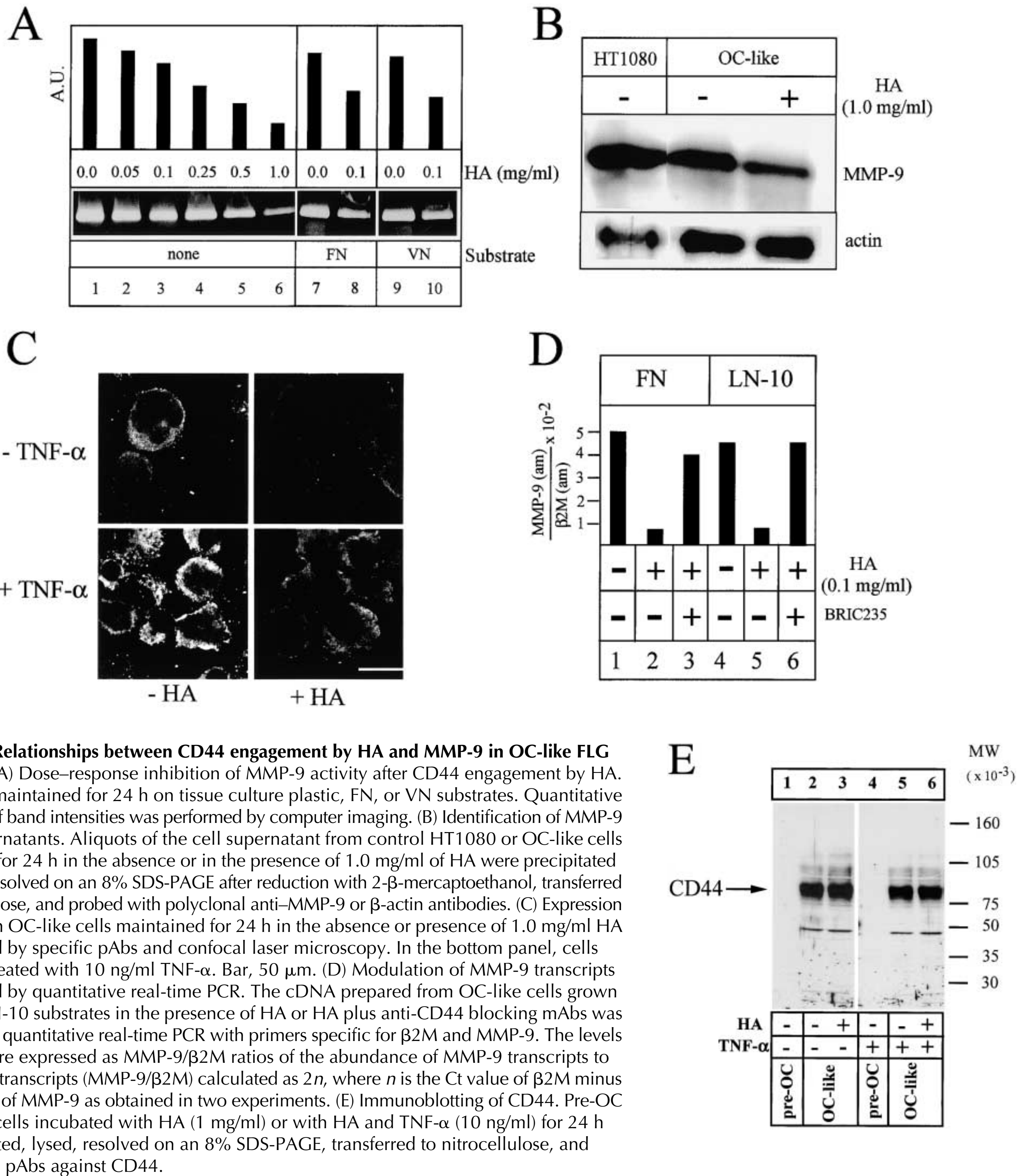

Figure 8. **Relationships between CD44 engagement by HA and MMP-9 in OC-like FLG 29.1 cells.** (A) Dose–response inhibition of MMP-9 activity after CD44 engagement by HA. Cells were maintained for 24 h on tissue culture plastic, FN, or VN substrates. Quantitative evaluation of band intensities was performed by computer imaging. (B) Identification of MMP-9 in cell supernatants. Aliquots of the cell supernatant from control HT1080 or OC-like cells maintained for 24 h in the absence or in the presence of 1.0 mg/ml of HA were precipitated with TCA, resolved on an 8% SDS-PAGE after reduction with 2-β-mercaptoethanol, transferred to nitrocellulose, and probed with polyclonal anti–MMP-9 or β-actin antibodies. (C) Expression of MMP-9 in OC-like cells maintained for 24 h in the absence or presence of 1.0 mg/ml HA as evaluated by specific pAbs and confocal laser microscopy. In the bottom panel, cells were also treated with 10 ng/ml TNF-α. Bar, 50 μm. (D) Modulation of MMP-9 transcripts as evaluated by quantitative real-time PCR. The cDNA prepared from OC-like cells grown on FN or LN-10 substrates in the presence of HA or HA plus anti-CD44 blocking mAbs was subjected to quantitative real-time PCR with primers specific for β2M and MMP-9. The levels of MMP-9 are expressed as MMP-9/β2M ratios of the abundance of MMP-9 transcripts to that of β2M transcripts (MMP-9/β2M) calculated as $2n$, where n is the Ct value of β2M minus the Ct value of MMP-9 as obtained in two experiments. (E) Immunoblotting of CD44. Pre-OC or OC-like cells incubated with HA (1 mg/ml) or with HA and TNF-α (10 ng/ml) for 24 h were collected, lysed, resolved on an 8% SDS-PAGE, transferred to nitrocellulose, and probed with pAbs against CD44.

