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BONE EXTRACELLULAR MATRIX STIMULATES INVASIVENESS OF ESTROGEN-RESPONSIVE HUMAN MAMMARY MCF-7 CELLS

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Bone is the most frequent site of metastasis in breast cancer. This causes destructive osteolytic lesions. To achieve metastasis to bone, breast cancer cells must proliferate in a new microenvironment, arrest on extracellular matrix and invade. Breast cancer cells progress in the invasive processes only if they destroy bone with the assistance of osteoclasts. In this work, we present data suggesting that MCF-7 cells, an estradiol receptor–positive cell line that exhibits modest invasive capacity, proliferate in the presence of soluble factors secreted by the osteogenic cell line SaOS-2. The cells acquire a more aggressive phenotype when cultured on an extracellular matrix produced by the same osseous cell line. Acquisition of the invasive phenotype appears to be related to the capacity of bone extracellular matrix to induce the expression of urokinase-like plasminogen activator by MCF-7 cells, which is specific for MCF-7 cells, given that MDA-231 cells, an estradiol receptor–negative and more aggressive cell line, did not show significant changes when cultured in the presence of soluble and insoluble bone factors. *Int. J. Cancer* 83:278–282, 1999.

Breast cancer, the most common malignancy in women, frequently involves bone metastasis (Mundy and Yoneda, 1995). These lesions are mainly of an osteolytic nature and lead to severe pain and fractures (Yoneda, 1996). Even though the propensity of breast cells to invade bone is well-documented, specific underlying cellular mechanisms have not been identified. In this regard, we know that breast cancer cells arrested in bone need to destroy bone to proliferate. In this function, tumoral cells are assisted by bone-resorbing osteoclasts (Yoneda, 1996).

Breast cancer develops 2 main different types, classified according to the expression of estrogen receptor (ER). These 2 kinds of tumor also differ in malignancy, hormone-resistant tumors (ER^-) exhibiting a more aggressive metastatic phenotype (Sheikh *et al.*, 1994). If ER^+ genes are transfected into cells belonging to this family, their aggressiveness diminishes considerably. The bases of such differences in malignancy are complex but probably due to altered expression of genes involved in differentiation and invasiveness (Garcia *et al.*, 1996).

Despite the more aggressive behavior of ER^- cells, ER^+ breast tumor cells spread easily to bone, while ER^- cells metastasize to viscera (Stewart *et al.*, 1981). *In vitro* studies support the hypothesis that the capacity of ER^+ breast cancer cells to release prostaglandins in response to 17β-estradiol is in part responsible for bone resorption (Valentin-Opran *et al.*, 1985)

Successful tumoral invasion strongly depends on the ability of metastatic cells to produce and recruit growth factors and proteolytic enzymes, to promote tumor growth and extracellular matrix (ECM) degradation (Stetler-Stevenson *et al.*, 1993). Our aim was to assess the effect of an ECM generated by bone-derived cells on the invasive and migratory capacity of ER^+ and ER^- breast cells. For this purpose, we have generated an ECM from the osteogenic sarcoma cell line SaOS-2 (MacQuillan *et al.* 1995) and evaluated the changes in invasive capacity of MCF-7 (ER^+) and MDA-231 (ER^-) cells.

Early studies have demonstrated that MCF-7 cells possess an intrinsic capacity to resorb bone that is independent of osteoclast activity (Eilon and Mundy, 1978). The resorptive capacity may be related to collagenolytic factors secreted by tumoral cells (Eilon and Mundy, 1981). In this study, we have analyzed the influence of SaOS-2 ECM on the acquisition of an invasive phenotype by measuring changes in cellular shape, *in vitro* invasive and migratory capacity and production of urokinase-like plasminogen activator (u-PA). This factor and its cellular receptor have been implicated in the progression of several types of malignancy, including breast cancer. Moreover, the u-PA system has been suggested as a possible therapeutic target to control cancer progression (Rabbani and Xing, 1998).

MATERIAL AND METHODS

Cells

Estrogen-responsive mammary MCF-7 cells were grown in a phenol red-free DMEM/F-12 mixture enriched with 7% FCS. Estrogen-unresponsive MDA-231 cells and osteosarcoma SaOS-2 cells were cultured in DMEM plus 10% FCS. Both cell lines were purchased from the ATCC (Manassas, VA).

