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Relationship Between Tumorigenicity, *In Vitro* Invasiveness, and Plasminogen Activator Production of Human Breast Cell Lines

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Two human breast epithelial cell lines (HBL-100, HMT-3522) of non-malignant origin and six (MCF-7, T47-D, ZR-75-1, CAMA-1, BT-20, HMT-3909) of malignant origin have been studied to evaluate whether *in vitro* invasion and/or secretion of urokinase-type plasminogen activator are related to tumorigenicity in athymic nude mice. Only the six cell lines of malignant origin were tumorigenic. Two of these were invasive in the *in vitro* assay. These two cell lines were oestrogen receptor negative. Three of the other four cell lines of malignant origin contained oestrogen receptors. The two cell lines of non-malignant origin were neither tumorigenic nor invasive. Thus tumorigenicity was correlated to malignant origin of the cell line whereas invasiveness *in vitro* was a property of the most undifferentiated cell lines of tumor origin. Secretion of urokinase-type plasminogen activator was neither correlated to tumorigenicity nor to invasion *in vitro* nor to malignancy of tissue origin.
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INTRODUCTION

IN THE study of human carcinogenesis, cultured normal cells have been used as targets for various carcinogenic agents. Methods for extended culture of human breast epithelial cells are available and the effect of infection with oncogenic viruses [1] or of carcinogens [2] has been studied in an attempt to transform the breast epithelial cells to cancer cells. To follow the sequence of events during malignant transformation it is essential that criteria for malignant transformation of cells in culture are established.

In vivo characterization of cancer cells is based on tumour formation, invasive growth, metastasis, and death of the patient, if untreated. These properties cannot be demonstrated in culture. The malignancy of cancer cells in culture may be characterized by animal models and *in vitro* assays. Tumour formation in athymic, nude mice has often been used as the ultimate criterion for malignancy of cultured cells. Unfortunately this assay has a low sensitivity for breast cancer, since tumour take of human breast cancer transplanted to athymic mice is generally 0–20% [3] although 85% tumour take was achieved in one study [4]. Furthermore observations of metastasis and invasion are rare in athymic mice, possibly because of the high concentration of natural killer cells in such mice [5]. Transplanted, benign human breast tissue is sometimes capable of growth in the nude mouse [6].

For mesenchyme-derived cancer cells (sarcomas), tumorigenicity in athymic mice is accompanied by several *in vitro* characteristics, such as altered morphology, immortalization, loss of contact inhibition, decreased serum requirement, anchorage independent growth and increased protease secretion [7]. This correlation does not exist for many epithelium-derived tumour cells, including breast carcinoma cells [7]. Thus means to characterize the malignant phenotype of human breast cells in culture are insufficient and mainly based on tumorigenicity in nude mice.

In vitro models for invasive growth of cultured cells have been described—the embryonic chick heart assay [8], the human amnion membrane assay [9] and the artificial membrane assay [10]. These assays may be included in the characterization of the malignant phenotype *in vitro* [11].

Cancer cells produce and secrete proteases and attention has been focused on the serine protease, urokinase-type plasminogen activator (uPA), which is thought to play an important role in tumour cell invasion [12]. In a series of sublines of the human breast cancer cell line, MCF-7, some correlation was demonstrated between invasion of tumors in athymic mice and uPA activity [7]: uPA production by cells may be a useful index to describe the malignant phenotype *in vitro*. We have investigated the relation between tumorigenicity in the athymic mouse, invasion in the embryonic chick heart assay and production and secretion of uPA in eight established human breast epithelial cell lines (six malignant and two benign).

MATERIALS AND METHODS

Cell culture

Eight breast epithelial cell lines were studied. Six came from carcinomas, either solid tumours or malignant effusions: MCF-7, passage 248–273; T-47D, passage 89–99; ZR-75-1, passage 94–102; CAMA-1, passage 41–48; BT-20, passage 301–312; and HMT-3909 [13], passage 10–14. Two cell lines were of non-malignant origin: HBL-100, passage 45–66, which was derived from cells of human milk and HMT-3522 [14], passage 26–53 which was established from fibrocystic disease of the breast (see ref. 15 for the origin of MCF-7, T-47-D, ZR-75-1, CAMA-1,

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BT-20, and HBL-100). MCF-7 and HBL-100 were supplied by the Breast Cancer Task Force Cell Culture Bank, Mason Research Institute (Worcester, Massachusetts); T-47D, ZR-75-1 and BT-20 were obtained from American Type Culture Collection (Rockville, Maryland); CAMA-1 was obtained from J. Fogh, Sloan–Kettering Institute (Rye, N.Y.); and HMT-3909 and HMT-3522 were established in our laboratory.

