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Original Paper

In Vitro Anti-proliferative and Anti-invasive Role of Amino-terminal Fragment of Urokinase-type Plasminogen Activator on 8701-BC Breast Cancer Cells

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8701-BC cells, derived from a primary carcinoma of the breast, constitutively express mRNA for urokinase-type plasminogen activator (uPA). In this paper, we demonstrated the presence of uPA in the conditioned medium, and of uPA-receptor (uPAR) on the cell surface of 8701-BC cells, which therefore have the potential for an autocrine mechanism of uPA-mediated stimulation. We examined whether exogenous addition of either intact uPA, or its amino-terminal fragment (uPA-ATF), which lacks catalytic activity but retains the uPAR binding site and a growth factor-like domain, or immunoneutralisation of endogenous uPA–uPAR interactions could exert any effect on the proliferative and invasive behaviour of 8701-BC cells. The data demonstrate that, while uPA promotes growth and invasion of 8701-BC cells, its effect reversed by blocking uPA–uPAR interactions, uPA-ATF not only fails to impart growth factor-like signals, but also restrains cell invasion *in vitro*. In the light of these and other data, an active participation of ATF in the complex cell–ECM network of interactions underlying cancer progression can be postulated. In addition, it appears worth considering the possibility of testing the effect of this uPA fragment *in vivo* for the therapy of breast (and possibly other) human invasive carcinomas. Copyright © 1996 Elsevier Science Ltd

Key words: 8701-BC cells, breast carcinoma, urokinase, cell proliferation, cell invasion
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INTRODUCTION

THE TWO-CHAIN serine protease urokinase-type plasminogen activator (uPA) is one of the major mediators of pericellular proteolysis in development, tissue repair, and malignant infiltration [1–7]. In breast cancer, in particular, the increased level of of uPA has been statistically correlated with poor prognosis [8–10]. Though recent evidence indicates that tumour-associated stromal and inflammatory cells are the main source of uPA production in human tumours [11], activated uPA, once bound to the specific receptor (uPAR) on the surface of cancer cells [12, 13], catalyses the formation of the broad-specificity protease, plasmin, from plasminogen and the initiation of the subsequent cascade proteolytic reaction. Increasing evidence has also shown that uPA can directly act on (and activate when latent) various extracellular matrix (ECM) substrates [5, 14]. Moreover, some investigators have demonstrated that both enzymatically-inactivated uPA and the (1–135) fragment of the non-catalytic "A" chain (uPA-ATF), which contains the "EGF-like" domain (encompassing the uPAR-binding site) and the "kringle" domain, are able to exert growth factor-like effects on different cell lines, both normal and neoplastic [15–22].

Recent studies [23] on the mRNA array for a number of soluble factors and enzymes, have indicated that, among others, the gene for uPA is constitutively switched-on in 8701-BC breast carcinoma cells. This finding prompted us to search for both the accumulation of the related protein product in the conditioned medium (CM) of 8701-BM cells and the presence of uPAR on the cell surface. Secondly, in consideration of the enzymatic and growth factor-like roles respectively described for uPA and its ATF, we have used 8701-BC cells as a model system to examine the role, if any, elicited by these molecules on those biological activities *in vitro* more closely related to the modulation of malignant phenotype, i.e. proliferation and invasion.

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MATERIALS AND METHODS

Cell cultures

The tumour cell line 8701-BC, derived from a biopsy fragment of a primary ductal infiltrating carcinoma (D.I.C.) of the human breast [24, 25] was cultured in RPMI 1640 medium (Gibco, Paisley, U.K.), with 10% fetal calf serum (FCS; Gibco) and antibiotics (100 U penicillin and 100 μg streptomycin/ml), at 37°C in a 3% CO_2 atmosphere. The mouse LB6 clone 19 cell line (a kind gift from Dr N. Pedersen, Univ. Institute of Microbiology, Copenhagen, Denmark) was derived from parental LB6 cells after transfection with pRSV-neo (conferring resistance to G-418) and *p*-uPAR-DNA containing the cDNA for the human uPAR expressed under the control of the SV40 promoter [26]. This cell line was cultured in Dulbecco's modified Eagle's medium (DMEM), with 10% FCS, glutamine, antibiotics and 0.4 mg/ml G-418.

