# Pro-collagen I COOH-terminal Trimer Induces Directional Migration and Metalloproteinases in Breast Cancer Cells*

**Daniela Palmieri‡, Silvia Poggi‡, Valentina Ulivi‡, GianLuigi Casartelli§, and Paola Manduca‡¶**

*From the ‡Genetica, Dipartimento di Oncologia, Biologia e Genetica, Università di Genova, 26 C. Europa and §Istituto per la Ricerca sul Cancro, Viale Benedetto XIV, Genova 16132, Italy*

Breast and prostatic carcinomas, melanoma, and endothelial cell lines are chemoattracted by medium conditioned by mature osteoblasts. The chemoattractant for endothelial cells was identified with C3, carboxyl-terminal trimer of pro-collagen type I. We report that C3 induces directional migration and proliferation, the expression of tissue inhibitor of metalloproteinases-2, pro-metalloproteinase-2 and –9, and their activation in MDA MB231 cells, without changing the expression of tissue inhibitor of metalloproteinases-1 and of metalloproteinase-14. Antiserum against metalloproteinase-2 or -9 or -14, tissue inhibitor of metalloproteinases-1, or GM6001 inhibits the C3-induced migration. Urokinase and its receptor are detected and unchanged upon exposure to C3. The antibody against urokinase or addition of plasminogen activator inhibitor inhibits migration. Blocking antibodies to integrins $\alpha_2$, $\alpha_6$, $\beta_1$, and $\beta_3$ inhibit chemotaxis and do not change urokinase and urokinase receptor expression. Blockage of $\alpha_2$, $\beta_1$, and $\beta_3$ integrins affect differently the induction by C3 of pro-metalloproteinase-2 and -9 and of tissue inhibitor of metalloproteinases-2. Chemotaxis to C3 is also inhibited by genistein, by pertussis toxin, which also inhibits C3-induced pro-metalloproteinase -2 and -9, but not urokinase expression. Wortmannin partially inhibits C3-induced cell migration. Other, but not all, breast carcinoma lines tested responded to C3 with migration and pro-metalloproteinase-2 induction. Presently C3 is the only agent known to induce migration specifically of both endothelial and breast carcinoma cells. The mitogenic and motogenic role of C3 *in vitro* might prefigure a role in *in vivo* carcinogenesis and in the establishment of metastasis.

Mature osteoblasts conditioned medium (CM),[1] collected when sustained biosynthesis of collagen type I occurs during osteogenesis *in vitro* from differentiating cultures of rat, rabbit, human osteoblasts, and conditionally transformed human osteoblast-like line HFO, induces directional migration of endothelial and melanoma and breast and prostatic carcinoma cells but not of normal cells or of transformed mesenchymal cells in the classical *in vitro* double chamber Boyden assay (1–4).

The molecule chemoattractant for endothelial cells was purified from rat osteoblast CM, and it identifies by sequence, immunoreactivity, and molecular size with the trimer carboxyl-terminal of type I pro-collagen, C3 (4). C3 is a by-product of the processing of pro-collagen by pro-collagenase C (or BMP-1), preliminary to the formation of collagen fibrils (5).

We investigated whether MDA MB231 breast carcinoma cells, which are stimulated by mature osteoblasts CM to directional migration, are induced by C3 to activate migratory and proliferation responses. It is recognized that the stroma of the tumors plays instructive and active roles toward tumor cells growth and invasiveness and that tumor-associated stromal fibroblasts undergo changes in the biosynthetic phenotype (6), assume the production of factors supportive for the proliferation of initiated tumor cells (7–10), and undergo precocious genotypic changes (11, 12).

We focused the study on a breast carcinoma-derived cell line because it is known that stromal fibroblasts of breast tumors synthesize type I collagen (6), and high expression of type I collagen in the stroma correlates with worse prognosis and high invasiveness *in vivo* (10). Molecules, produced and localized in the microenvironment of the stroma shared by tumor and endothelial cells and capable of inducing in both cell types directional migration and/or cell proliferation and peri-cellular proteolysis, might behave as promoters for the overall development of primary tumors and for the establishment of metastasis. No such molecule has been yet described, and C3 presents itself as a likely candidate for this role.

Tumor cells invasion and angiogenesis share mechanistic analogies and the common feature of presenting high expression of metalloproteinases (MMP) and/or serine proteinases, in particular the urokinase (uPA) system, and/or their activation. In both tumor and endothelial cells the enhancement of peri-cellular proteolysis and the acquisition of the migratory phenotype can occur in response to various extracellular matrix components or to factors stored within it, mediated through engagement of integrins, growth factors receptors, and activation of heterotrimeric G-proteins (13, 14). Increased expression and activation of pro-MMPs play multiple roles during angiogenesis and tumor growth and invasion as follows: modulation of tight cell-cell and cell-ECM relationships, control of tissue homeostasis, proteolysis, release from the ECM of growth factors, and of protein fragments with distinct biological activities (15–17). MMPs regulate tumor cell growth, at both primary and metastatic sites. Increased expression of pro-MMP-2 and -9 is associated to the metastatic behavior of most tumors, including breast carcinoma, and *in vitro* the production of active forms of MMPs is associated with the induction of migratory behavior in cancer cells (18–22). The migration of mouse

[1] The abbreviations used are: CM, conditioned medium; C3, carboxyl-terminal trimer of pro-collagen type I; ER, estrogen receptor; MMP, metalloproteinase; TIMP, tissue inhibitor of MMP; uPA, urokinase-uPA; uPAR, urokinase receptor; PAI, plasminogen activator inhibitor; PTX, pertussis toxin; ECM, extracellular matrix; PI3K, phosphatidylinositol 3-kinase; FCS, fetal calf serum; PTK, protein-tyrosine kinase; SFM, serum-free medium.

*Pro-collagen I COOH-terminal Trimer Chemoattracts Tumor Cells*

melanoma, human breast, and prostatic carcinoma cell lines induced by CM from mature rat and rabbit osteoblasts is accompanied by induction of MMPs (2, 3). Tissue inhibitors of metalloproteinases (TIMP) -1 and -2 play a regulatory role on proteolysis by MMPs (16–19, 23). Modulation of cell adhesion and cell migration can depend on the direct interaction of MMP-14 and MMP-2 with integrins and ECM components (24–26). The uPA system determines the controlled proteolysis of ECM, regulated by a complex network of feedback mechanisms and by the balancing in amount of proteinases (tPA and uPA), their inhibitors (PAIs), and the uPA receptor (uPAR). It is involved in the activation of secreted and ECM-associated growth factors, in cell adhesion, migration, cytoskeleton reorganization, in MMP-9 and -14 activation (27), and in proliferation (28–29). The uPA system has an important and possibly causal role in the development of the metastatic phenotype. Free uPA is a prognostic marker for human breast malignancy (30–31), and the expression of uPAR is associated with invasiveness in breast tumors and is absent in normal breast tissue (32–36). Proteolytic and non-proteolytic mechanisms have been implied for the effects of uPA on cell migration. In prostatic carcinoma cells lines the uPA system was shifted by the exposure to the CM collected from mature osteoblasts toward a balance favoring proteolysis (3). The intrinsic basal mobility *in vitro* of the breast carcinoma cell line MDA MB231 is regulated by the level of uPA, via activation of phosphatidylinositol 3-kinase (PI3K) (37). Modulation of cell migration is dependent on the direct interaction of uPAR with integrins and ECM components (28, 38).