teraction may impair migration of OC-like FLG 29.1 cells toward ECM substrates. HA was previously reported to inhibit macrophage migration in cross-linked fibrin gels also containing FN (Lanir et al., 1988), although the underlying mechanism had not been elucidated. In this paper, we investigated the possibility that the CD44-HA interaction could prevent cell movement toward ECM ligands (a) by directly masking the substrates and thus hampering the access to their specific receptors, or (b) by modifying the affinity/avidity and/or the expression levels of specific integrin receptors. Several observations argue against these possibilities. First, soluble HA did not prevent cell adhesion onto FN, VN, and LN-10. Second, growing OC-like FLG 29.1 cells in the presence of an excess of HA before performing the CAFCA assay did not change the adhesion strength of specific integrin receptors via an inside-out mechanism. Consistently, the expression levels of specific integrins were not modified by pretreatment with soluble HA. In addition, the cytoplasmic domain of CD44, as expressed in FLG 29.1 cells, has the wild-type sequence (unpublished data), thus excluding the possibility that the lack of migration toward HA might depend on a mutated serine codon (Peck and Isacke, 1998) in this domain. Finally, OC-like FLG 29.1 cells expressed almost exclusively the hemopoietic form and very low amounts of larger isoforms, thus making it unlikely that the inhibitory effect exerted by

HA on cell migration could be due to the expression of atypical CD44 isoforms (Jiang et al., 2002).

Once established that the unexpected lack of migration on HA and the HA-mediated inhibition of motility toward ECM components were not due to intrinsic deficits of CD44 nor to other effects on integrin expression and/or affinity/avidity, we hypothesized that HA might rather actively participate in some specific regulatory aspects of OC-like cell motility. Both cysteine proteinases and MMPs are essential factors in the regulation of specific OC functions, including migration to the bone surface and activation of bone resorption processes (Blavier and Delaisse, 1995; Vu et al., 1998; Lee et al., 1999; Engsig et al., 2000). MMPs have been reported to play a role in OC migration through collagen in the periosteum (Sato et al., 1998) or within the developing marrow cavity of long bones (Blavier and Delaisse, 1995; Engsig et al., 2000). On the other hand, MMP inhibitors do not seem to affect, or only marginally reduce, the resorptive activity of isolated OCs seeded onto slices of devitalized bone or dentine (Hill et al., 1994). Conversely, cysteine proteinase inhibitors, although active on bone resorption, are unable to impair OC migration through collagen (Blavier and Delaisse, 1995; Sato et al., 1998; Lerner, 2000). Thus, cysteine proteinases seem mainly involved in bone resorption processes, whereas MMPs are the proteinases more likely responsible for OC motility (Everts et al., 1999; Lerner, 2000). In complete agreement with this notion, migration of OC-like FLG 29.1 cells toward various ECM components, including FN, VN, and LNs, was strongly dependent on MMP-9, a proteinase expressed by normal OCs (Tezuka et al., 1994; Okada et al., 1995), and resulted in the major if not unique MMP detected in conditioned media and cell lysates of fully differentiated OC-like FLG 29.1 cells. Although MMP-9 was mainly released by OC-like cells in its proenzyme form, active MMP-9 protein was detected in OC-like cell lysates; these low amounts being significantly increased upon induction by TNF-α (Zhang et al., 1998). Interestingly, OC-like FLG 29.1 cells express high levels of TNF-α mRNA, secrete discrete amounts of TNF-α, and exogenous TNF-α has been shown to increase OC-like cell migration (unpublished data). Formal proof that MMP-9 is involved in OC-like FLG 29.1 cell migration was obtained by experiments with MMP inhibitors and MMP-9 antisense oligonucleotides, which specifically knocked out MMP-9 expression and greatly reduced migration of OC-like cells toward FN substrates without affecting CD44 expression. In full accordance with this scenario, the expression of MMP-9 was strikingly down-regulated both at mRNA and protein levels upon engagement of CD44 by soluble HA, suggesting that the CD44-HA interaction interfered with OC-like FLG 29.1 cell migration by inhibiting the expression and/or function of the promigratory proteinase MMP-9.

That MMPs act by promoting cell migration has often been associated with a direct cleavage of specific ECM components. In this context, MMP-2 has been thought to be responsible for the migration of carcinoma cells toward ECM substrates by degrading LN-5 (Giannelli et al., 1997). In particular, because for cell migration to occur, ECM located at the migratory direction has to be degraded, membrane-bound MMPs seem to be optimally placed for pericellular proteolysis associated with cell motility (Nagase and Woessner, 1999; Seiki, 1999). Among membrane-bound MMPs, MT-MMPs are directly tethered to the plasma membrane; soluble MMPs also may act as membrane-bound MMPs by using specific transmembrane receptor systems as docking molecules. In this regard, CD44 has been demonstrated to serve as a docking molecule to retain MMP-9 proteolytic activity at the cell surface (Yu et al., 1997; Bourguignon et al., 1998). Interestingly, no evidence for other gelatinolytic activities in membrane extracts or for MT-MMPs mRNA expression were found in OC-like FLG 29.1 cells (unpublished observation). Therefore, it is also conceivable that in the FLG 29.1 cell system, migration via integrins toward ECM is dependent on MMP-9 either associated to CD44 molecules at the cell surface (Bourguignon et al., 1998; Yu and Stamenkovic, 1999) or released in tightly regulated pulses of very small amounts of activated enzyme, resulting in focalized matrix degradation (Espaza et al., 1999). The notion that in OC-like FLG 29.1 cells CD44 and MMP-9 were both evenly distributed on cell membranes and cytoplasms, whereas the involved integrins are polarized (Martin et al., 2002), does not argue against our interpretation of the data.