Growth media

T-47D: RPMI-1640 supplemented with 8 μg/ml insulin and 10% fetal calf serum (FCS). BT-20: modified Eagle's medium supplemented with 0.1 mmol/l non-essential aminoacids (NEA) and 20% FCS. HBL-100: McCoy-5a supplemented with 0.5% FCS; MCF-7, ZR-75-1, and CAMA-1: see ref 15. HMT-3909 and HMT-3522 were propagated in chemically defined medium [13, 14]. All media were supplemented with 2 mmol/l glutamine. Penicillin (a gift from Leo Pharmaceuticals, Ballerup) and streptomycin were added to experimental cultures only.

Invasiveness in vitro

To study invasiveness of the breast epithelial cell lines *in vitro* we used the embryonic chick heart assay [8]. A slight modification of the method [16] was used. Multicellular aggregates were prepared by suspending 6–12 × 10^5 cells in 6 ml Leibowitz phosphate-buffered L-15 medium (Flow) supplemented with 2 mmol/l glutamine, 0.1 mmol/l NEA, 10 μg/ml adenosine, guanosine, uridine, and cytidine, and 10% FCS. The cell suspension was incubated for 1–7 days at 37°C on a gyratory shaker to form aggregates. After confrontation of aggregates with precultured heart fragments on semisolid agar plates, complexes were transferred individually to 5 ml Erlenmeyer flasks containing 1.5 ml medium and rotated at 37°C for up to 20 days. Every 2–3 days complexes were selected at random, fixed with Bouin's, and embedded in paraffin. Sections were stained with haematoxylin/eosin. The interaction of cells with precultured heart fragments was classified into four grades [17]: (I) cells were found at periphery of outer fibroblast-like layers; (II) cells occupied outer fibroblast-like layers; (III) cells replaced less than 50% of heart tissue; and (IV) cells replaced more than 50% of heart tissue. Cells in grade III and IV may be considered invasive.

Tumorigenicity in athymic mice

Tumorigenicity was tested in 6–8 week-old female inbred BALBc athymic nude mice. Bilateral ovariectomy was done 1 week before the inoculation. Each cell line was tested in six mice, three of which were treated with oestradiol 1 mg every 3 weeks intramuscularly as a depot ('Progynone', Schering). 10^7 cells suspended in 100 μl culture medium were inoculated subcutaneously in the fourth mammary gland and the mice were examined weekly for at least 3 months or till death. The test was positive only if progressively growing tumours were formed. The tumours were fixed in 3.6 % formaldehyde buffer (pH 6.9) and embedded in paraffin. Sections were stained with haematoxylin/eosin and examined by light microscopy. From most tumours, tissue was taken for oestrogen and progesterone receptor assay.

Oestrogen and progesterone receptor assay

Tumour tissue from the nude mice was assayed for steroid receptors. The dextran-charcoal technique [18] was used for measurement of free oestrogen (ER) and progesterone (PR) receptors and the hydroxylapatite method (18) for assaying chromatin-bound filled ERs.

Urokinase-type plasminogen activator

For study of uPA secretion, each cell line was plated in a 6-well multidish (Teknunc, Roskilde, Denmark). The medium was renewed in all 6 wells at day 1. At day 3 the cells in 3 wells were washed twice with phosphate-buffered saline (PBS) and the protein content of the cells in each well was determined by Lowry's method modified by Oyama *et al.* [19]. Medium in the remaining 3 wells was renewed. At day 5, the medium from these wells was pooled and centrifuged at 1500 *g* at 4°C for 15 min to remove cell debris. The supernatant (conditioned medium) was stored at −20°C until uPA assay. The protein content of the cells was measured. The uPA concentration was assayed by enzyme immunoassay with a monoclonal antibody (provided by Dr. Lars Skriver) against human uPA as solid-phase antibody. A biotinylated, rabbit polyclonal antibody and avidine-conjugated peroxidase (Kem-En-Tech, Hellerup, Denmark) were used for detection of bound uPA [20]. Human pro-uPA was used as both external and internal standard. UPA secretion was calculated assuming exponential growth between days 3 and 5.