Integrin receptors mediate multiple cell responses to the ECM and the peri-cellular microenvironment, adhesion, migration, apoptosis, and proliferation (39). Different integrins interact on the cell surface with multiple transmembrane proteins and with membrane-associated receptors as well as and depending on interactions with the ECM (31, 38, 40, 41). Expression and utilization of the integrin chains $\beta_1$ and $\beta_4$ in breast epithelium controls cell mobilization, concomitant to ECM-derived and growth factor-associated stimuli (42). $\alpha_6\beta_1$ expression is associated with the migratory behavior of early differentiating normal breast cells and invasive breast carcinoma, in the regulation of mammary epithelial cells survival, and in the apoptotic response of breast carcinoma cells (43–44). $\alpha_6\beta_4$ is involved in the ECM-independent migratory behavior and in the survival of breast epithelial cells (45). $\alpha_3\beta_1$ (46) and $\alpha_v\beta_3$ integrins interact with uPAR, uPA, PAI, and ECM components (29). $\alpha_v\beta_3$ enters in complexes with MMP-14 and MMP-2, functional to the enhancement of peri-cellular proteolysis and to inside-in signaling in a transfected cell line of breast carcinoma (29, 40, 47) and in melanoma (24). The effects of these interactions are the control of localized proteolysis, through regulation of proteinase function and/or directional migration. In ovarian carcinoma cells in collagen gels $\beta_1$ integrin controls the expression of MMP-14 and MMP-2 (48); in melanoma cells $\beta_3$ integrin signaling regulates MMP-1 expression (24); in MDA MB231 cells $\alpha_3\beta_1$-tetraspanin complexes regulate the expression of MMP-2 via PI3K-mediated signaling (41), and in osteosarcoma cells plated in collagen, $\alpha_2\beta_1$ integrin regulates the expression of MMP-1 (49). In tumor cells different integrins interacting with different ECM proteins can therefore regulate the expression of secreted and transmembrane MMPs, and supermolecular complexes of integrins with transmembrane and/or secreted MMPs, and/or uPA and uPAR, can regulate in turn both peri-cellular proteolysis, cell migration, and cell proliferation.

In endothelial cells the directional migration induced by C3 requires the function of $\alpha_1$, $\beta_1$, and $\beta_3$ integrins and is inhibited by pertussis toxin (PTX) (4), and the cell signaling pathway(s) activated through these integrins involve phosphotyrosine kinase and heterotrimeric G-proteins (4, 39). In general, the signaling machinery involved in the induction of cell migratory behavior includes pathways mediated through activation of phosphotyrosine kinases (inhibited by genistein), heterotrimeric G-proteins (13, 14) (inhibited by PTX) (4, 50), and PI3K (inhibited by wortmannin) (37, 48). All of these signaling pathways are also, directly or in a cooperative fashion, capable of affecting the progression of the cell cycle and/or the cell death by apoptosis (39, 52). The mechanistic aspects of this signaling are still largely unraveled.

In this study we investigated if purified C3 causes the induction of directional migration and proliferation and/or apoptosis of human breast carcinoma cell lines. We used inhibitors and antibodies to investigate which proteinases and receptors and which elements of the molecular machinery of cell signaling are involved in the induction of the migratory process by C3.

EXPERIMENTAL PROCEDURES

*Cell Cultures*—Rat tibial osteoblasts cultures (ROB) were obtained as described (1) and expanded in Coon's-modified F-12 supplemented with 10% fetal calf serum (FCS, Seromed, Italy). Cultures at 46–70 cumulative population doublings were utilized for the preparation of conditioned medium in differentiation medium (100 $\mu$g/ml ascorbic acid and 10 mM $\beta$-glycerophosphate) (53). MDA MB231, MDA MB435, MCF-7, and ZR75.1 breast cancer cell line (courtesy of Prof. S. Toma, Università di Genova, Italy) and endothelial cells, EA hy926, were cultured in Dulbecco's modified Eagle's medium, 10% FCS.
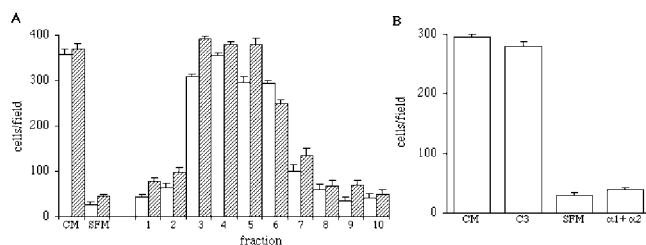
*Conditioned Media from Osteoblasts, Purification of C3*—Conditioned media were collected from mature rat osteoblasts in the second phase of osteogenesis (after 7–10 days of culture in differentiation medium) *in vitro*, and C3 was purified as described (4). The fractions obtained from the heparin-Sepharose column were tested in chemotaxis with endothelial and MDA MB231 cells.

*Cell Proliferation, Mitotic and Apoptotic Indexes*—Cells were plated in the presence of serum, and after allowing cell attachment, the medium was removed, and cultures were rinsed with serum-free medium (SFM) and maintained in SFM, with or without purified C3 (13 $\mu$g/ml), or 10% FCS. In the presence of C3, cells loosened their attachment to the plastic. We therefore analyzed separately at each time point cells easily detaching and detached and those adhering to the plastic for the numbers of mitosis and apoptosis. Because no significant differences were detected in their frequency in the two fractions, we report the sum of the two fractions. Mitotic and apoptotic indexes were determined, after 48 or 72 h, in cells fixed and stained in 50 $\mu$g/ml Hoechst for 20 min. At least 1000 cells/sample were counted in-blind by two observers, and standard error was calculated. Experiments were performed in duplicate.

*Chemotaxis Assay and Treatment with C3*—Migration assay was performed in Boyden chambers as described (4). $12 \times 10^4$ cells were placed in the upper compartment of each Boyden chamber, and unfractionated CM, purified C3 (13 $\mu$g/ml), or SFM for negative control were placed in the lower compartment. In the study of the effects on migration of antibodies against the C3 chains $\alpha_1$ and $\alpha_2$, these were added to the lower compartment of the Boyden chamber. To study the effects on migration of antibodies against MMPs, uPA, and integrins, and in that of TIMP-1, GM6001, PAI, PTX, wortmannin, and genistein, these were added with the cells in the upper compartment. The concentrations of antibodies against $\beta_1$, $\beta_3$ integrin chains, against MMP-2 and -9, and against the $\alpha_1$ and $\alpha_2$ chains of type I collagen were chosen as effective in the inhibition of C3-induced endothelial cell migration. After migration five random fields of the filters were counted for each sample; the standard deviation was calculated and indicated in the figures. Duplicate samples were run in each experiment, and the experiments were repeated at least twice. To study the expression by cells of MMPs and uPA upon induction with C3, the media conditioned during the chemotaxis by the tumor cancer cells (the upper compartment of the Boyden chamber) were collected.

In other experiments tumor cells grown to confluence on plastic were challenged with C3 (13 $\mu$g/ml) in SFM for 6 h or cultured for the same time in SFM, and the CM were collected. In 6 h attached cells do not lose their attachment to the plastic. Cells lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS).

Fig. 1. **Purification of the chemoattractant from medium conditioned by mature rat osteoblasts.** *A*, Boyden chamber assay of the fractions eluted from heparin-Sepharose column, tested with endothelial (EA hy926, *open column*) and breast tumor cells (MDA MB 231, *dashed column*). *B*, Boyden chamber assay with MDA MB 231 cells of mature osteoblasts CM, purified C3 (13 $\mu$g/ml), C3 in the absence or presence of antibodies against $\alpha_1$, and $\alpha_2$ chains of the trimer carboxyl terminus of pro-collagen type I, and SFM. *Error bars* refer to S.D.
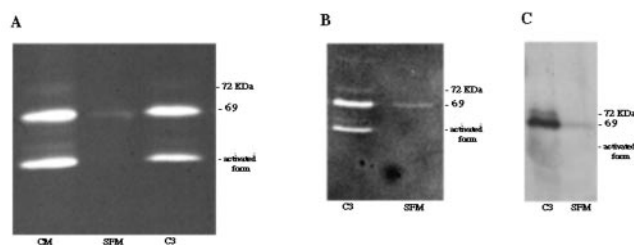


Fig. 2. **C3 induction of pro-MMP-2 expression and activation.** *A*, zymogram of media collected after 6 h from MDA MB 231 cells incubated in Boyden chambers and exposed to CM, SFM, or C3. *B*, zymogram of media collected after 6 h of exposure to C3 or SFM from MDA MB 231 cells plated on plastic. *C*, Western blot with antiserum against MMP-2 of the media in *B*.