Indirect immunofluorescence assay

MCF-7 mammary cells were plated at a density of 5.5×10^3 cells/cm^2 on plastic or coated with SaOS-2-derived ECM (SaOS-2 ECM) coverslips and cultured for 72 hr. Thereafter, cells were washed 3 times with PBS and treated for 30 sec with a solution of 1% Triton X-100 in PBS. Then, cells were fixed with cold methanol for 15 min, followed by incubation with 1% BSA for 30 min, to block non-specific sites. Thereafter, fixed cells were incubated with an anti-β-actin monoclonal antibody (MAb) (Sigma, St. Louis, MO), diluted 1:1,000, for 60 min. Cells were washed 3 times with PBS and incubated with a second antibody, anti-mouse IgG conjugated with FITC, for 60 min. Finally, cells were washed with PBS, mounted on slides and processed for immunofluorescence. Cells were examined under a Zeiss (Oberkochen, Germany) axioscope microscope, equipped with an epifluorescence attachment, and a series of microphotographs was taken using 400 ASA film.

Proliferation assay

To test the effect of the conditioned media from SaOS-2 (SaOs-2 CM) on mammary cell proliferation, MCF-7 and MDA-231 cells were plated in the presence or in the absence of CM. SaOs-2 CM was prepared by culturing confluent cultures of SaOS-2 cells in serum-free medium for 24 hr. Cells were plated on 16-mm plates (Nunc, Roskilde, Denmark), at a density of 5×10^4 cells per well for 48 hr. At the end of this period, a 4-hr pulse with 2 µCi/ml ^{3}H-thymidine (67 Ci/mmol; ICN, Irvine, CA) was performed. After the pulse, cell monolayers were rinsed 3 times with PBS and incubated 2 times with 5% (w/v) TCA for 5 min. Cellular proteins

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were solubilized in 0.5 ml 10% SDS and counted in a scintillation counter.

ECM preparation

Bone cell–derived extracellular matrices were prepared according to the procedure described by Globus *et al.* (1989). Briefly, 16-mm plates were coated with a solution of 1 ml of gelatin (193 µg/ml) and 1 ml of carbodiimide (4.2 µg/ml) at 32 µg of gelatin/cm^2 for 3 hr at room temperature. Thereafter, plates were washed 3 times with distilled water and sterilized under UV light for 2 hr. On coated plates, 5×10^4 SaOS-2 cells in DMEM + 10% FCS were seeded for 10 days. After confluence (day 4), cultures were fed with media plus FCS which also contained 50 µg/ml of ascorbic acid. After 10 days of culture, cell monolayers were washed once with PBS and incubated for 8 min with 20 mM NH_4OH at 37°C to lyse the cells. Matrices were then washed 5 times with serum-free culture medium and maintained at 4°C for further use.

In experiments in which type I collagen was used, plates were initially incubated with a solution of 10 µg/ml overnight at 4°C. Plates were then washed 3 times with PBS + 0.1% BSA, followed by incubation with 1% BSA for 30 min to block non-specific sites.

Wounding analysis

For wounding experiments, MCF-7 and MDA-231 cells cultured on plastic and SaOS-2 ECM were grown to confluence. Cell monolayers were wounded by making linear incision cuts with a 20-µl tip, which generated a small line devoid of cells. Wounded cultures were further incubated with media plus serum for 24 hr (MDA-231) and 72 hr (MCF-7), then stained with crystal violet and photographed under the microscope with a magnification of 40× (Ando and Jensen, 1996).

Invasion assay

To test *in vitro* invasive potential, mammary cells were cultured on 3 different matrices: plastic, SaOS-2 ECM and type I collagen. Invasion assays were carried out using Transwell chambers. Invasion capacity was defined as the ability of cells to penetrate an 8-µm-pore polycarbonate membrane in Transwell chambers coated with 30 µg of Matrigel (Collaborative Research, Bedford, MA). Cells were cultured on different matrices for 72 hr and then seeded (10^5) in the upper chamber of the Transwell in 200 µl of medium plus FCS. The lower chamber was filled with 700 µl of the same medium. After 48 hr of incubation, the number of cells present in both chambers was assayed with the MTT test (Sigma). After 2 hr of incubation, media from both chambers were collected and the cells attached to both sides of the filter were removed with 0.05% trypsin/0.02% EDTA in PBS. Cells were collected and centrifuged and pellets treated with a mixture of DMSO/isopropanol 3:2, to dissolve the formazan product. Absorbance was read at 505 nm (Santibáñez *et al.*, 1997).