Cleavage of CD44 molecules can result either in promotion or impairment of cell migration (Espaza et al., 1999; Okamoto et al., 1999; Ahrens et al., 2001; Kajita et al., 2001). According to Kajita et al. (2001), cleavage of CD44 may be required to detach cells from HA and promote their migration. However, in other cell systems, CD44 cleavage can impair cell migration by disrupting the migration promoting CD44–MMP-9 clusters (Espaza et al., 1999) or, through the production of soluble fragments acting as decoy receptors for HA, by reducing the number of HA molecules directly bound to cells (Ahrens et al., 2001). In addition, as shown for the first time by us in the OC-like FLG 29.1 model, impairment of cell migration may be independent from CD44 cleavage but rather dependent on HA engagement of CD44 followed by down-regulation of the promigratory MMP-9 proteinase. Cleavage of CD44, as reported by Kajita et al. (2001), may promote cell migration also by rescuing cells from the inhibitory effect exerted by HA–CD44 interactions on cell migration through MMP-9 down-regulation.

The recent finding that human metastatic breast cancer cells (Bourguignon et al., 2000) and murine epithelial cells activate GTP-binding proteins (Oliferenko et al., 2000) upon CD44 engagement through HA suggested that the status of these proteins may be important for cell migration (Bourguignon et al., 2000). Whether GTP-binding proteins should similarly have a role in migrating OC-like cells remains to be investigated. However, it should be pointed out that because both integrins and CD44 are actively engaged at the same time, the migration system analyzed by us with OC-like cells is totally different from that studied in polarized epithelial cells (Bourguignon et al., 2000; Oliferenko et al., 2000). In fact, epithelial cells were investigated while migrating either chemotactically toward soluble HA (Bourguignon et al., 2000) or after being locally stimulated by microinjections of HA (Oliferenko et al., 2000), under experimental conditions in which only CD44 was engaged.

OC-like cells migrating toward ECM molecules are actively engaging cognate integrins as well as CD44, and the active integrin-dependent migration is counteracted by the addition of soluble HA and its binding to CD44.

After the generation of preOCs from hemopoietic progenitors (Roodman, 1996), MMP-9 plays an important permissive role in facilitating OC invasion into primitive long bones of the marrow cavity (Blavier and Delaisse, 1995); a process that is down-regulated by MMP inhibitors (Sato et al., 1998). In the OC-like FLG 29.1 model, migration was almost fully abrogated by TIMP-1 or GM6001, as well as by specific antisense oligonucleotides, indicating that MMP-9 plays a fundamental promigratory function and that this activity can be physiologically or pharmacologically modulated. Our results suggest a mechanistic framework for the observed association between engagement of CD44 by HA and negative regulation of migration processes, and implies that the regulation of pro- and antimigratory activity might be a mechanism also playing a role in OC activity in vivo. An attractive possibility is that other cells of the bone microenvironment, including osteoblasts and/or stromal or inflammatory cells (Roodman, 1996) may be stimulated to secrete HA by environmental cues or that stromal HA is released from ECM. Soluble high molecular mass HA binds then to CD44 and inhibits MMP-9 production. Such process would eventually help avoiding excess ECM degradation and prevent migration of OCs to nearby sites to perform additional bone degradation. This hypothesis is in accordance with the prevalent localization of CD44 on the microvilli of the basolateral plasma membrane of OC cells rather than in the area in direct contact with the bone surface (Nakamura and Ozawa, 1996). The recent identification of a peptide inhibitor of HA-mediated leukocyte trafficking using phage display technology can provide a pharmacological reagent that might also result useful in balancing excessive bone resorption by inhibiting the interactions between HA and OC precursors, thus preventing their migration toward bone sites (Mummert et al., 2000).

Materials and methods

Source and characteristics of antibodies and flow cytometry

Expression of various cell surface components was analyzed by single-color direct or indirect immunofluorescence by using mAbs recognizing the following: β1/CD29, α2/CD49b, α3/CD49c, α4/CD49d, α5/CD49e, α6/CD49f, β2/CD18, β3/CD61 (Beckman Coulter/Immunotech S.A.), α9β1 complex (CHEMICON International, Inc.), β4, and β7. These latter mAbs, as well as mAbs recognizing the standard CD44 isoform, were obtained through the "Adhesion Structures" panel of the Fifth International Workshop of Leukocyte Typing (Denning et al., 1995). As second step reagents, isotype-matched control mAbs and phycoerythrin-conjugated $F(ab')_2$ fragments of goat anti–mouse Igs were purchased from Jackson ImmunoResearch Laboratories. Viable, antibody-labeled cells were identified according to their forward and side scatter, electronically gated, and assayed for surface fluorescence on a FACScan™ flow cytometer (Immunocytometry Systems, Becton Dickinson). In some instances, cells were labeled with FITC-conjugated HA and similarly analyzed. Function-blocking mAbs recognizing specific CD44 epitopes were from the Fifth International Workshop of Leukocyte Typing (Denning et al., 1995), whereas anti-β1 (clone 4B4) was from Coulter/Immunotech S.A., anti-α1 (clone FB12), anti-α3 (clone P1B5), anti-αvβ3 (clone LM 609), and anti-αvβ5 (clone P1F6) were obtained from CHEMICON International, Inc., and anti-α6 (clone GoH3) was provided by Dr. Arnoud Sonnenberg (Netherlands Cancer Institute, Amsterdam, Netherlands). Antibodies against MMP-9 were obtained from Santa Cruz Biotechnology, Inc. (M-17) or from CHEMICON International, Inc. (AB805).