*Antibodies and Inhibitors*—Anti-human antibodies utilized for inhibition of migration were to integrin $\beta_1$ (monoclonal AIIB2, courtesy of Dr. C. Damsky, University of California), integrin $\beta_1$ (monoclonal antibody 6S6, Chemicon, Temecula, CA), integrin $\beta_3$ (monoclonal antibody B212, courtesy of Dr. G. Tarone, University of Torino, Italy), integrin $\alpha_2$ (monoclonal antibody A2-IIE10, Sigma), integrin $\alpha_6$ (monoclonal antibody 135-13C, courtesy of Dr. Kennel, Ornel Life Science Division, Oak Ridge, TN), $\alpha_1$ and $\alpha_2$ chains of the COOH-terminal of procollagen type I (polyclonal antibody, courtesy of Dr. A. Veis, Northwestern University, Chicago), MMP-2 and -9 (polyclonal antibody, courtesy of Dr. W. Stettler Stevenson, National Institutes of Health, Bethesda), MMP-14 (polyclonal antibody, Biotrend, Koln, Germany), and uPA (monoclonal antibody, Calbiochem). Anti-human uPAR (monoclonal antibody R4, courtesy of Dr. F. Blasi, S. Raffaele, Milano, Italy), TIMP-1 (polyclonal antibody, Chemicon), and TIMP-2 (monoclonal antibody 13446, Chemicon) were utilized for Western blotting. Inhibitors utilized were TIMP-1 (human recombinant, Chemicon), PAI (human recombinant, Chemicon), and GM6001 (Chemicon). All other chemicals, unless otherwise stated, were from Sigma.

*Zymograms*—Samples of CM were concentrated by cold ethanol precipitation (46:100, v/v) for 1.5 h on ice, collected by centrifugation, and resuspended in sample buffer for electrophoresis. Protein equivalent amounts (by Lowry) from CM were loaded on SDS-acrylamide gel. The gels for detection of uPAs were cast with 1% non-fat dry milk and 10 $\mu$g/ml human plasminogen and run at $6-8$ °C in a water-cooled box. After electrophoresis, gels were washed twice in 2.5% Triton X-100, incubated 16–18 h at 37 °C in 100 mM glycine, pH 8.0, stained with 0.2% Coomassie Blue in 50% methanol, 10% acetic acid, and destained in 50% methanol, 10% acetic acid. The gels for detection of MMPs were cast with 0.28% w/v gelatin (type A), run at $6-8$ °C in a water-cooled box, rinsed twice for 30 min in 2.5% Triton X-100, incubated 16–18 h at 37 °C in 40 mM Tris-HCl, 0.2 M NaCl, 10 mM CaCl$_2$, stained, and destained as above.

*Western Blotting*—50 $\mu$g of proteins from cells lysed in RIPA buffer were run after reduction on SDS-acrylamide gels. The samples were electrotransferred to nitrocellulose, and the membranes were saturated at room temperature for 1 h in TTBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.01% Tween 20), 5% bovine serum albumin. The membranes were incubated 16 h in TTBS added with monoclonal R4 anti-uPAR, polyclonal anti-MMP-14, polyclonal anti-MMP-2, polyclonal anti-TIMP-1, or monoclonal anti-TIMP-2 antibodies, followed by 1 h in TTBS with horseradish peroxidase-secondary antibody, and the bands were visualized by ECL chemiluminescence (Amersham Biosciences).

## RESULTS

C3, the inducer of directional migration specific for endothelial cells, is purified from medium conditioned by mature rat osteoblasts. Fig. 1*A* shows that the chemoattractant for both endothelial (EA hy926) and breast carcinoma (MDA MB231) cells is found in the same fractions of the last purification step, a heparin-Sepharose column. A pool of these fractions yields on SDS-PAGE a single band at 120 kDa, unreduced, and a doublet between 33 and 35 kDa, upon reduction, corresponding to purified C3 (not shown). Purified C3 is, therefore, the major single inducer of directional migration present in the osteoblasts CM for both EA hy926 and MDA MB231. To confirm further that

C3 is the only inducer of directional migration of MDA MB231 in these fractions, we have added in the lower compartment of the Boyden chamber antibodies against $\alpha_1$ and $\alpha_2$ COOH-terminal chains of pro-collagen type I, at a concentration previously shown to block the C3-induced migration of endothelial cells. The addition of the antibodies causes full inhibition of the directional migration of MDA MB231 cells (Fig. 1*B*).

The induction of directional migration by CM and by C3 is positively correlated with the enhancement in the expression of pro-MMP-2, of the 69-kDa form of MMP-2 and with its activation. This is seen as a marked increase in the relative amounts of activated forms of MMP-2 at lower molecular size in zymograms on gelatin of both the media conditioned by the migrating cells (Fig. 2*A*) and those collected from cells adhering to plastic (Fig. 2*B*) and identified by Western blot (Fig. 2*C*).

The basal level of expression of MMP-9, detected by zymograms on gelatin as pro-enzyme at 92 kDa, is increased in the cells upon exposure to C3 both during migration in a Boyden chamber (Fig. 3*A*) and while adhering to plastic (Fig. 3*B*), and in both conditions the activated form of MMP-9 at 82 kDa is detected. The level of TIMP-1 is unchanged (Fig. 3*C*) and that of TIMP-2 is up-modulated upon exposure to C3 (Fig. 3*D*), as detected by Western blot in the CM from adhering cells and detected in the CM from migrating cells by zymography and reverse zymography (not shown). MMP-14, the transmembrane metalloproteinase activator of MMP-2, is expressed constitutively in MDA MB231, and its level is not significantly changed upon exposure to C3 and during induction of MMP-2 and -9 and their activation, as measured in zymograms of cell lysates (not shown) and in Western blots from cell lysates (Fig. 3*E*). Each specific antiserum, against MMP-2 or MMP-9 or MMP-14, inhibits the migration of MDA MB231 in Boyden assay. Migration is also inhibited by a generic MMP inhibitor, GM6001, or by addition of exogenous TIMP-1 (Fig. 4), indicating that the induction and activation of both pro-MMP-2 and -9 are required for the acquisition of the migratory phenotype.

MDA MB231 cells express constitutively, and in a fashion unaffected by exposure to C3, the serine proteinases of the uPA system, here shown in zymograms on plasminogen of the medium collected from cells exposed to C3 while adhering to plastic (Fig. 5*A*) or collected after chemotaxis with C3 (Fig. 5*B*). Lesser amounts of low molecular forms of uPA are found in the media collected after chemotaxis, compared with that collected from adhering cells, possibly due to diffusion to the lower chamber. MDA MB231 also express constitutively the uPAR, shown by Western blot, and its expression is unchanged upon treatment with C3 (Fig. 5*C*). The uPA system is involved in the chemotactic response of MDA MB231 to C3, as shown by the inhibition of directional migration obtained by addition of antibodies against uPA or by addition of PAI during chemotaxis (Fig. 5*D*).
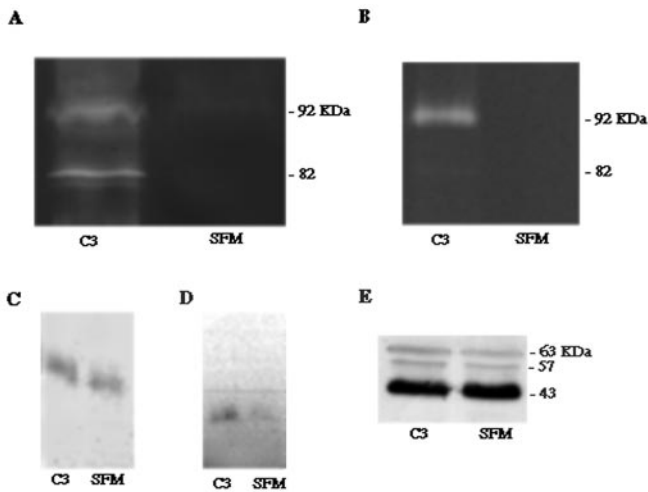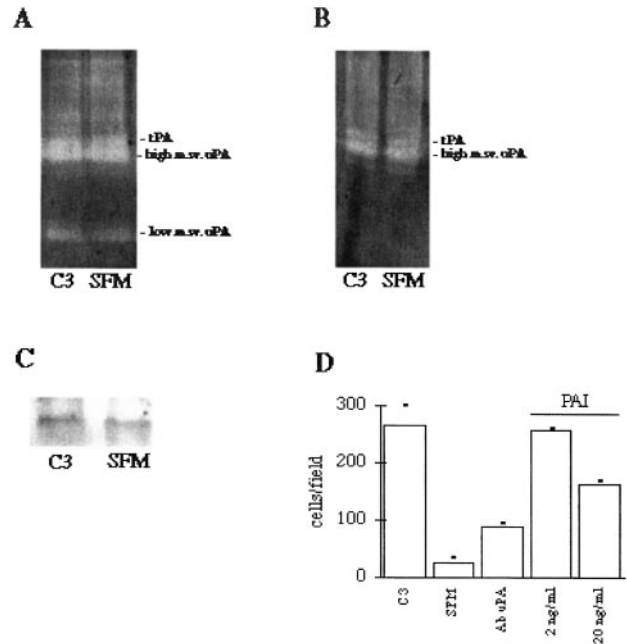
FIG. 3. **C3 induction of pro-MMP-9 expression and activation and determination of the effect of C3 on the levels of TIMP-1 and -2 and MMP-14.** *A*, zymogram of media collected after 6 h from MDA MB 231 cells incubated in Boyden chambers and exposed to C3 or SFM. *B*, zymogram of media collected after 6 h from cells plated on plastic and exposed to C3. *C*, TIMP-1. *D*, TIMP-2 expression upon exposure of the cells for 6 h to C3. TIMP-1 and -2 are detected by Western blot in conditioned medium. *E*, detection by Western blot of the expression of MMP-14 upon culture of cells for 6 h in presence of C3 or in SFM. MMP-14 is detected in the cell lysates.
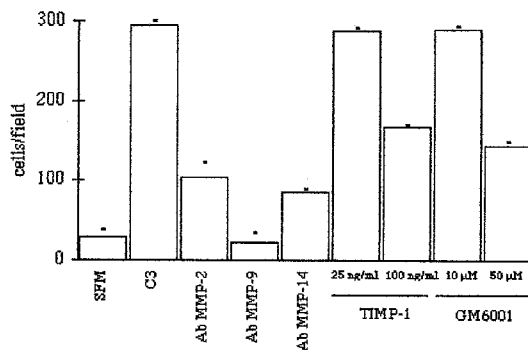