For assay of uPA content in cell lysates, the cells in exponential growth phase were washed twice with PBS and detached from the culture wells by incubation at 37°C for 30 min in Ca^{2+} and Mg^{2+} free PBS containing 1 mmol/l EDTA. The cell suspension was washed twice with PBS. Cell lysis was performed with 0.5% 'Triton-X-100' in 0.1 mmol/l Tris pH 8.1 on ice and verified by light microscopy. The uPA concentration was measured as before. Protein content in the cell lysate was assayed by a modified Lowry's method [21].

RESULTS

In vitro *invasiveness (Table 1)*

Each cell line was tested three times. All experiments were reproducible. During precultivation, the heart fragment became rounded and an outer fibroblast-like layer was formed. During co-cultivation of the mammary cell aggregates with precultured heart fragments, the fragment was sometimes surrounded by the mammary cells. This event, called encirclement, has been described by others [16] and is not considered a reliable criterion of malignancy.

The MCF-7 cell line showed encirclement of the heart fragment (Fig. 1A) around day 6 with a sharp borderline between the aggregate and the fragment. However, in one experiment, at days 8 and 11 we found a group of 5–10 cells placed a few cell diameters inside the fragment. This cell infiltration did not lead to any further replacement of the heart tissue, and the MCF-7 was therefore evaluated as non-invasive and scored as grade I.

The T-47D cell aggregates encircled the heart fragments

Table 1. Invasion of human breast cell lines in embryonic chick heart fragment invasion assay

Cell lines	Invasion	Grade
MCF-7	–	I
T-47D	–	I
ZR-75-1	–	II
CAMA-1	–	II
BT-20	+	IV
HMT-3909	+	IV
HBL-100	–	II
HMT-3522	–	II

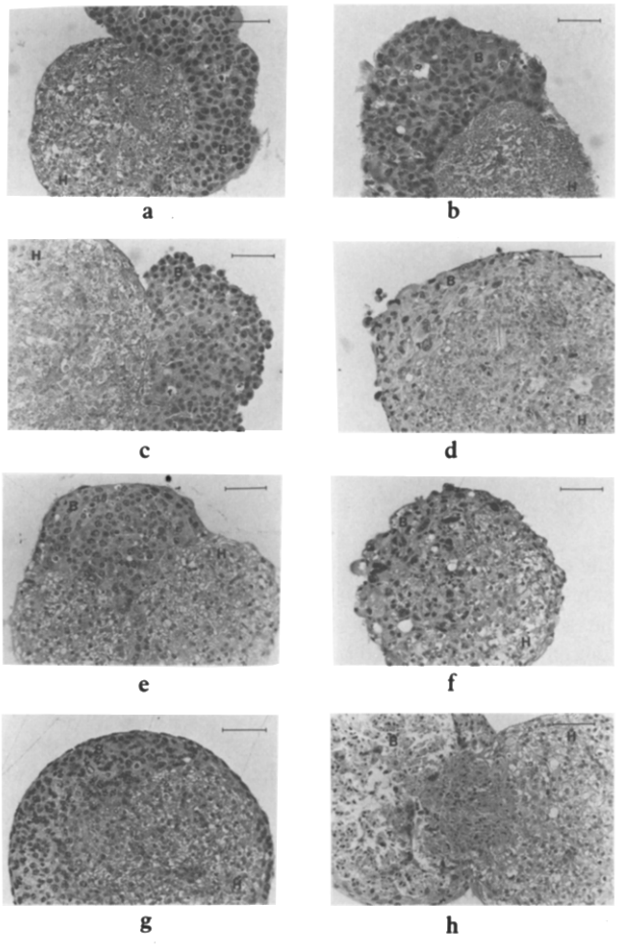

Fig. 1. Light microscopy of sections from complexes of cell aggregates co-cultured with precultured heart fragments. a = MCF-7, day 11; b = T-47D, day 10; c = ZR-75-1, day 9; d = CAMA-1, day 7; e = BT-20, day 10; f = HMT-3909, day 12; g = HBL-100, day 8; and h = HMT-3522, day 10. B = breast epithelial cells, H = embryonic chick heart tissue. Haematoxylin/eosin, bar = 20 μm.

around day 9 (Fig. 1B), but during the entire observation period there was a sharp borderline between aggregate and fragment and no sign of invasiveness. However, necrosis of the heart tissue starting around day 2 was observed in all experiments. T-47D was scored as grade I.