ECM molecule

FN was purchased from Calbiochem-Novabiochem. VN was purified from human plasma according to the procedure of Yatohgo et al. (1988). Rat tail collagen type I was obtained from Collaborative Research Biochemicals. Native LN-1 nidogen complex from EHS mouse tumor, LN-5, and LN-8 were obtained as described previously (Spessotto et al., 2001). LN-10 from human placenta was purchased from CHEMICON International, Inc. Several preparations of HA were purchased from Sigma-Aldrich (H1504, H1751, H7630, and H1876) and Calbiochem-Novachem (385902).

Cells and culture conditions

The human preOC cell line FLG 29.1 was maintained in Iscove's modified Dulbecco medium (IMDM; Seromed, Biochrom) supplemented with L-glutamine (Seromed) and 10% FBS (Seromed) at 37°C and 5% CO_2, and induced into mature OC-like cells by treatment with 1.0×10^{-7} M 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich) for 72 h. Specific OC differentiation was determined by a number of morphological, immunochemical, and molecular features (Gattei et al., 1992, 1996; Fiorelli et al., 1994, 1995). In this paper, we refer to the uninduced FLG 29.1 cells as preOC and to the differentiated cells as OC-like cells.

Confocal laser scanning microscope

OC-like FLG 29.1, cultured in the presence or absence of 1 mg/ml HA, were (a) stimulated with 10 ng/ml TNF-α for 24 h and centrifuged onto ethanol-cleaned slides; or (b) allowed to adhere onto FN-coated slides. In both cases, slides were air dried and cells were fixed in PBS for 10 min at RT with 3.7% formaldehyde and then permeabilized with 0.1% Triton X-100 for 2 min. Slides were extensively washed with 0.1% BSA/PBS and blocked for 30 min with 2% BSA/PBS before application of antibodies. Incubation with both primary (anti–MMP-9 M-17 and anti-CD44 F-10–44–2) antibodies and secondary (matched FITC-conjugated anti-Ig; Jackson ImmunoResearch Laboratories) antibodies, diluted in 0.1% BSA/PBS (1:100) was performed for 1 h at RT in a moist chamber. Actin staining was performed using Texas red phalloidin (Molecular Probes, Inc.). Slides were then mounted in MOWIOL® 4-88 (Calbiochem-Novabiochem) containing 2.5% 1,4-diazabicyclo[2,2,2]octane (Sigma-Aldrich) as antifading agent. Immunofluorescence-labeled cells were studied using a confocal laser scanning microscope (Diaphot 200 [Nikon]; MRC-1024 [Bio-Rad Laboratories]).

RT-PCR, Southern blotting, and nucleotide sequencing

1μg total RNA, extracted from FLG 29.1 cells under different experimental conditions by the guanidium-thiocyanate method, was reverse-transcribed using avian myeloblastosis virus reverse transcriptase (Promega) for 1 h at 42°C in a 20-μl mix containing 0.4 μg hexadeoxyribonucleotides random primers. All cDNA preparations were checked for first-strand synthesis as described previously (Gattei et al., 1996). 2 μl of the cDNA preparations was amplified in a 50-μl final volume containing 25 pmol of primers specific for α8 (sense, 5′-ACACCACCAACAACAGAAAGA-3′; antisense, 5′-AACTCCCCAGCAGCAACT-3′) and α9 (sense, 5′-TTCCAAATACAGCC-CTTCAGT-3′; antisense, 5′-AAATATAAACCTTGCCGATGC-3′) integrin chains in a thermal cycler (PTC200; MJ Research, Inc.). Amplification conditions were as follows: 35 cycles of 30 s at 94°C, 45 s at 62°C, 90 s at 72°C, and a final extension of 5 min at 72°C. For CD44 studies, we chose a common sense primer, located on exon 5 (5′-GCAGCACTTCAGGAGGT-TACA-3′), used in conjunction with different antisense primers annealing to exon 15 (5′-GGGTGGAATGTGTCTTGGTC-3′), exon 10/v6 (5′-TCTG-TTGCCAAACCACTGTTC-3′), or exon 12/v8 (5′-GCTGCGTTGTCAT-TGAAAGAG-3′). For all primer pairs, the PCR amplification protocol was a two-step procedure consisting of 3 min at 94°C followed by 35 cycles of 30 s at 94°C and 75 s at 68°C. For Southern blotting, 10 μl of amplified cDNA was run on 1.5% agarose gel, blotted onto nylon membranes (Boehringer) and hybridized with 2×10^6 cpm/ml of a common ^{32}P 5′-end-labeled oligoprobe recognizing specific sequences within the exon 5 of CD44 (5′-ATGGGAGTCAAGAAGGTGGAGCAA-3′). After washing to a final stringency of 0.2× standard sodium cytrate-0.1× SDS, filters were exposed for 1.5 h to XAR-5 film (Kodak) at −80°C. For MMP-9 detection, cDNAs were amplified with specific primers (sense, 5′-CACCATGAGCCTCTGGCAGC-3′; antisense, 5′-GCCCAGGGACCACAACTCGT-3′) along with SYBR® Green PCR core reagents (Applied Biosystems) according to the manufacturer's protocol. The incorporation of the SYBR® Green dye into the PCR products was monitored in real time with a sequence detection system (ABI PRISM 7700; Applied Biosystems), resulting in the calculation of threshold cycle (Ct value) that defines the PCR cycle number at which an exponential growth of PCR product begins. The Ct values for β2-microglobulin (β2M) and MMP-9 were used to calculate the abundance of MMP-9 transcripts relative to that of β2M mRNA. The oligonucleotide primers for β2M cDNA

were as follows: sense, 5′-TCCAGCGTACTCCAAAGATTCA-3′; antisense, 5′-AGATTAACCACAACCATGCCTTACT-3′.