FIG. 4. **The inhibitory effect on MDA MB231 cells migration in Boyden chamber assay of antisera against MMPs, of MMPs inhibitor GM6001, and of addition of TIMP-1.** Cells were treated for 6 h during Boyden chamber assay.



FIG. 5. **Detection of uPA, tPA, and uPAR expressed in MDA MB231 in response to C3, and inhibition of the migration induced by C3 by the antibody against uPA and by PAI.** Zymogram on plasminogen shows uPA and tPA secreted by the cells in the medium collected from cultures treated with C3 or in SFM while adhering to plastic (*A*), and in the medium collected during chemotaxis in the presence or absence of C3 (*B*). *C*, detection by Western blot of the uPA receptor in cells cultured in the presence or absence of C3. *D*, migration of MDA MB231 induced by C3 in the presence or absence of antibodies against uPA or in presence of PAI during chemotaxis induced by C3.

A direct connection between the acquisition of migratory phenotype upon exposure to C3 and the expression of pro-MMP-2 and pro-MMP-9 is established utilizing PTX, an inhibitor of heterotrimeric G-proteins and related signaling pathways. PTX inhibits the directional migration of MDA MB231 cells (Fig. 6*A*); the induction of pro-MMP-2, and its activation associated with the exposure to C3 and the induction of pro-MMP-9 (Fig. 6*B*). Nonetheless, PTX does not affect the expression of uPA (Fig. 6*C*). The inhibition occurs at concentrations of PTX that do not affect the viability of the cells, as judged by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (not shown). Other factors required for migration of MDA MB231 induced by C3 are integrin chains $\alpha_2$, $\alpha_6$, $\beta_1$, and $\beta_3$. Each of the corresponding antibodies, utilized in standard Boyden assay, is inhibitory to the migration to a different extent. Antibodies against the human integrin chains $\beta_1$ or $\beta_3$, at a concentration that severely inhibits migration of endothelial cells induced by C3, only partially inhibit the migration of MDA MB 231 but blocking antibodies to $\alpha_2$, $\alpha_6$, and to $\beta_1$ chains inhibit fully the C3-induced chemotaxis, whereas no inhibition is observed with a nonspecific IgG (Fig. 7*A*). Blocking antibodies for these specific integrins were utilized in the presence or

absence of C3 also on cells adhering to plastic, and the expression of MMPs (Fig. 7*B*), TIMP-2 (Fig. 7*C*), uPA (Fig. 7*D*), and uPAR (Fig. 7*E*) was detected. The exposure to antibody to $\alpha_2$ reduces slightly only the induction of pro-MMP-9 and exposure to anti-$\beta_3$ antibodies, and one of the two anti-$\beta_1$ monoclonal antibodies tested inhibits induction of pro-MMP-2 and -9 upon exposure to C3, whereas the other anti-$\beta_1$ monoclonal antibody has no effect on MMPs expression, also confirmed by Western blot for MMP-2 (not shown). The conditioned medium of MDA MB 231, analyzed by zymograms after exposure to $\alpha_6$ antibody, also in absence of C3, showed MMPs in large amounts, and testing of the antibody preparation showed these were present in the antibody itself as contaminants (Fig. 7*B*). TIMP-2 level in the medium is unaffected by treatment with anti-$\alpha_2$ antibody, and it is decreased upon treatment with anti-$\alpha_6$, both the anti-$\beta_1$ monoclonal and anti-$\beta_3$ antibodies, in the presence and absence of C3. uPA and uPAR expression are unaffected by exposure to blocking antibodies. Addition to the cells during Boyden chamber assay of genistein inhibits, in dose-dependent fashion, cell migration indicating that PTK-mediated signaling is required in the induction of the migratory phenotype by C3. Induction of migration by C3 also requires the function of PI3K, as shown by the inhibitory effect, exerted in dose-dependent fashion, by addition of wortmannin during Boyden chamber assay (Fig. 8). Parallel cultures were treated with genistein or wortmannin for 6 h to certify the absence of cytotoxicity (not shown).

The effects of C3 on cell growth and death has been analyzed at time intervals over 5 days in MDA MB231 cultured in SFM, without or with C3 or 10% FCS. Over five days, after a lag, cell numbers increase in the cultures supplemented with serum and less in the cultures in SFM, with or without C3 (not shown). Hoechst-stained cells, cultured in the three conditions utilized above, were analyzed at successive time points over 3
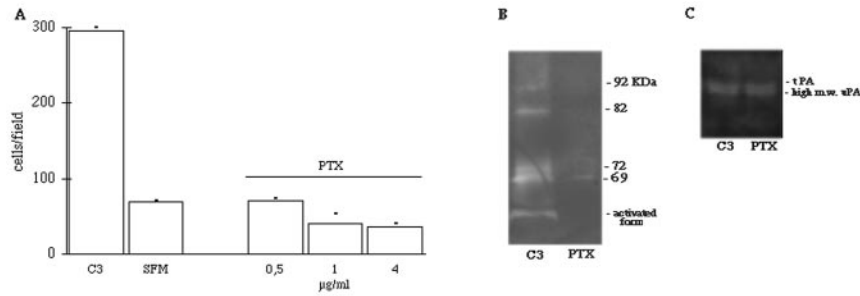
FIG. 6. **PTX inhibits both migration and MMP-2 and -9 expression in MDA MB231 in response to C3 but not uPA expression.** Cells were induced to migrate by C3 in Boyden chambers assay for 6 h, in the presence or absence of different concentrations of PTX (detailed below) (*A*), and the media conditioned by these cells were analyzed for detection of MMPs by zymogram on gelatin (*B*) or by zymogram on plasminogen (shown here for the lowest PTX concentration, 0.5 μg/ml) (*C*).
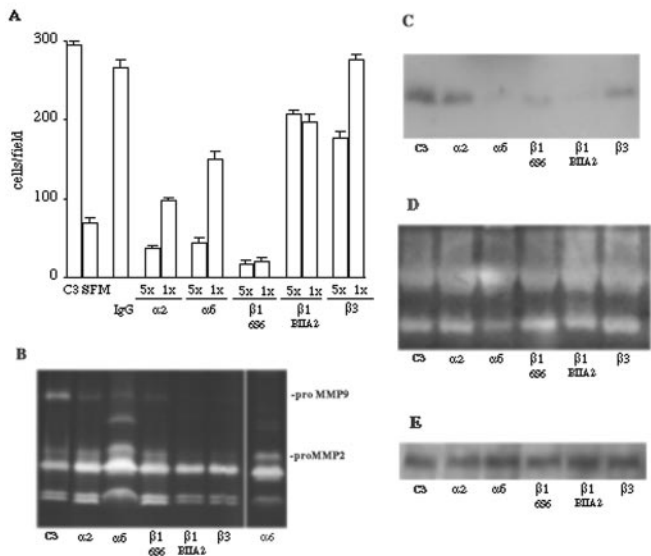