In a parallel experiment using both mammary cell lines, 20 µg/ml of media conditioned by SaOS-2 cells were added to both chambers to test whether a soluble factor generated by bone-derived cells was able to stimulate *in vitro* invasiveness.

u-PA secretion assay

MCF-7 and MDA-231 mammary cells (2×10^5) were seeded on plastic 16-mm plates coated with SaOS-2 ECM for 72 hr in medium containing FCS. Cells were then cultured with serum-free media for 24 hr. Media conditioned by cells were collected and concentrated by lyophilization, dialyzed and assayed for u-PA activity by reverse diffusion assay (Santibáñez and Martínez, 1993).

Northern blot analysis

Cells (6×10^6) were cultured on SaOS-2 ECM or plastic in 80-cm^2 culture flasks for 6 and 24 hr. Total RNA from each group was extracted with Trizol according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). Once isolated, RNA was fractionated in a 0.8% agarose gel and transferred to nylon membranes (Bio-Rad, Hercules, CA). For detection of u-PA mRNA, a [^{32}P]-labeled cDNA of 429 bp obtained from TIGR/ATCC, a special collection of human cDNA clones, was used as a probe. To control the amount of RNA loaded, the same membrane was hybridized with a 7 S rRNA probe.

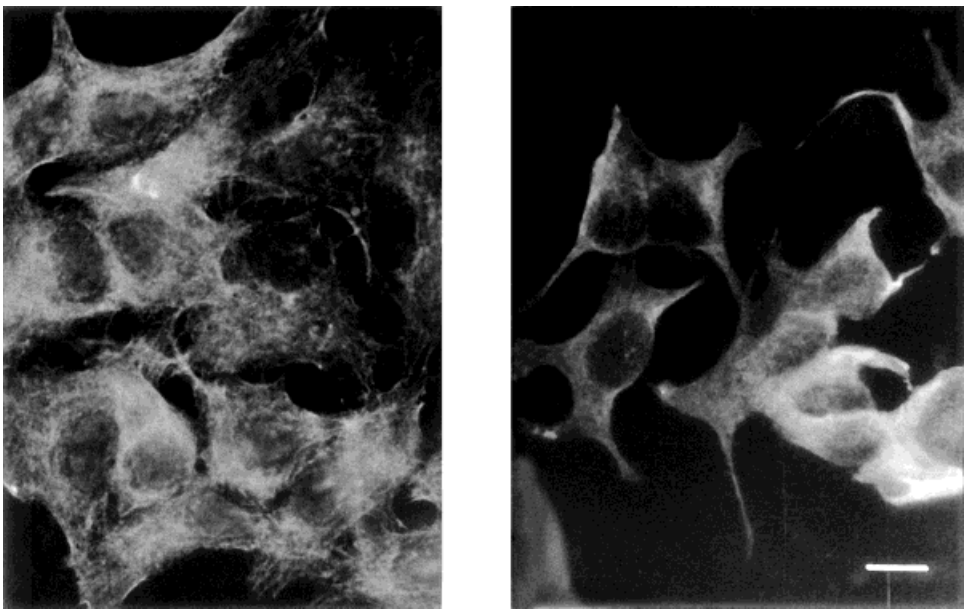

FIGURE 1 – Actin indirect immunofluorescence. MCF-7 mammary cells were plated at a density of 5.5×10^3 cells/cm^2 on intact plastic (left) or SAOS-2 ECM-coated coverslips (right). After 72 hr of culture, cells were treated as described in Material and Methods. Scale bar = 5 µm.

RESULTS

Morphological assay

The effect on cytoskeletal architecture of plating cells on SaOS-2 ECM was examined by immunofluorescence, using an anti-α-actin MAb (Fig. 1). Control plastic-seeded cells displayed the expected flat and extended epithelial morphology. A very different picture arose when MCF-7 cells were cultured on SaOS-2 ECM: cells exhibited a dispersed array with abundant processes. MDA-231 cells treated in a similar way did not express any morphological difference when seeded on the different substrates (data not shown).