The ZR-75-1 cells replaced the outer fibroblastic layer within 5–7 days, which was unchanged to the end of the experiments at day 16. In one experiment, at days 7 and 9 (Fig. 1C), single cell infiltration of the heart was observed but this infiltration did not lead to progressive destruction of the heart tissue. ZR-75-1 was, therefore, considered as non-invasive at grade II.

The CAMA-1 cells replaced the outer fibroblastic layer around day 6, and in two of the experiments encirclement at days 11–12 was observed (Fig. 1D). Because of no further replacement and no destruction of heart tissue, the CAMA-1 was considered non-invasive at grade II.

After 3 days the BT-20 cells showed encirclement and at day 4 the fibroblastic layer of the PHF was replaced by BT-20 cells. At days 8–10, we observed single cell infiltration which was followed by 'en block' invasion (Fig. 1E), and from day 10–12, almost the entire heart fragment was replaced by the cancer cells. BT-20 was therefore considered invasive at grade IV.

Because of difficulties with aggregate formation of HMT-3909, only one experiment with this cell line was successful. At day 6 we observed encirclement and single cell infiltration followed by a more extensive invasion (Fig. 1F), resulting in total replacement and destruction of the heart tissue. At day 14, only a small area of necrotic heart tissue remained in the centre of the co-culture complex. HMT-3909 was thus invasive at grade IV.

The HBL-100 cell line caused a replacement of the fibroblast-like layer of the heart fragment within 3 days, and after 6 days the HBL-100 cells encircled the fragment. In some sections single cell infiltration was observed (Fig. 1G). Since this situation did not develop into a progressive replacement of the heart tissue, the HBL-100 cells were classified as non-invasive at grade II.

Contrary to the other cell lines, the *in vitro* invasion assay with HMT-3522 cells resulted in central necrosis in the contact area between the cell aggregate and the heart fragment. The area of necrosis was already apparent at day 3 and increased throughout the test period to include most of the contact zone and extended into both the HMT-3522 aggregate and the fragment (Fig. 1H). This course was seen in all the experiments with HMT-3522. Despite the atypical result we considered HMT-3522 as non-invasive (grade I) since we believe that an invasive potential could have been expressed in the non-necrotic area.

Tumorigenicity in athymic mice

Only results from experiments done at the same time as the invasion assay are included in Table 2. All cell lines were transplanted to athymic mice in at least two more experiments and in all cases the same results were found. MCF-7, T-47D, ZR-75-1, CAMA-1, BT-20 and HMT-3909 were all tumorigenic. However, MCF-7, T-47D, and ZR-75-1 only gave rise to tumours in mice treated with oestradiol. By histological examination the tumours were verified as carcinomas. HBL-100 and HMT-3522 were non-tumorigenic; a few mice survived 4–6 months after inoculation without palpable tumour.

Oestrogen and progesterone receptors (Table 3)

In the tumours (MCF-7, T47D, ZR-75-1) which only grew in mice treated with oestrogen, ERs were measured as chromatin-bound filled receptors and the PgR levels were high, reflecting that fact that the tumours grew as oestrogen responsive tumours. One tumour of ZR-75-1, observed in an untreated, castrated mouse was found to be ER and PgR negative. Tumours of CAMA-1, BT-20, and HMT-3909 were all ER and PgR negative except one tumour of CAMA-1 cells that contained low amounts of PgR.

*Table 2. Tumorigenicity of human breast cell lines in nude mice**

Cell lines	With oestrogen	Without oestrogen
MCF-7	3	0
T-47D	2	0
ZR-75-1	3	1
CAMA-1	3	3
BT-20	3	3
HMT-3909	3	3
HBL-100	0	0†
HMT-3522	0	0

*Six mice were inoculated and three were treated with oestrogen. Results are no. of animals with tumour out of three (except for †, n = two).