FIG. 7. **Antibodies against integrins inhibit the migration of MDA MB231 induced by C3.** Blocking antibodies for specific integrins were utilized in the presence or absence of C3 during chemotaxis on subconfluent cells adhering to plastic. *A*, migration of MDA MB231 induced by C3 in the presence or absence of antibodies against integrins or a specific IgG. Each of the blocking antibodies to $\beta_1$, $\beta_3$, $\alpha_2$, and $\alpha_6$ inhibits chemotaxis. *B*, effect of the exposure to antibodies on pro-MMP-9 and -2. The *last lane* shows anti-$\alpha_6$ antibody preparation directly loaded on gel. *C*, TIMP-2 in the medium. *D*, uPA; *E*, uPAR expression.
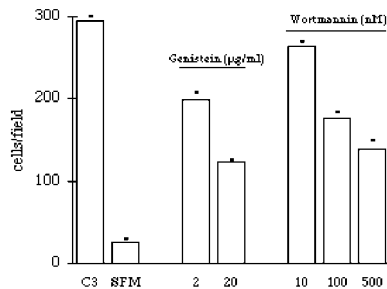


FIG. 8. **PTK and PI3K signaling pathways are involved in directional migration induced by C3.** Migration of MDA MB231 induced by C3 in the presence or absence of genistein or wortmannin (concentrations as detailed below).

days for morphological evidence of mitotic and apoptotic cells. C3 has a mitogenic effect in the first 48 h of culture comparable with that of 10% FCS. During this length of time in culture the cells in the presence of C3 become loosely attached and easily detach from the plastic. Both adhering and detached cells were collected and analyzed. The frequency of mitotic and apoptotic cells in the two populations are not significantly different, and

TABLE I
*Mitotic and apoptotic indices of MDA MB231*

Cells were stained with Hoechst. 1000 or more cells were scored for each experimental time point. Data are reported as percentages.

| Culture condition | SFM+FCS 10% | SFM+C3 | SFM |
|---|---|---|---|
| % mitosis | | | |
| 48 h | 3.4 | 3.0[c] | 0.7[a] |
| 72 h | 4.0 | 1.1[b] | 0.9[a] |
| % apoptosis | | | |
| 48 h | 3.2 | >0.1[a] | >0.1[a] |
| 72 h | 3.5 | 1.4[c] | 0.5[b] |

[a] $p < 0.001$.
[b] $p < 0.015$.
[c] $p > 0.3$, not significant.

the data from the two have been pooled in Table I. At 72 h the serum-supplemented cultures maintain the same frequency of mitosis as at 48 h, and it decreases in the C3-supplemented cultures, indicating that addition of C3 alone is not sufficient to support in time the proliferation and that other factors are required to maintain over time a level of cell duplication similar to that supported by 10% FCS. Apoptotic cells are more frequently found in cultures supplemented with serum than in SFM or SFM + C3 at 48 h. At 72 h the frequency of apoptotic cells in SFM + C3 becomes not significantly different from that of the cultures in FCS and exceeds that of the control in SFM. The estrogen receptor (ER) expression varies in different lines of breast carcinoma, and its loss has been often associated with the progression to malignancy of the tumor cells. We have tested for the migratory response to C3 and for the induction of MMPs three other breast carcinoma-derived cell lines, beside MDA MB231, in order to assess whether a correlation existed between C3-inducible migratory behavior, MMPs induction, and ER expression. The human breast carcinoma MDA MB435 (ER−) and MCF-7 (ER+) lines challenged with C3 are induced to migrate directionally by C3 (Fig. 9*A*), and the induction of directional migration by CM and by C3 is positively correlated with the enhancement of expression of MMP-2 and with its activation, as shown in zymograms on gelatin of the media conditioned by the migrating cells (Fig. 9*B*). Each cell line has a different basal pattern of expression of pro-MMP-2 and a different extent of induction. The increase in the expression of pro-gelatinase and of the 69-kDa form of MMP-2, and a marked increase in the relative amounts of activated forms of MMP-2, commonly detected in the three cell lines chemoattracted by CM and by purified C3 so far identified, associate migratory phenotype with MMP-2 activity. Nonetheless, the line ZR75.1 (ER+), which has undetectable basal levels of expression of pro-MMP-2 and is not induced by C3 to migrate and to express pro-MMP-2, upon exposure to CM is induced to express pro-MMP-2 and activated forms of MMP-2 but not to migrate (Fig. 9, *A* and *B*), suggesting that MMP-2 induction and activation

FIG. 9. **C3 is a chemoattractant and induces MMP-2 expression and activation in breast carcinoma cell lines regardless of their ER status and in association with induction of MMP-2 expression and function.** *A,* Boyden chamber assay of cell lines in SFM in the presence or absence of CM or C3. *B,* zymograms on gelatin of the media collected after chemotaxis.
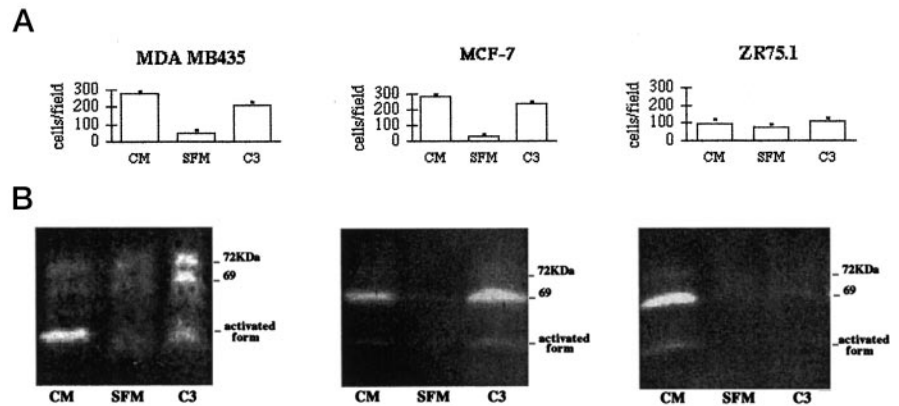
are not the only required factor for the acquisition of the migratory phenotype. Moreover, other factors must contribute or be responsible for the MMP-2 induction by CM in ZR75.1 cells.

DISCUSSION

C3, the major chemoattractant component for endothelial cells in the conditioned medium collected from mature osteoblasts (4), induces directional migration in breast tumor cells, associated with induction of pro-MMP-2 and its activation but not unequivocally associated with their ER status. Recent studies (54) by microarray of the gene expression of breast cancer also suggested that there is not an absolute association between the ER expression by tumor cells and the expression of an invasive phenotype. The induction of migration by C3 is associated in all responding lines with the induction of MMP-2 activity within the 6 h of the experimental time and, in the line MDA MB231 where it was sought with the induction of transient proliferation, of MMP-9 activity and of TIMP-2. The induction of MMPs and TIMP-2 occur upon the exposure of MDA MB231 cells to C3 while adhering to plastic or in suspension during chemotaxis, and it is a direct effect of the cell interaction with C3. The induction of pro-MMP-2 and -9 requires G-proteins activity, because it is abolished in presence of PTX. In similar fashion, migration of endothelial cells induced by C3 is also inhibited by PTX (4). The induction of pro-MMP-2 and -9 and their functionality is a prerequisite for the acquisition of the migratory behavior, and migration is inhibited by the MMPs inhibitor GM6001, by TIMP-1, and by each of the specific antibodies against MMP-2, -9, and -14. The results obtained by inhibition experiments with these antibodies are consistent with the hypothesis that active MMP-9, possibly the end product of an activation cascade involving pro-MMP-14 and pro-MMP-2 (or alternatively activated by uPA), plays an essential role in migration of the tumor cells, because inhibition of migration by antibodies against MMP-9 is total. These results are also compatible with the possibility not the alternative that MMP-2, -9, and -14 are all required at the same time (or place) for the promotion of migratory phenotype. Similar conclusions with regard to the requirement for the essential function of MMP-9 can be drawn also from the fact that ZR75.1 cells, although induced by osteoblasts CM to express activated forms of MMP-2 but not of MMP-9 (not shown), do not activate a migratory phenotype, thus indicating that production and activation of pro-MMP-2 (by MMP-14) can be dissociated from or is not sufficient in itself to activate migration. The induction of pro-MMP-2 and -9 occurs in MDA MB231 in a background where MMP-14 is constitutively produced and active. This is not changed by exposure to C3 of cells both adhering on plastic and during migration. Given that MMP-14, the proteolytic activator of pro-MMP-2, as well as its potential extracellular activator uPA are constitutively expressed in