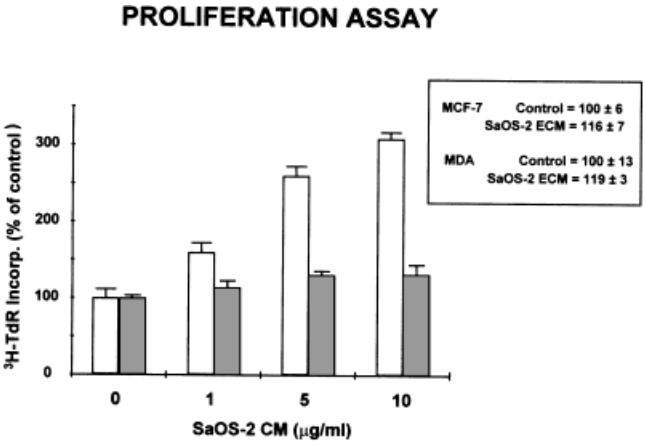

FIGURE 2 – Effect of SaOS-2-derived CM on proliferation capacity of breast tumor cells. MCF-7 cells (white columns) and MDA-231 cells (shaded columns) were plated in media plus FCS in the presence of increasing concentrations of SaOS-2 CM, and their proliferative capacity was assayed as indicated in Material and Methods. Values are expressed as mean ± SD of 3 determinations. The effect on cell proliferation of culturing both cell lines on SaOS-2 ECM is shown in the inset.

Proliferation studies

To test whether factors secreted by SaOS-2 cells were able to stimulate cellular proliferation, we incubated MCF-7 and MDA-231 cells in the presence of SaOS-2 CM. As Figure 2 shows, increasing concentrations of SaOS-2 CM stimulated, in a dose-dependent manner, the proliferation of MCF-7 cells, while bone cell–derived media did not stimulate the proliferation of MDA-231 cells. Furthermore, plating both cell lines on SaOS-2 ECM did not stimulate proliferation (Fig. 2, inset).

Wounding assay

The morphological changes exhibited by cells plated on SaOS-2 ECM clearly suggest a more motile phenotype. To test this possibility, confluent cultures of MCF-7 and MDA-231 cells were wounded and treated as explained in ''Material and Methods''. As Figure 3 shows, only MCF-7 cells plated on bone cell–derived ECM displayed an increased migratory capacity. In contrast, MDA-231 cells did not express any mobility increase after being seeded on ECM. MCF-7 cells migrated after an experimental period of 48 hr, while MDA-231 cells, which constitutively express a more motile phenotype, needed only 24 hr to cover the cell-free wound.

Invasion assay

The invasive capacity of mammary cells plated on plastic or SaOS-2 ECM was assayed in Matrigel-coated Transwell chambers. As Table I shows, MCF-7 cells plated onto SaOS-2 ECM expressed an *in vitro* invasive potential 6 times higher than control cells plated onto plastic. However, MDA-231 cells (almost 9 times more invasive than MCF-7) increased only 1.6 times their invasive capacity when cultured on SaOS-2 ECM. Prior culturing of both cell lines on type I collagen did not induce significant changes in invasive capacity. In addition, the presence of SaOS-2 CM in both