Table 3. ERs and PRs in tumours of human breast cancer cell lines in nude mice

Cell line	Tumour†	ER (free)	K_d (ER)	ER (bound)	PgR (free)	K_d (PgR)
MCF-7*	A	22	40	175	480	4.0
MCF-7*	B	<10	—	196	838	2.5
T-47D*	A	<10	—	126	8311	24.8
ZR-75-1*	A	94	8.4	143	3951	7.5
ZR-75-1*	B	<10	—	125	2412	6.4
ZR-75-1*	C	<10	—	241	4259	10.4
ZR-75-1	D	<10	—	<10	<10	—
CAMA-1*	A	<10	—	<10	<10	—
CAMA-1*	B	<10	—	<10	23	2.5
CAMA-1	C	<10	—	<10	<10	—
CAMA-1	D	<10	—	<10	<10	—
BT-20*	A	<10	—	<10	<10	—
BT-20*	B	<10	—	<10	<10	—
HMT-3909*	A	<10	—	<10	<10	—
HMT-3909	B	<10	—	<10	<10	—

*These mice received oestradiol.
ER and PR are expressed as fmol/mg protein.
Kd = dissociation constant × 10^{-10} mol/l.
†A–D indicates individual tumours.

Urokinasetype plasminogen activator

The plasminogen activator may be measured as enzyme activity or as amount of enzyme protein. Measuring the enzyme activity in the conditioned medium does not reflect the enzyme activity in the microenvironment around the invading cell due to the presence of inhibitors from the serum or secreted by the cells. Therefore, we chose to measure the amount of uPA by enzyme immunoassay. UPA release from HMT-3522 cells was several fold higher than that of any of the other cell lines (Fig. 2). CAMA-1 and HMT-3909 released moderate amounts of uPA whereas the remaining cell lines showed little (HBL-100, MCF-7, T-47D) or no detectable release (ZR-75-1, BT-20). It might be suggested that the high release—especially from HTM-3522 and HMT-3909 grown in serum-free medium—was due to leakage from dead or damaged cells rather than to secretion. There was, however, no indication of any significant cell loss or damage by phase-contrast microscopy. The moderate concentration of uPA in the cell lysate also speaks against such an explanation.

The great difference in uPA release to the medium between the cell lines was not reflected in the uPA content of cell lysates (Fig. 3). The HMT-3522 cells that released substantial amounts of uPA contained a moderate concentration of uPA in the cell lysate. Of the three cell lines with high release of uPA to the medium, only CAMA-1 contained a high amount in the lysate.

DISCUSSION

The eight human breast cell lines were classified as six carcinoma cell lines and two non-carcinoma cell lines according to the tissue of origin and tumorigenicity in nude mice. Three of the six carcinoma cell lines were hormone-dependent (MCF-7, T-47D, ZR-75-1), based on the need for oestrogen treatment to support growth of tumours after transplantation to nude mice. ERs and PRs have been found in these lines [22] as well as in their tumours, as demonstrated in the present study.

Only two of the six carcinoma cell lines were invasive. These two lines were derived from primary breast carcinomas whereas

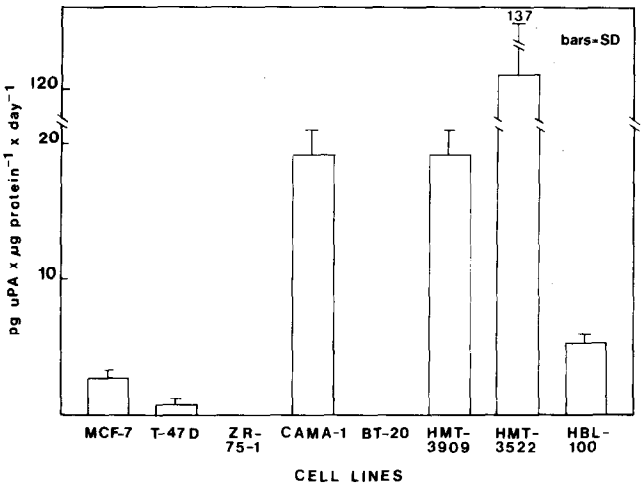

Fig. 2. uPA secretion by human breast cell lines. Mean (S.D.).