MDA MB231 cells, the increase in activated forms of MMP-2, observed upon exposure to C3, might be simply consequent to the induction of the expression of the pro-enzyme. In addition, upon exposure to C3 we observed up-modulation of TIMP-2, the co-factor of pro-MMP-2 activation. The change in levels of TIMP-2 might suffice to enhance activation of secreted pro-MMP-2 by pre-existent MMP-14. Activated MMP-2 can activate pro-MMP-9, also induced by exposure to C3. The fact that inhibition of migration is obtained with antibodies against either MMP-2 or MMP-14 suggests that the migratory phenotype requires the simultaneous function of all three MMPs. Experiments of inhibition with GM 6001 and TIMP-1 also confirm that the functions of MMPs are required for the directional migration of MDA MB231 in response to C3. In establishing the requirement for MMPs, our data agree with those showing that MMP-2 and -9 functionality is required for primary and metastatic carcinogenesis in breast tumors (17) and enhances both primary and metastatic tumor growth (18). Nonetheless, pro-MMP-9 might also be processed by uPA, which is produced constitutively by MDA MB231 and is required for the induction of the migratory phenotype, as discussed below.

After 48 h of exposure of MDA MB231 to C3, we observe a mitogenic effect, which is not sustained for more prolonged culture times. The association of a growth-promoting effect to the induction of the activity of the endogenous MMPs, which is sustained for at least 24 h (the longest time tested by us, data not shown), suggests the possibility that these last might be involved in the mitogenic effect of C3, directly or through the production of mitogenic stimuli by proteolysis of the ECM, as current evidence suggests to occur *in vivo* (18). If the mitogenic effect of C3 was indeed mediated by proteolysis of ECM, its transience *in vitro* might be due to the presence of lesser and different ECM than that surrounding tumor cells *in vivo*. Other pathways for the mitogenic effect of C3 are equally possible, because the machinery that controls cell proliferation is at the end point of all the signaling pathways implied in the response to C3 by the experiments with inhibitors discussed below.

uPA and uPAR are constitutively expressed, both unchanged upon exposure to C3, by MDA MB231. We have not investigated if upon exposure to C3 the levels of expression of the specific inhibitors of the uPA system, PA-1 and -2, change. Their levels might regulate the availability of uPA and its interactions with the receptor (31), and this point deserves further studies.

The migratory phenotype is inhibited partially by treatment with up to 20 μg/ml of PAI and to about 80% by anti-uPA antibody, showing that uPA is required for the acquisition of the migratory phenotype in response to C3. uPA might be required for the activation of pro-MMP-9 and -14 or it might

affect signaling via uPAR. One of the known signaling mechanisms affected by uPAR occupancy involves PI3K function. Studies with the PI3K inhibitor wortmannin indicate that C3 induction of the migratory phenotype is partly dependent on PI3K function. The maximum inhibition we could obtain with wortmannin at concentrations up to 600 nM (not shown) was 55%, suggesting that the PI3K pathway is not the only one that cells utilize for the acquisition of the migratory phenotype. Signaling through uPAR is known to involve multiple interactions between members of the uPA system and with integrins and ECM components (28). Although the signaling pathways involved in the response to occupancy of uPAR are complex and still not fully untangled (33, 34), evidence has accumulated that they also include heterotrimeric G-proteins and PTK (34), which are also required by MDA MB231 cells to migrate in response to C3.

Taken together, the inhibition experiments mentioned above, and those with genistein, show the involvement of all these pathways in the acquisition of the migratory phenotype induced by C3 in MDA MB231. They indicate that the function of both the uPA system and of at least 3 members of the MMPs family converge to activate fully the migratory phenotype of MDA MB231 in response to C3. The complementary action of the families of serine and metalloproteinase for the manifestation of migratory phenotype was reported previously (55, 56).

The extracellular interactions that modulate the proteolytic and signaling functions of the uPA system include the activation of uPAR by specific MMPs (55). Basal cell mobility of MDA MB231 is regulated by MMPs and uPA levels via PI3K (37). Notwithstanding the implications mentioned above, we are not yet in a position to trace a mechanistic picture of the events involved in signaling induced by C3.

Fibroblasts bind to C3 via integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (57, 58), and the directional migration of endothelial cells in response to C3 requires $\beta_1$ and $\beta_3$ integrins (4). MDA MB231 cells do not normally express the $\alpha_1$ integrin chain. The expression of migratory phenotype through gelatin of MDA MB231 in response to C3 is here shown, by inhibition studies with the specific antibodies, to involve $\alpha_2$, $\alpha_6$, $\beta_1$, and $\beta_3$ integrin chains. In Boyden chambers experiments it is nonetheless impossible to distinguish whether integrins are required for the recognition of the chemoattractant or for the adhesion to and traversing the gelatin coating. The inhibition of migration upon treatment with genistein is consistent with either or both possibilities.

Integrins play a role in determining the regulation of expression and activation of MMPs in many tumor cells and in the regulation of the function of uPA system in concurrence with the interaction with the ECM (29). These interactions, in general, are functional to enhanced localized proteolysis and are often associated with acquisition of a migratory phenotype. The usage of integrins by each cell type is dependent on the nature of the ECM and varies between different cell types for the same ECM protein. Through the dissection of new and/or cell-specific mechanistic responses of different cell types to different ECM components, to growth factors, to transmembrane and pericellular proteinases and their receptors (39), different signaling pathways can be activated. Signals activated through integrins are shown to be of a great complexity. $\alpha_6$ integrin expression is involved in the migratory process in breast carcinoma cells and was specifically associated with the metastatic phenotype (42, 43). Engagement by ligands of dimers containing the integrin $\beta_1$ (including $\alpha_6\beta_1$) and the $\beta_3$ chains causes the activation of signaling through PTK but can also involve, according to the ECM components engaged and to the presence of growth factors, different signal transduction molecules, whose activation (as for most if not all integrins, except $\alpha_6\beta_4$) converges in the

activation of a core signaling machinery affecting the actin cytoskeleton and activating the c-Jun NH$_2$-terminal kinase and thus regulating cell cycle and ultimately cell growth (39). This signaling machinery involves Rac and Ras, whose activation is required also for induction and activation of MMPs and for the acquisition of invasiveness in transformed cell lines (40, 52, 60). Exchange toward Ras and Rac is increased by the activity of PI3K, a kinase involved in the processes of focal adhesion disassembly and migration. Substrate-independent signaling through $\alpha_6\beta_4$ is wortmannin-sensitive, involves the functionality of PI3K, and the activation of SOS exchange toward Rac and Rho GTPases (48), and ultimately also affects growth regulation (52). $\beta_4$ integrin is also uniquely capable of direct activation of Shc, which couples integrins to the control of cell cycle (61).

The evidence gathered to this time by inhibition of signaling pathways establishes that the acquisition of the full migratory phenotype induced by C3 in MDA MB231 is mediated by signaling requiring the activities of heterotrimeric G-proteins, PTK, also shown to inhibit the MMPs induction by C3, and PI3K. The requirement for PI3K (perhaps through its role of increasing the exchange between SOS and Rho family members) represents a potential link between uPA-responsive and integrin-PTK-mediated pathways. The activation of PI3K can also play a role in the control of growth regulation through its effect on the p70S6K and cdk2-cycline-E pathway (51), an aspect presently under study in our system.