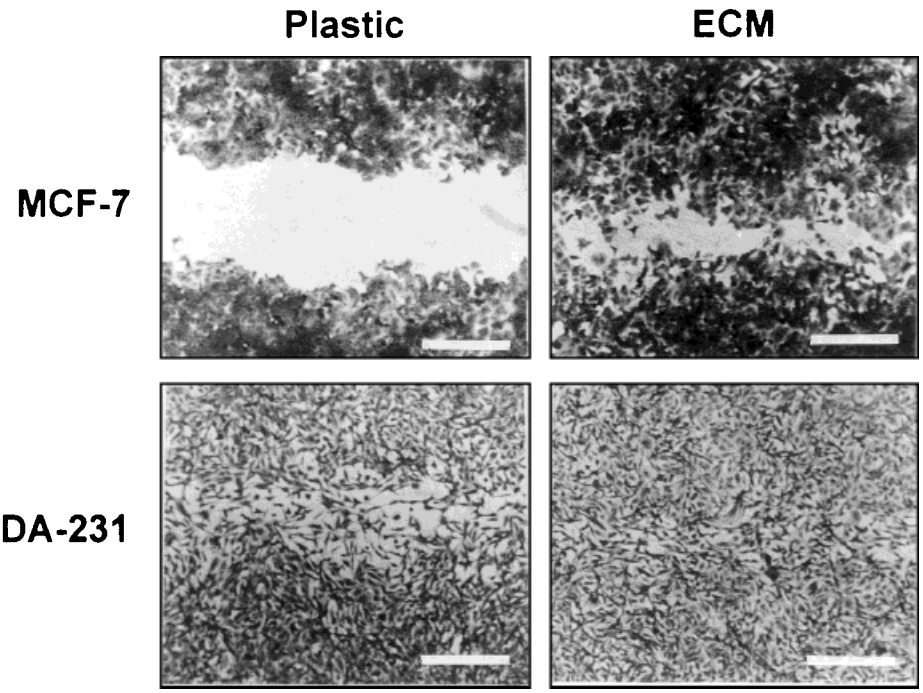

FIGURE 3 – Effect of SaOS-2 ECM on migratory capacity of cancer breast cells. Confluent cultures of MCF-7 and MDA-231 cells plated on plastic or on SAOS-2 ECM were wounded with a yellow pipet tip and allowed to migrate, as described in Material and Methods. Scale bar = 100 μm.

TABLE I – EFFECT OF SaOS-2 ECM ON INVASIVE CAPACITY OF CANCER BREAST CELLS

	% Invasion	Increment
MCF-7		
Control	1.08 ± 0.4	1
SaOS-2 ECM	6.52 ± 0.9	6.06
SaOS-2 CM	1.58 ± 0.5	1.46
Type I collagen	1.89 ± 0.4	1.75
MDA-231		
Control	9.50 ± 0.97	1
SaOS-2 ECM	15.60 ± 1.4	1.64
SaOS-2 CM	10.62 ± 1.3	1.12
Type I collagen	10.52 ± 1.3	1.10

MCF-7 and MDA-231 cells, previously cultured on plastic, type I collagen or SaOS-2 ECM, were assayed in matrigel-coated Transwell chambers, as described in Material and Methods. Data are expressed as percentage of cells in the lower chamber related to the total number of viable cells present at the end of the assay. Values are expressed as mean ± SD of 3 determinations.

chambers did not produce any relevant effect on the invasive potential of both cell lines.

u-PA production assay

The capacity of individual mammary cells to produce and secrete u-PA into culture media was assayed. As Figure 4*a* shows, the modest amount of active u-PA secreted to media by MCF-7 cells increased significantly when cells were seeded on SaOS-2 ECM. MDA-231 cells, cultured on plastic, secreted a higher amount of u-PA compared with MCF-7 cells. However, they did not increase the level of secreted u-PA after being cultured on bone cell–derived ECM. In both cases, a single active species of 50 kDa was secreted. In addition, changes in the expression level of u-PA mRNA from MCF-7 cells cultured on plastic and SaOS-2 ECM were evaluated by Northern blotting. As Figure 4*b* shows, a unique band of hybridization of 2.4 kb, representing u-PA mRNA, was expressed in cells cultured for 24 hr on SaOS-2 ECM, while cells cultured on plastic did not express u-PA mRNA at detectable levels.