Antisense oligonucleotide treatment

An antisense oligodeoxyribonucleoside methylphosphonate probe targeted to the 5′-region of MMP-9 mRNA (from nucleotides −19 to +4: 5′-CUC-AUGGUGAGGGCAGAGGUGUCU-3′) and a control scrambled oligonucleotide (5′-CUACGGGUAGGUGCGGAAUGGGUC-3′) were synthesized. PreOC cells were induced with TPA for 4 h, washed, and appropriate amounts (2–10 μM) of oligonucleotides were added for 24 h in the presence of TPA. At the end of the incubation, an aliquot of the cells was solubilized, total RNA was extracted, and the expression of MMP-9 was evaluated by real-time PCR. Other cells were allowed to migrate toward FN-coated wells (see Fluorescence-assisted transmigration invasion and motility assay [FATIMA]) for 24 h and the fraction of migrated cells was then evaluated.

Zymography and Western blotting

PreOC and OC-like FLG29.1 cells were washed, resuspended (10^6 cells/ml) in IMDM, and incubated with 10 ng/ml TNF-α for 24 h at 37°C. The supernatants were collected, centrifuged at 15,000 rpm for 20 min at 4°C, and the cells were lysed in 1 ml Tris buffer containing 1% Triton X-100. Supernatants and cell lysates (150 μl/well) were loaded onto SDS–acrylamide-gelatin gels (8% polyacrylamide, 0.1% gelatin). The gels were then washed twice for 30 min with 50 mM Tris-HCl, pH 7.4, containing 2.5% Triton X-100, incubated overnight at 37°C in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 10 mM $CaCl_2$, stained for 30 min with 30% methanol/10% acetic acid containing 0.5% Coomassie brilliant blue R-250, destained, and finally photographed.

Supernatants, precipitated with TCA, were subjected to electrophoresis on an 8% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. Membranes were incubated with rabbit anti–MMP-9 antibodies (AB805), and finally with peroxidase-labeled goat anti–rabbit antibodies (Zymed Laboratories) followed by enhanced chemiluminescence (ECL Plus; Amersham Biosciences). To visualize CD44, cell lysates obtained from preOC and OC-like cells exposed to HA and TNF-α, were subjected to 8% SDS-PAGE and Western blotting with anti-CD44 pAbs.

CAFCA

The quantitative cell adhesion assay used in this study has been described extensively (Spessotto et al., 2000, 2001). In brief, polyvinyl chloride 6-well plates, CAFCA strips (Whatman Inc.), were coated with ECM molecules. Cells were labeled with the vital fluorochrome calcein AM (Molecular Probes, Inc.) for 15 min at 37°C, and then aliquoted into the bottom CAFCA miniplates at a density of $20–50 \times 10^4$ cells/ml. Cell adhesion to substrates was assayed in PBS containing 0.5% polyvinyl pyrrolidone (PVP 360; Sigma Chemical Co.), 1 mmol/liter $MgCl_2$, 1 mmol/liter $CaCl_2$, and 2% India ink as a fluorescence quencher. In some instances, cells were preincubated with function-blocking anti-integrin mAbs (1–10 μg/ml) for 20 min at 37°C, and then plated onto the substrate. CAFCA miniplates were centrifuged at 142 *g* for 5 min at 37°C to synchronize the contact of the cells with the substrate, incubated for 20 min at 37°C, and mounted together with a similar CAFCA miniplate to create communicating chambers for subsequent reverse centrifugation (45 *g*). In some experiments, different detachment forces were applied to measure the strength of adhesion (from 45 *g* up to 400 *g*). The relative number of cells bound to the substrate and cells that fail to bind to the substrate were estimated by top/bottom fluorescence detection in a computer-interfaced microplate fluorometer (SPECTRAFluor Plus; Tecan), and fluorescence values were then elaborated.

Fluorescence-assisted transmigration invasion and motility assay (FATIMA)

Migration experiments involving haptotactic movement of the cells through a porous membrane were performed according to the FATIMA assay (Spessotto et al., 2000). The procedure is based on the use of Transwell-like inserts, carrying a fluorescence-shielding porous PET membrane (polycarbonate-like material; 8-μm pores) (HTS FluoroBlok™ inserts; Becton Dickinson) or Transwells (Corning Costar Corporation) in cases where there was an interest in examining the morphology of the migrated cells or counting the number of cells per field under the microscope. The underside of the insert membrane was coated with the various purified ECM molecules in bicarbonate buffer at 4°C overnight and blocked with 1% BSA for 1 h at RT. Cells were fluorescently tagged with a lipophilic dye (DiI; Molecular Probes, Inc.) at a final 5-μg/ml concentration for 10–15 min at 37°C, resuspended in RPMI with 0.1% BSA, and aliquoted into the upper side of each insert unit (10^5 cells/insert), with and without blocking or unrelated control mAbs. In some cases, TIMP-1 (R & D Systems Europe Ltd.) and the MMP inhibitor GM6001 (CHEMICON International, Inc.) were added to the upper chamber. Conditioned medium from the giant cell tumor–derived stromal cell line C433 or NIH-3T3 cells was in some cases added to the lower chamber to generate chemotactic effects. The time-dependent migratory behavior of the cells was monitored by the SPECTRAFluor Plus microplate fluorometer from the top (nonmigrated cells) and bottom (migrated cells) side of the porous membrane. In some cases, migrated cells were counted under inverted microscopy (magnification of 20). Results are expressed as cells per field (minimum of 5–10 microscopy fields counted).