Experiments utilizing blocking antibodies to $\alpha_2$, $\alpha_6$, $\beta_1$, and $\beta_3$ integrin chains show that these inhibit cell migration through gelatin and have no effect on the level of the secreted uPA and of the uPAR. The pattern of expression of pro-MMPs and TIMP-2 varied with the antibody utilized from inhibition of the induction by C3 of the MMPs to no effect. The MMPs detected upon the block of cell migration determined by exposure to the anti-$\alpha_6$ antibody were shown to be contaminants of the antibody preparation. We cannot therefore know if the blocking of $\alpha_6$ integrin chain has an effect on MMP expression, although obviously exogenous MMPs added with the antibody do not seem to interfere with its blocking the migration of cells.

In general, there is an intrinsic difficulty in the interpretation of the data of inhibition of function with antibodies, due to the impossibility to dissect the effect of treatment with each antibody on the interaction of the cells with the gelatin necessary for enacting migration, from the effect on the interaction with C3 necessary for initiating the migratory phenotype. Moreover, as well known, inhibition with antibodies of proteins with multiple bindings are intrinsically complex to decline mechanistically without further studies with deletion mutants and/or panels of monoclonal antibodies targeted to multiple epitopes. Such a dissection will have to be done.

So we have evidence that for the two different $\beta_1$ monoclonal antibodies utilized, the epitope that they interact with might elicit different responses in the MDA MB231. The blocking by one antibody blocks completely the expression of migratory phenotype not accompanied by inhibition of the C3-mediated induction of pro-MMP-2 (only a slight inhibition of the induction of pro-MMP-9 and of TIMP-2 is observed), as if the interaction with the antibody still allowed the interaction with C3 and the consequent induction of MMPs although creating a contest where denatured collagen (gelatin) is not recognized as substratum. A different monoclonal antibody only partially inhibits the expression of migratory phenotype, and therefore we assume that it permits recognition by the cells of gelatin, whereas it seriously inhibits pro-MMPs and TIMP-2 modulation by C3, and therefore it possibly affects the binding of C3. These data are consistent with involvement of $\beta_1$ chains in

mediating the signaling from C3 that induces modulation of the MMP system and its relevance in the enactment of migration through gelatin. A monoclonal antibody to $\beta_3$ blocks both cell migration and the C3-mediated induction of MMPs. The involvement of $\beta_3$ chains in the recognition of C3 is in analogy with results in inhibition studies in endothelial cells (4). A blocking antibody to $\alpha_2$ acts similarly to the first mentioned monoclonal antibody to $\beta_1$, inhibiting the expression of migratory phenotype and not affecting C3-induced pro-MMPs and TIMP-2 modulation, leaving open the possibility that $\alpha_2$ chains might not be critical for the C3-mediated induction of the MMPs-TIMP system. In summary, these data support that $\beta_1$ and $\beta_3$ integrins are involved in the signaling to the cells of the presence of C3 which causes changes not involving the uPA system, but affecting the MMPs-TIMP system, consistent with reports that implied $\beta$ chain interaction with collagenous ECM in modulation of MMPs in this and other cell lines (24, 41, 48). Treatment with anti-$\alpha_6$ antibody is highly inhibitory for cell migration, even in presence of contaminating exogenous MMPs in the antibody preparation (which also make irrelevant the data on TIMP-2 level). Blocking of the $\alpha_6$ chain is capable to block migration through gelatin, but we cannot presently unravel if the functionality of the cell motility machinery (such as mobility signaling molecules and phosphorylation of structural proteins of the cytoskeleton), known to be directly affected by $\alpha_6$ occupancy, might be blocked by antibody binding independently from MMP induction.

The multistep progression that epitomizes carcinogenesis is presently viewed as involving genetic alteration in the epithelia as well as epigenetic contributions from the surrounding stromal tissue. Collagen type I, from which C3 originates, is a major product of tumor stroma fibroblasts, and high synthesis of collagen in stromal fibroblasts is positively correlated to the metastatic outcome of breast carcinomas (6). The production by tumor-associated fibroblasts of processed collagen fragments and the half-life of C3 in the stroma associated with tumors has not been yet investigated, although it is known that carcinoma-associated fibroblasts acquire a different biosynthetic phenotype than their normal counterpart, involving ECM proteins, abnormal actin expression, and inappropriate secretion of proteolytic molecules (6, 8, 9). They also acquire the capability to stimulate tumor progression in initiated epithelial prostatic cells *in vivo* and to induce *in vitro* an increase in cell proliferation and a decrease in cell death (59).

Although quite aware that caution must be used in extending results obtained *in vitro* to the *in vivo* situation, and for the value these considerations might have in directing further investigations, we hypothesize that regulation of the level of C3 might be one of the early epigenetic changes in tumor stromal fibroblasts associated with permissivity to the growth of primary tumors. The relevance of C3 in this process is its unique capability to promote the migration and to attract both endothelial and primary tumor cells and thus to favor their encounter within the stroma and to allow for the known reciprocally inductive interactions to occur (*e.g.* vascular endothelial growth factors are produced by tumor cells that promote proliferation of endothelial cells and morphogenesis of vessels, and organization of new vessels is permissive for tumor growth (15)). The adjacency of the two cell types would establish a productive cycle toward the successive steps of malignancy. MMPs, induced by exposure to C3 in tumor cells, could contribute to the acquisition of the migratory phenotype of both kinds of cells and to the enhancement of growth of tumor cells (17).

The production of C3 in tissues with high levels of collagen biosynthesis, as in bone, could attract preferentially circulating breast carcinoma cells and eventually other tumor cells that are attracted by C3, such as melanoma[2] and possibly prostatic carcinoma cells. The endogenous production of MMPs in tumor cells would be sustained by the presence of C3 in the homing site and this might favor their growth and the neoangiogenesis, in a fashion similar as that hypothesized above for primary tumors and with the end effect of promoting the colonization by C3-responsive cancer cells of C3-rich homing sites. This hypothesis is consistent with the current opinion that the preferential homing site of different tumors depends on the local composition of the ECM and on the molecules produced upon its proteolysis and gives a testable rationale for the preferential homing of breast carcinoma cells to bone (59). In an experimental set up where C3 with breast carcinoma cells was implanted subcutaneously in nude mice, we obtained a preliminary confirmation of the pro-angiogenic role of C3 and of its promoting role on tumor growth.[2] A consequence of our hypothesis, presently under test, is that a subset of tumors, those that preferentially metastasize to collagen-rich tissues like bone, will be most chemoattracted *in vitro* by C3 and induced to the synthesis and activation of MMPs.