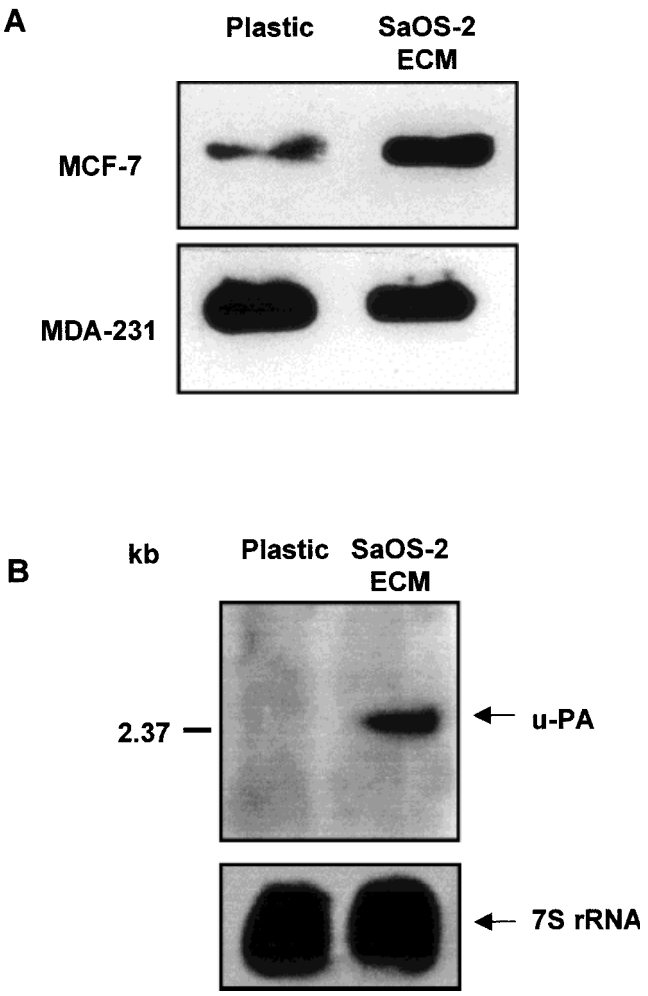

FIGURE 4 – Effect of SaOS-2 ECM on u-PA production by breast cancer cells. *(a)* u-PA activity. MCF-7 and MDA-231 cells were seeded on plastic and SaOS-2 ECM-coated plates. After 24 hr of culturing in serum-free medium, soluble u-PA was assayed by reverse diffusion, as described in Material and Methods. *(b)* u-PA mRNA. Northern blot of total RNA extracted from MCF-7 cells cultured on plastic or SaOS-2 ECM-coated plates for 24 hr. Blots were hybridized with a [^{32}P]-labeled cDNA probe, as described in Material and Methods. Approximately 7.7 µg of RNA were loaded per lane. An RNA-loading control (the same blot reprobed with a cDNA from 7 S rRNA) is shown in the bottom panel.

DISCUSSION

The establishment of cancer metastases represents the end point of a sequence of steps involving tumor–host interactions. Successful formation of metastases in distant organs requires that tumor cells attach, proliferate and operate a degradative machinery, to establish the environmental conditions needed to develop a secondary tumor (Radinsky, 1995).

The skeleton is one of the 3 most common sites of cancer metastasis. Bone (and bone marrow) harbors metastatic cells since it is a well-irrigated site and can produce a variety of growth factors (Yoneda *et al.,* 1996). The specific interactions of cancer cells with the host microenvironment are characterized by a constant ''cross-talk'' among tumor and host cells (Nicholson, 1989; Heiss *et al.,* 1995). A relevant example is represented by the invasive fate of melanoma cells in the brain environment, which shows that the organ microenvironment can profoundly influence the pattern of gene expression and the biological phenotype of metastatic tumor cells (Radinsky, 1995)

Our results are in line with previous evidence and represent an attempt to verify whether the bone microenvironment can modify the invasive/migratory behavior of breast cancer cells. Our results show that MCF-7 cells (belonging to the ER^+ family) exhibit an increased proliferative response when cultured in the presence of soluble factors produced by the osteoblastic cell line SaOS-2. The invasive cell line MDA-231 (ER^-) did not increase its proliferative rate in these conditions (Fig. 2). Soluble factors that stimulate cellular proliferation appear to be unable to deposit on insoluble ECM since cells plated on SaOS-2 ECM do not increase their proliferative rate (Fig. 2, inset). In attempts to identify the factor(s) involved in this proliferative effect, we and others did not succeed in detecting IGF-1 (one of the most conspicuous factors involved in MCF-7 proliferation) as a component of SaOS-2 CM (Ström *et al.,* 1994).