This work was supported in part by grants from the Associazione Italiana per la Ricerca sul Cancro (V. Gattei), the FSN-Ricerca Finalizzata 1999–2000 (V. Gattei) and 2000–2001 (V. Gattei and A. Colombatti) of the Ministero della Sanità, and by research grants from the University of Parma (R. Perris).

Submitted: 26 February 2002
Revised: 16 July 2002
Accepted: 5 August 2002

References

Ahrens, T., J.P. Sleeman, C.M. Schempp, N. Howells, M. Hofmann, H. Ponta, P. Herrlich, and J.C. Simon. 2001. Soluble CD44 inhibits melanoma tumor growth by blocking cell surface CD44 binding to hyaluronic acid. *Oncogene.* 20:3399–3408.

Arai, F., T. Miyamoto, O. Ohneda, T. Inada, T. Sudo, K. Brasel, T. Miyata, D.M. Anderson, and T. Suda. 1999. Commitment and differentiation of osteoclast precursor cells by the sequential expression of c-Fms and receptor activator of nuclear factor κB (RANK) receptors. *J. Exp. Med.* 190:1741–1754.

Aruffo, A. 1996. CD44: one ligand, two functions. *J. Clin. Invest.* 98:2191–2192.

Bartolazzi, A., A. Nocks, A. Aruffo, F. Spring, and I. Stamenkovic. 1996. Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. *J. Cell Biol.* 132:1199–1208.

Blavier, L., and J.M. Delaisse. 1995. Matrix metalloproteinases are obligatory for the migration of preosteoclasts to the developing marrow cavity of primitive long bones. *J. Cell Sci.* 108:3649–3659.

Bord, S., A. Horner, C.A. Beeton, R.M. Hembry, and J.E. Compston. 1999. Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) distribution in normal and pathological human bone. *Bone.* 24:229–235.

Borland, G., J.A. Ross, and K. Guy. 1998. Forms and functions of CD44. *Immunology.* 93:139–148.

Bourguignon, L.Y., Z. Gunja-Smith, N. Iida, H.B. Zhu, L.J. Young, W.J. Muller, and R.D. Cardiff. 1998. CD44v(3,8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *J. Cell. Physiol.* 176:206–215.

Bourguignon, L.Y., H. Zhu, L. Shao, and Y.W. Chen. 2000. CD44 interaction with Tiam1 promotes Rac1 signaling and hyaluronic acid-mediated breast tumor cell migration. *J. Biol. Chem.* 275:1829–1838.

Denning, S.M., M.J. Telen, L.P. Hale, H.X. Liao, and B.F. Haynes. 1995. CD44 and CD44R cluster report. *In* Leucocyte Typing. V.S.F. Schlossman, L. Boumsell, W. Gilks, J.M. Harlan, T. Kishimoto, C. Morimoto, J. Ritz, S. Shaw, R. Silverstein, T. Springer, T.F. Tedder, and R.F. Todd, editors. Oxford University Press, Inc., Oxford. 1713–1719.

Duong, L.T., P. Lakkakorpi, I. Nakamura, and G.A. Rodan. 2000. Integrins and signaling in osteoclast function. *Matrix Biol.* 19:97–105.

Engsig, M.T., Q.J. Chen, T.H. Vu, A.C. Pedersen, B. Therkidsen, L.R. Lund, K. Henriksen, T. Lenhard, N.T. Foged, Z. Werb, and J.M Delaisse. 2000. Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. *J. Cell Biol.* 151:879–889.

Espaza, J., C. Vilardell, J. Calco, M. Juan, J. Vives, A. Urbano-Marquez, J. Yague, and M.C. Cid. 1999. Fibronectin upregulates gelatinase B (MMP-9) and induces coordinated expression of gelatinase A (MMP-2) and its activator MT1-MMP (MMP-14) by human T lymphocyte cell lines. A process repressed through RAS/MAP kinase signaling pathways. *Blood.* 94:2754–2765.

Everts, V., W. Korper, D.C. Jansen, J. Steinfort, I. Lammerse, S. Heera, A.J. Docherty, and W. Beertsen. 1999. Functional heterogeneity of osteoclasts: matrix metalloproteinases participate in osteoclastic resorption of calvarial bone but not in resorption of long bone. *FASEB J.* 13:1219–1230.

Fiorelli, G., R.T. Ballock, L.M. Wakefield, M.B. Sporn, F. Gori, L. Masi, U. Frediani, A. Tanini, P.A. Bernabei, and M.L. Brandi. 1994. Role for autocrine TGF-β 1 in regulating differentiation of a human leukemic cell line toward osteoclast-like cells. *J. Cell. Physiol.* 160:482–490.

Fiorelli, G., F. Gori, M. Petilli, A. Tanini, S. Benvenuti, M. Serio, P. Bernabei, and

M.L. Brandi. 1995. Functional estrogen receptors in a human preosteoclastic cell line. *Proc. Natl. Acad. Sci. USA.* 92:2672–2676.