the non-invasive cell lines originated from effusions. This was somewhat unexpected since cells of malignant effusions are already selected *in vitro* for invasive growth and they may, therefore, also be expected to be invasive *in vitro*. However, the observed differences in invasiveness among the investigated cell lines rather reflect differences in differentiation grade of the lines. The less differentiated phenotype of the two *in vivo* invasive cell lines is indicated by the lack of ERs [13, 22] and the ability to grow in nude mice without oestrogen supplementation. Three out of the four non-invasive carcinoma cell lines were ER positive [22]. The fourth, CAMA-1, though ER negative in our laboratory [15] and capable of tumour growth in nude mice without oestrogen, has been found to contain ER and to respond to oestrogen [23]. In our study one out of three CAMA-tumours weakly responded to oestrogen by expression of PgR. The lack of *in vitro* invasiveness of the oestrogen-dependent cell lines is hardly due to a lack of sufficient oestrogen since the *in vitro* invasion assay was done in medium with 10% FCS, which contains sufficient oestradiol for biological responses such as induction of PRs and growth [18]. Furthermore, addition of oestradiol during co-cultivation did not result in invasion of the MCF-7 cells *in vitro* (A.E. Lykkesfeldt, Fibiger Institute).

The embryonic chick heart *in vitro* invasion assay was developed to study the mechanism of invasion. The results were evaluated on histological sections of the co-cultures with a

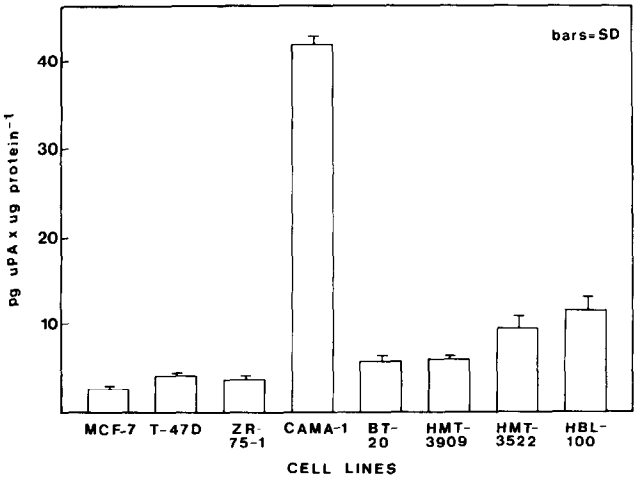

Fig. 3. uPA content in cell lysates of human breast cell lines. Mean (S.D.).

grading system [17]. The assay was slightly modified for the study of human bladder cell lines [16], which were classified into transformation grades according to growth, tumorigenicity in nude mice and *in vitro* invasion [11]. In that study, all tumorigenic cell lines were also scored as invasive in the embryonic chick heart *in vitro* assay. Two cell lines, although immortalized, were neither tumorigenic nor invasive *in vitro*. In our study, a third category was demonstrated: four cell lines that were immortalized and tumorigenic though not invasive in the *in vitro* invasion assay.

All the investigated cell lines derived from breast carcinoma tissue were tumorigenic in nude mice, in accordance with observations from other groups [13, 24, 25]. The two cell lines of non-malignant origin did not form tumours in nude mice. The HBL-100 cell line was investigated in passages 45–66 and found to be non-tumorigenic. In passage 103 tumorigenicity of this cell line has been demonstrated and the transformation of this line has been related to the presence of the SV-40 genome in the cellular DNA and expression of SV40 large T-antigen [26].

In human breast carcinomas the PA activity, mainly due to uPA, is higher than that in normal breast tissue [27]. From our study uPA was expressed and secreted from cultured cell lines in quantities unrelated to tissue of origin, tumorigenicity in nude mice and invasiveness *in vitro*. This discrepancy between results in tumour tissue and in tissue cultures may have several explanations. The uPA content does not necessarily reflect the PA activity. The presence in the tissue of specific PA inhibitors may decrease the proteolytic activity without affecting the uPA content. Furthermore, several proteases including uPA are induced by extracellular matrix components [28] only represented in the cultures by collagen. Finally, membrane receptors for uPA have been demonstrated [29] implying that locally (at the receptor sites) high concentrations of uPA may exist which are not reflected in the average concentration of uPA in the medium or in the lysate.

In conclusion, in several human breast cell lines, tumorigenicity was closely related to the tissue of origin. The *in vitro* invasion assay characterized a subgroup of breast carcinoma cell lines of lower differentiation grade whereas uPA content and secretion did not correlate with tissue origin, tumorigenicity or to invasion *in vitro*.

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