When mammary cells were cultured on an ECM generated by SAOS-2 cells, a different response was observed: while MCF-7 cells expressed a new set of morphological and functional properties, MDA-231 cells appeared to be more refractory to these changes. The acquisition of such new functional properties generated, in the case of MCF-7 cells, a more aggressive phenotype. MCF-7 cells cultured on SaOS-2 ECM acquired a less epithelial morphology, along with the appearance of cellular processes that evoked a motile phenotype (Fig.1). This was confirmed by experiments showing that MCF-7 cells plated on SaOS-2 ECM expressed an enhanced migratory capacity compared with cells plated on plastic. MDA-231 cells did not undergo morphological

changes (not shown) or increase their migratory capacity when plated on SaOS-2 ECM (Fig. 2). The migratory phenotype was obtained *via* acquisition of enhanced *in vitro* invasive properties, which in turn were a consequence of increased production of u-PA. This included expression of both protein and mRNA (Fig. 4).

As a control experiment, we subjected both cell lines to an initial culture on type I collagen, the main component of SaOS-2 ECM. The results (Table I) led to the conclusion that a non-collagen component of SaOS-2 ECM should be responsible for the increasing invasive capacity of MCF-7 cells. From our data, it is also possible to verify that a prior plating of MCF-7 cells on SaOS-2 ECM represents a more efficient stimulus to achieve an enhanced invasive phenotype than the presence of media conditioned by SaOS-2 in both Transwell chambers during the invasion assay (Table I).

It has been proposed that cell adhesion to ECM molecules activates many intracellular signaling pathways (Gumbiner, 1996). Binding of u-PA to its cellular receptor in MCF-7 cells also promotes cell migration and motility. This appears to depend on a u-PAR-dependent signaling pathway that provokes ERK phosphorylation and is neutralized by the MAP kinase inhibitor PD 098059 (Nguyen *et al.,* 1998). Our results, among others, show that MCF-7 cells cultured in the absence of any external stimuli synthesize marginal amounts of u-PA. However, as shown in this work, MCF-7 cells cultured on SaOS-2 ECM are able to increase the production of u-PA in an amount which is probably sufficient to activate u-PA-dependent adhesion to ECM and the above-mentioned signaling pathway.

u-PA appears to be a strong prognostic marker in breast cancer patients (Duffy *et al.,* 1990). In disseminated forms of breast cancer, patients whose tumoral cells have migrated to bone and express detectable amounts of u-PA have a worse prognosis than those who do not have detectable amounts of u-PA (Solomayer *et al.,* 1997). The presence of u-PA-positive cells in bone marrow is important for detecting micrometastatic cells. This can be interpreted in 2 ways: on the one hand, u-PA-positive cells in bone marrow may correspond to a subgroup of tumoral cells that have the phenotypical and functional capacity to reach the bone due to their high metastatic potential, including over-production of proteases (mainly u-PA); on the other hand, these cells acquire u-PA positivity during the housing of cells in the bone environment.

Based on our data, we speculate that the bone ECM might be in part responsive to the acquisition of an aggressive phenotype of breast cancer cells. However, whether the increase of u-PA production represents an important contribution to such phenotypic conversion or is only part of a more complex cellular response remains open to investigation.

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REFERENCES

ANDO,Y. and JENSEN, P., Protein kinase C mediates up-regulation of urokinase and its receptor in migrating keratinocytes of wounded cultures, but urokinase is not required for movement across a substratum *in vitro*. *J. cell. Physiol.,* **167,** 500–511 (1996).

DUFFY, M.J., REILLY, D., O'SULLIVAN, D., O'HIGGINS, N., FENNELY, J.J. and ANDREASEN, P., Urokinase plasminogen activator, a new and independent prognostic marker in breast cancer. *Cancer Res.,* **50,** 6827–6829 (1990).

EILON, G. and MUNDY, G.R., Direct resorption of bone by human breast cancer cells *in vitro. Nature (Lond.),* **276,** 726–728 (1978).

EILON, G. and MUNDY, G.R., Effects of inhibition of microtubule assembly on bone mineral release and enzyme release by human cancer breast cells. *J. clin. Invest.,* **67,** 69–76 (1981).

GARCIA, M., DEROCQ, D., FREISS, G. and ROCHEFORT, H., Activation of estrogen receptor transfected into a receptor negative breast cancer cell line decreases the metastatic and invasive potential of the cells. *Proc. nat. Acad. Sci. (Wash.),* **89,** 11538–11542 (1992).