Gattei, V., P.A. Bernabei, A. Pinto, R. Bezzini, A. Ringressi, L. Formigli, A. Tanini, V. Attadia, and M.L. Brandi. 1992. Phorbol ester induced osteoclast-like differentiation of a novel human leukemic cell line (FLG 29.1). *J. Cell Biol.* 116:437–447.

Gattei, V., D. Aldinucci, J.M. Quinn, M. Degan, M. Cozzi, V. Perin, A. De Iuliis, S. Juzbasic, S. Improta, N.A. Athanasou, et al. 1996. Human osteoclasts and preosteoclast cells (FLG 29.1) express functional c-kit receptors and interact with osteoblast and stromal cells via membrane-bound stem cell factor. *Cell Growth Differ.* 7:753–763.

Giannelli, G., J. Falk-Marzillier, O. Schiraldi, W.G. Stetler-Stevenson, and V. Quaranta. 1997. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science.* 277:225–228.

Hill, P.A., G. Murphy, A.J. Docherty, R.M. Hembry, T.A. Millican, J.J. Reynolds, and M.C. Meikle. 1994. The effects of selective inhibitors of matrix metalloproteinases (MMPs) on bone resorption and the identification of MMPs and TIMP-1 in isolated osteoclasts. *J. Cell Sci.* 107:3055–3064.

Holly, S.P., M.K. Larson, and L.V. Parise. 2000. Multiple roles of integrins in cell motility. *Exp. Cell Res.* 261:69–74.

Ilangumaran, S., B. Borisch, and D.C. Hoessli. 1999. Signal transduction via CD44: role of plasma membrane microdomains. *Leuk. Lymphoma.* 35:455–469.

Inaoka, T., G. Bilbe, O. Ishibashi, K. Tezuka, M. Kumegawa, and T. Kokubo. 1995. Molecular cloning of human cDNA for cathepsin K: novel cysteine proteinase predominantly expressed in bone. *Biochem. Biophys. Res. Commun.* 206:89–96.

Jiang, H., R.S. Peterson, W. Wang, E. Bartnick, C.B. Knudson, and W. Knudson. 2002. A requirement for the CD44 cytoplasmic domain for hyaluronan binding, pericellular matrix assembly, and receptor-mediated endocytosis in COS-7 cells. *J. Biol. Chem.* 277:10531–10538.

Kajita, M., Y. Itoh, T. Chiba, H. Mori, A. Okada, H. Kinoh, and M. Seiki. 2001. Membrane-type 1 matrix metalloproteinase cleaves CD44 and promotes cell migration. *J. Cell Biol.* 153:893–904.

Kania, J.R., T. Kehat-Stadler, and S.R. Kupfer. 1997. CD44 antibodies inhibit osteoclast formation. *J. Bone Miner. Res.* 12:1155–1164.

Lacey, D.L., E. Timms, H.L. Tan, M.J. Kelley, C.R. Dunstan, T. Burgess, R. Elliott, A. Colombero, G. Elliott, S. Scully, et al. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* 93:165–176.

Ladeda, V., J.A. Aguirre Ghiso, and E. Bal de Kier Joffe. 1998. Function and expression of CD44 during spreading, migration, and invasion of murine carcinoma cells. *Exp. Cell Res.* 242:515–527.

Lanir, N., P.S. Ciano, L. van de Water, J. McDonagh, A.M. Dvorak, and H.F. Dvorak. 1988. Macrophage migration in fibrin gel matrices. II. Effects of clotting factor XIII, fibronectin, and glycosaminoglycan content on cell migration. *J. Immunol.* 140:2340–2349.

Lee, E.R., G. Murphy, M. El-Alfy, M.A. Davoli, L. Lamplugh, A.J. Docherty, and C.P. Leblond. 1999. Active gelatinase B is identified by histozymography in the cartilage resorption sites of developing long bones. *Dev. Dyn.* 215:190–205.

Lee, J.Y., and A.P. Spicer. 2000. Hyaluronan: a multifunctional, megaDalton, stealth molecule. *Curr. Opin. Cell Biol.* 12:581–586.

Leppert, D., E. Waubant, R. Galardy, N.W. Bunnett, and S.L. Hauser. 1995. T cell gelatinases mediate basement membrane transmigration in vitro. *J. Immunol.* 154:4379–4389.

Lerner, U.H. 2000. Osteoclast formation and resorption. *Matrix Biol.* 19:107–120.

Lesley, J., and R. Hyman. 1998. CD44 structure and function. *Front. Biosci.* 3:D616–D630.

Mangeat, P., C. Roy, and M. Martin. 1999. ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol.* 9:187–192.

Martin, K.H., J.K. Slack, S.A. Boerner, C.C. Martin, and J.T. Parson. 2002. Integrin connections map: to infinity and beyond. *Science.* 296:1652–1653.

Mummert, M.E., M. Mohamadzadeh, D.I. Mummert, N. Mizumoto, and A. Takashima. 2000. Development of a peptide inhibitor of hyaluronan-mediated leukocyte trafficking. *J. Exp. Med.* 192:769–779.

Nagase, H., and J.F. Woessner, Jr. 1999. Matrix metalloproteinases. *J. Biol. Chem.* 274:21491–21494.

Nakamura, H., and H. Ozawa. 1996. Immunolocalization of CD44 and the ERM family in bone cells of mouse tibiae. *J. Bone Miner. Res.* 11:1715–1722.

Naor, D., R.V. Sionov, and D. Ish-Shalom. 1997. CD44: structure, function, and association with the malignant process. *Adv. Cancer Res.* 71:241–319.