REFERENCES

1. Stringa, E., Filanti, C., Giunciuglio, D., Albini, A., and Manduca, P. (1995) *Bone* (*New York*) **16,** 663–670
2. Giunciuglio, D., Cai, T., Filanti, C., Manduca, P., and Albini, A. (1995) *Cancer Lett.* **97,** 69–74
3. Festuccia, C., Giunciuglio, D., Guerra, F., Villanova, I., Angelucci, A., Manduca, P., Teti, A., Albini, A., and Bologna, M. (1999) *Oncol. Res.* **11,** 17–31
4. Palmieri, D., Camardella, L., Ulivi, V., Guasco, G., and Manduca, P. (2000) *J. Biol. Chem.* **275,** 32658–32663
5. Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D. S. (1996) *Science* **271,** 360–362
6. Kauppila, S., Stenback, F., Risteli, J., Jukkola, A., and Risteli, L. (1998) *J. Pathol.* **186,** 262–268
7. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tisty, T. D., and Cunha, G. R. (1999) *Cancer Res.* **59,** 5002–5011
8. Bourhis, X. D. L., Berthois, Y., Millot, G., Degeorges, A., Sylvi, M., Martin, P. M., and Calvo, F. (1997) *Int. J. Cancer* **71,** 42–48
9. Ito, A., Nakajiama, S., Sasaguri, Y., Nagase, H., and Mori, Y. (1995) *Br. J. Cancer* **71,** 1039–1045
10. Jukkola, A., Tahtela, R., Tholix, E., Vuorinen, K., Blanco, G., Risteli, L., and Risteli, J. (1997) *Cancer Res.* **57,** 5517–5520
11. Moinfar, F., Man, Y. G., Arnould, L., Bratthauer, G. L., Ratschek, M., and Tavassoli, F. A. (2000) *Cancer Res.* **60,** 2562–2566
12. Picard, O., Rolland, Y., and Poupon, M. F. (1986) *Cancer Res.* **46,** 3290–3294
13. Parent, C. A., Blacklock, B. J., Froehlich, W. M., Murphy, D. B., and Devreotes, P. N. (1998) *Cell* **95,** 81–91
14. Maghazachi, A. A. (2000) *Int. J. Biochem. Cell Biol.* **32,** 931–943
15. Hanahan, D., and Weinber, R. A. (2000) *Cell* **100,** 57–70
16. Hanemaaijer, R., Verheijen, J. H., Maguire, T. M., Visser, H., Toet, K., McDermott, E., O'Higgins, N., and Duffy, M. J. (2000) *Int. J. Cancer* **86,** 204–207
17. Curran, S., and Murray, G. I. (1999) *J. Pathol.* **189,** 300–308
18. Chambers, A. F., and Matrisian, L. M (1997) *J. Natl. Cancer Inst.* **89,** 1260–1270
19. McCawley, L. J., and Matrisian, L. M. (2001) *Curr. Opin. Cell Biol.* **13,** 534–540
20. Hua, J., and Muschel, R. J. (1996) *Cancer Res.* **56,** 5279–5284
21. Kawamata, H., Kameyama, S., Kawai, K., Tanaka, Y., Nan, L., and Barch, D. H. (1995) *Int. J. Cancer* **63,** 568–575
22. Tsunezuka, Y., Kinoh, H., Takino, T., Watanabe, Y., Okada, Y., and Shinagawa, A. (1996) *Cancer Res.* **56,** 5678–5683
23. Fassina, G., Ferrari, N., Brigati, C., Benelli, R., Santi, L., Noonan, D. M., and Albini, A. (2000) *Clin. Exp. Metastasis* **18,** 111–120
24. Hofmann, U. B., Westphal, J. R., Van Kraats, A. A., Ruiter, D. J., and Van Muijen, G. N. P. (2000) *Int. J. Cancer* **87,** 12–19
25. Kubota, S., Ito, H., Ishibashi, Y., and Seyama, Y. (1997) *Int. J. Cancer* **70,** 106–111
26. Morini, M., Mottolese, M., Ferrari, N., Ghiorzo, F., Buglioni, S., Mortarini, R., Noonan, D. M., Natali, P. G., and Albini, A. (2000) *Int. J. Cancer* **87,** 336–342
27. Okumura, Y., Sato, H., Seiki, M., and Kido, H. (1997) *FEBS Lett.* **402,** 181–184
28. Ossowski, L., and Aguirre-Ghiso, J. A. (2000) *Curr. Opin. Cell Biol.* **12,** 613–620
29. Degryse, B., Orlando, S., Resnati, M., Rabbani, S. A., and Blasi, F. (2001)

---

[2] P. Manduca, unpublished data.

*Oncogene* **20,** 2032–2043
30. Wang, Y. (2001) *Med. Res. Rev.* **21,** 146–170
31. Yebra, M., Goretzki, L., Pfeifer, M., and Mueller, B. M. (1999) *Exp. Cell Res.* **250,** 231–240
32. Duffy, M. J., Reilly, D., O'Sullivan, C., O'Higgings, N., Fennelly, J. J., and Andreasen, P. A. (1990) *Cancer Res.* **50,** 6827–6829
33. Pedersen, A. N., Christensen, I. J., Ross, W. S., Briand, P., Mouridsen, H. T., Dano, K., and Brunner, N. (2000) *Cancer Res.* **60,** 6927–6934
34. Andreasen, P. A., Kioller, L., Cristensen, L., and Duffy, M. J. (1997) *Int. J. Cancer* **72,** 1–22
35. de Witte, J. H., Foekens, J. A., Brunner, N., Heuvel, J. J. T. M., van Tienoven, T. H., Look, M. P., Klijn, J. G. M., Geurts-Moespot, A., Grebenchtchikov, N., Benraad, T. J., and Sweep, C. G. J. (2001) *Br. J. Cancer* **85,** 85–92
36. Bianchu, E., Cohn, R. L., Thor, A. T., Todd, R. F., Mizukami, F., Lawrence, D. A., Ljung, B. M., Shuman, M. A., and Smith, H. S. (1994) *Cancer Res.* **54,** 861–866
37. Sliva, D., Rizzo, M. T., and English, D. (2002) *J. Biol. Chem.* **277,** 3150–3157
38. Khatib, A. M., Nip, J., Fallavollita, L., Lehmann, M., Jensen, G., and Brodt, P. (2001) *Int. J. Cancer* **91,** 300–308
39. Giancotti, F. G. (2000) *Nat. Cell Biol.* **2,** E13–E14
40. Deryugina, E. I., Ratnikov, B. I., Postnova, T. I., Rozanov, D. V., and Strongin, A. J. (2002) *J. Biol. Chem.* **277,** 9749–9756
41. Sugiura, T., and Berditchevski, F. (1999) *J. Cell Biol.* **146,** 1375–1389
42. Varner, J. A., and Cheresh, D. A. (1996) *Curr. Opin. Cell Biol.* **8,** 724–730
43. Shaw, L. M. (1999) *J. Mammary Gland Biol. Neoplasia* **4,** 367–376
44. Falcioni, R., Antonini, A., Nisticò, P., Di Stefano, S., Crescenzi, M., Natali, P. G., and Sacchi, A. (1997) *Exp. Cell Res.* **236,** 76–85
45. Mercurio, A. M., Rabinovitz, I., and Shaw, L. M. (2001) *Curr. Opin. Cell Biol.* **13,** 541–545
46. Wei, Y., Eble, J. A., Wang, Z., Kreidberg, J. A., and Chapman, H. A. (2001) *Mol. Biol. Cell* **12,** 2975–2986
47. Deryugina, E. I., Bourdon, M. A., Jungwirth, K., Smith, J. W., and Strogin, A. Y. (2000) *Int. J. Cancer* **86,** 15–23
48. Shaw, L. M., Rabinowitz, I., Wang, H. H., Toker, A., and Mercurio, A. M. (1997) *Cell* **91,** 949–960
49. Riikonen, T., Westermarck, J., Koivisto, L., Broberg, A., Kahari, V. M., and Heino, J. (1995) *J. Biol. Chem.* **270,** 13548–13552
50. Degryse, B., Resnati, M., Rabbani, S. A., Villa, A., Fazioli, F., and Blasi, F. (1999) *Blood* **94,** 649–662
51. Koyoama, H., Reinesen, E. W., Bornfeldt, K. E., Roberts, J. M., and Ross, R. (1996) *Cell* **87,** 1069–1078
52. Mettouchi, A., Klein, S., Guo, W., Lopez-Lago, M., Lemichez, E., Westwick, J. K., and Giancotti, F. (2001) *Mol. Cell* **8,** 115–127
53. Manduca, P., Palermo, C., Caruso, C., Brizzolara, A., Sanguineti, C., Filanti, C., and Zicca, A. (1997) *Bone (New York)* **21,** 31–39
54. Gruvberger, S., Ringner, M., Chen, Y., Panavally, S., Saal, L. H., Borg, A., Ferno, M., Peterson, C., and Meltzer, P. S. (2001) *Cancer Res.* **61,** 5979–5984
55. Koolwijk, P., Sidenius, N., Peters, E., Sier, C. F., Hanemaaijer, R., Blasi, F., and van Hinsbergh, V. W. (2001) *Blood* **97,** 3123–3131
56. Mignatti, P., and Rifkin, D. B (1996) *Enzyme & Protein* **49,** 117–137
57. Davies, D., Tuckwell, D. S., Calderwood, D. A., Weston, S. A., Takigawa, M., and Humphries, M. J. (1997) *Eur. J. Biochem.* **246,** 274–282
58. Weston, S. A., Hulmes, D. J., Mould, A. P., Watson, R. B., and Humphries, M. J. (1994) *J. Biol. Chem.* **269,** 20982–20986
59. Olumi, A. F., Dazin, P., and Tisty, T. D. (1998) *Cancer Res.* **58,** 4525–4530
60. Liu, E., Thant, A., Kikkawa, F., Kurata, H., Tanaka, S., Nawa, A., Mizutani, S., Matsuda, S., Hanafusa, H., and Hamaguchi, M. (2000) *Cancer Res.* **60,** 2361–2364
61. Wary, K. K., Mainiero, F., Isakoff, S. J., Marcantonio, E. E., and Giancotti, F. G. (1996) *Cell* **87,** 733–743