GLOBUS, R.K., PLOUET, J. and GOSPODAROWICZ, D., Cultured bovine bone cells synthesize basic fibroblast growth factor and store it in their extracellular matrix. *Endocrinology,* **124,** 1539–1547 (1989).

GUMBINER, B.M., Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell,* **84,** 345–357 (1996).

HEISS, M.M., ALLGAYER, H., GRUETZNER, K.U., FUNKE, L., BABIC, R., JAUCH, K.W. and SHILDBERG, F.W., Individual development and u-PA receptor expression of disseminated tumor cells in bone marrow: a reference to early systemic disease in solid cancer. *Nature (Med.),* **1,** 1035–1039 (1995).

MCQUILLAN, D.J., RICHARDSON, M.D. and BATEMAN, J.F., Matrix deposition by calcifying human osteogenic sarcoma cell line (SAOS-2). *Bone,* **16,** 415–426 (1995).

MUNDY, R.G. and YONEDA, T., Facilitation and suppression of bone metastasis. *Clin. Orthopaed. rel. Dis*., **312,** 34–44 (1995).

NGUYEN, D.H.D., HUSSAINI, I.M. and GONIAS, S.L., Binding of urokinase-type plasminogen activator to its receptor in MCF-7 cells activates extracellular signal–regulated kinase 1 and 2 which is required for increased cellular motility. *J. biol. Chem*., **27,** 8502–9507 (1998).

NICHOLSON, G.L., Organ specificity of tumor metastasis: role of preferential adhesion, invasion and growth of malignant cells at specific secondary sites. *Cancer Metastasis Rev.,* **7,**143–188 (1989).

RABBANI, S.A. and XING, R.H., Role of urokinase (uPA) and its receptor (uPAR) in invasion and metastasis of hormone-dependent malignancies. *Int. J. Oncol.,* **12,** 911–920 (1998).

RADINSKY, R., Modulation of tumor cell gene expression and phenotype by the organ-specific metastatic environment. *Cancer Metastasis Rev.,* **14,** 323–338 (1995).

SANTIBÁÑEZ, J.F. and MARTÍNEZ, J., Membrane-associated procollagenase of leukemic cells is activated by urokinase-type plasminogen activator. *Leukemia Res.,* **17,** 1057–1062 (1993).

SANTIBÁÑEZ, J.F., NAVARRO, A. and MARTÍNEZ, J., Genistein inhibits proliferation and *in vitro* invasive potential of human prostatic cancer cell lines. *Anticancer Res*., **17,** 1199–1204 (1997).

SHEIKH, M.S., GARCÍA, M., PUJOL, P., FONTANA, J.A. and ROCHEFORT, H., Why are estrogen-receptor-negative breast cancers more aggressive than the estrogen-receptor-positive breast cancers? *Invasion Metastasis,* **14,** 329–336 (1994).

SOLOMAYER, E.F., DIEL, I.J., WALLWIENER, D., BODE, S., MEYBERG, G., SILLEM, M., GOLLAN, C., KRAMER, M.D., KRAINICK, U. and BASTERT, G., Prognostic relevance of urokinase plasminogen activator detection in micrometastatic cells in bone marrow of patients with breast cancer. *Brit. J. Cancer,* **76,** 812–818 (1997).

STETLER-STEVENSON, W.G., AZNAVOORIAN, S. and LIOTTA, L.A., Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Ann. Rev. Cell Biol*., **9,** 541–573 (1993).

STEWART, J.F., KING, R.J.B., SEXTON, S.A., MILLS, R.R., RUBENS, R.D. and HAYWARD, J.L., Oestrogen receptors, sites of metastatic disease and survival in recurrent breast cancer. *Europ. J. Cancer,* **17,** 449–453 (1981).

STRÖM, P., LJUNGHALL, S. and MELHUS, H., Adult human osteoblast-like cells do not express insulin-like growth factor-I. *Biochem. biophys. Res. Comm*., **199,** 78–82 (1994).

VALENTIN-OPRAN, A., EILON, G., SAEZ, S. and MUNDY, G.R., Estrogens and antiestrogens stimulate release of bone resorbing activity by cultured human breast cancer cells. *J. clin. Invest.,* **75,** 726–731 (1985).

YONEDA, T., Mechanisms of preferential metastasis of breast cancer to bone. *Int. J. Oncol*., **9,** 103–109 (1996).