Noonan, K.J., J.W. Stevens, R. Tammi, M. Tammi, J.A. Hernandez, and R.J. Midura. 1996. Spatial distribution of CD44 and hyaluronan in the proximal tibia of the growing rat. *J. Orthop. Res.* 14:573–581.

Okada, H., J. Yoshida, M. Sokabe, T. Wakabayashi, and M. Hagiwara. 1996. Suppression of CD44 expression decreases migration and invasion of human glioma cells. *Int. J. Cancer.* 66:255–260.

Okada, Y., K. Naka, K. Kawamura, T. Matsumoto, I. Nakanishi, N. Fujimoto, H. Sato, and M. Seiki. 1995. Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: implications for bone resorption. *Lab. Invest.* 72:311–322.

Okamoto, I., Y. Kawano, H. Tsuiki, J. Sasaki, M. Nakao, M. Matsumoto, M. Suga, M. Ando, M. Nakajima, and H. Saya. 1999. CD44 cleavage induced by a membrane-associated metalloprotease plays a critical role in tumor cell migration. *Oncogene.* 18:1435–1446.

Oliferenko, S., I. Kaverina, J.V. Small, and L.A. Huber. 2000. Hyaluronic acid (HA) binding to CD44 activates Rac1 and induces lamellipodia outgrowth. *J. Cell Biol.* 148:1159–1164.

Peck, D., and C.M. Isacke. 1998. Hyaluronan-dependent cell migration can be blocked by a CD44 cytoplasmic domain peptide containing a phosphoserine at position 325. *J. Cell Sci.* 111:1595–1601.

Rochman, M., J. Moll, P. Herrlich, S.B. Wallach, S. Nedvetzki, R.V. Sionov, I. Golan, D. Ish-Shalom, and D. Naor. 2000. The CD44 receptor of lymphoma cells: structure-function relationships and mechanism of activation. *Cell Adhes. Commun.* 7:331–347.

Roodman, G.D. 1996. Advances in bone biology: the osteoclast. *Endocr. Rev.* 17:308–332.

Sato, T., M. del Carmen Ovejero, P. Hou, A.M. Heegaard, M. Kumegawa, N.T. Foged, and J.M. Delaisse. 1997. Identification of the membrane-type matrix metalloproteinase MT1-MMP in osteoclasts. *J. Cell Sci.* 110:589–596.

Sato, T., N.T. Foged, and J.M. Delaisse. 1998. The migration of purified osteoclasts through collagen is inhibited by matrix metalloproteinase inhibitors. *J. Bone Miner. Res.* 13:59–66.

Seiki, M. 1999. Membrane-type matrix metalloproteinases. *APMIS.* 107:137–143.

Sohara, Y., N. Ishiguro, K. Machida, H. Kurata, A.A. Thant, T. Senga, S. Matsuda, K. Kimata, H. Iwata, and M. Hamaguchi. 2001. Hyaluronan activates cell motility of v-Src-transformed cells via ras-mitogen-activated protein kinase and phosphoinositide 3-kinase-Akt in a tumor-specific manner. *Mol. Biol. Cell.* 12:1859–1868.

Spessotto, P., E. Giacomello, and R. Perris. 2000. Fluorescence assays to study cell adhesion and migration in vitro. *Methods Mol. Biol.* 139:321–343.

Spessotto, P., Z. Yin, G. Magro, R. Deutzmann, A. Chiu, A. Colombatti, and R. Perris. 2001. Laminin isoforms 8 and 10 are primary components of the subendothelial basement membrane promoting interaction with neoplastic lymphocytes. *Cancer Res.* 61:339–347.

Teitelbaum, S.L. 2000. Bone resorption by osteoclasts. *Science.* 289:1504–1508.

Tezuka, K., K. Nemoto, Y. Tezuka, T. Sato, Y. Ikeda, M. Kobori, H. Kawashima, H. Eguchi, Y. Hakeda, and M. Kumegawa. 1994. Identification of matrix metalloproteinase 9 in rabbit osteoclasts. *J. Biol. Chem.* 269:15006–15009.

Thomas, L., T. Etoh, I. Stamenkovic, M.C. Mihm, Jr., and H.R. Byers. 1993. Migration of human melanoma cells on hyaluronate is related to CD44 expression. *J. Invest. Dermatol.* 100:115–120.

Trochon, V., C. Mabilat, P. Bertrand, Y. Legrand, F. Smadja-Joffe, C. Soria, B. Delpech, and H. Lu. 1996. Evidence of involvement of CD44 in endothelial cell proliferation, migration and angiogenesis in vitro. *Int. J. Cancer.* 66:664–668.

Vu, T.H., J.M. Shipley, G. Bergers, J.E. Berger, J.A. Helms, D. Hanahan, S.D. Shapiro, R.M. Senior, and Z. Werb. 1998. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell.* 93:411–422.

Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13:281–292.

Yu, Q., B.P. Toole, and I. Stamenkovic. 1997. Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. *J. Exp. Med.* 186:1985–1996.

Yu, Q., and I. Stamenkovic. 1999. Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. *Genes Dev.* 13:35–48.

Zhang, Y., K. McCluskey, K. Fujii, and L.M. Wahl. 1998. Differential regulation of monocyte matrix metalloproteinase and TIMP-1 production by TNF-α, granulocyte-macrophage CSF, and IL-1-β through prostaglandin-dependent and –independent mechanisms. *J. Immunol.* 161:3071–3076.

Zhao, W., M.H. Byrne, B.F. Boyce, and S.M. Krane. 1999. Bone resorption induced by parathyroid hormone is strikingly diminished in collagenase-resistant mutant mice. *J. Clin. Invest.* 103:517–524.