Radioiodination of uPA-ATF and crosslinking assay

uPA-ATF (a kind gift from Dr J. Henkin, Abbott Labs, Abbot Park, Illinois, U.S.A.) was subjected to radioiodination with ^{125}I-Na using Iodogen (Pierce Eurochemie B.V., Holland) according to manufacturer's instructions. The specific activity obtained in the preparation used in this study was 60 μCi/μg. Cross-linking assays for the identification of uPAR were carried out as described by Del Rosso and associaties [17, 18]. Acidification was performed with 0.1 M NaCl/50 mM glycin, pH 3 and, after neutralisation, confluent cells in 25 cm^2 flasks (approximately 2×10^6) were lysed by adding 500 μl of lysis buffer (0.1 M Tris–HCl, pH 8, plus 10 mM EDTA, 1% aprotinin, 1% Triton X-114 and 1 mM phenylmethylsulphonyl fluoride (PMSF)). The cell extract was incubated with iodinated uPA-ATF, with or without unlabelled competitor, the crosslinking performed in the presence of 1 mM *N-N'*-disuccinimidyl-suberate (DSS, Pierce) for 15 min, and stopped by addition of ammonium acetate to 10 mM. The samples were analysed by 12.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions and autoradiography.

uPA assays

Dot-blot. CM from 8701-BC cells was prepared as already described [27], concentrated 100× and 100 μl were spotted onto nitrocellulose paper (Hybond C, Amersham, U.K.). The monoclonal antibody (MAb) used was anti-human uPA 5B4 (1:200; a kind gift from Dr M.L. Nolli, Dow Lepetit, Varese, Italy), specific for the "kringle" domain, previously used in other studies [28]. The immunoreaction was visualised with the protein A-gold solution, followed by silver enhancement according to the manufacturer's instructions (BioRad, Richmond, U.S.A.).

Colorimetric determination. The assay system utilises the uPA present in CM to convert the exogenously-added plasminogen to active plasmin, which subsequently digest the substrate and liberate the free chromophore 4-nitranilide. For this purpose, aliquots of CM were diluted in 60 mM Tris–saline, pH 7.5, and incubated in microtitre plates in the presence of 0.65 μg/ml plasminogen (Boehringer, Mannheim, Germany) and 0.5 μmol/ml of Chromozym-PL (Tosyl-glycyl-prolyl-lysine-4-nitranilide acetate; Boehringer) at 37°C in the dark for 30 min. The samples were read at 405 nm in an automated microplate reader. Human kidney cell uPA, purchased from Sigma (St. Louis, Missouri, U.S.A), was used as control to construct a standard absorbance versus units curve, which was linear between 45 and 217 mU uPA/ml (not shown). According to manufacturer's definition, one unit of uPA is able to activate that amount of porcine plasminogen which will produce a ΔA_{275} of 1.0 per ml per min at pH 7.5 at 37°C, when measuring perchloric acid soluble products from α-casein (1 cm light path).

Proliferation assay

Cells were plated at a concentration of 2.5×10^4/well in four-well plastic dishes (Nunc, Roskilde, Denmark) with 10% FCS–RPMI. After overnight incubation to allow adhesion, cells were cultured with serum-free RPMI medium, with or without supplementation with either uPA (Sigma), uPA-ATF or MAbs. The latter were anti-human uPA MAb 5B4 and anti-human uPAR MAb R3 (kindly provided by Prof. F. Blasi), which were used at a 1:200 dilution. MAb 5B4 impeded sterically uPA–uPAR interaction as did MAb R3, developed against the uPA-binding site of uPAR [29]. After culture for 1 day, the medium was refreshed and after 24 h, the cells were trypsinised and counted using a haemocytometer. Each assay was conducted at least in triplicate.

Chemotaxis assay

Cell chemotactic behaviour was evaluated by a modified Boyden chamber test [30], employing polyvinylpyrrolidone-free polycarbonate filters (13 mm filter diameter, 8 μm pore diameter; Nucleopore, Pleasanton, California, U.S.A.) and "bind well" chambers with an upper compartment of 800 μl and a lower compartment of 200 μl (Neuro Probe, Cabin John, Maryland, U.S.A.) [31, 32]. For chemotaxis assay, cells were trypsinised, washed once with RPMI plus 10% FCS to inactivate trypsin and then twice with 0.1% bovine serum albumin (BSA)–RPMI, and seeded on to the filter at a concentration of 3×10^5 cells/chamber. The chemoattractants tested were uPA and uPA-ATF, individually dissolved at various concentrations either in 0.1% BSA–RPMI medium or in 8701-BC CM. We also tested the chemoattractive influence of 8701-BC cell CM treated with anti-uPA 5B4 MAb, used at 1:100 dilution. The assay was carried out for 6 h at 37°C in a 3% CO_2 atmosphere. At the end of the incubation, the cells attached to the upper surface of the filter were removed mechanically, whilst those which had migrated to the lower surface were fixed, stained with toluidine blue and counted by selecting 10–15 random fields of the filter at a 200× magnification. Each determination was conducted at least in triplicate. Negative controls were run using 0.1% BSA–RPMI or untreated CM as chemoattractants.

Chemoinvasion assay

For the chemoinvasion assay, the test filters were coated with 25 μg of Matrigel (Collaborative Res., Bedford, Massachusetts, U.S.A.), dried within the sterile cabinet and rehydrated with 0.1% BSA–RPMI just before utilisation. The experimental design and procedure followed those described for the chemotaxis assay. In a parallel set of experiments, tissue inhibitor of metalloproteinase (TIMP)-1 (10 μg/ml; kindly provided by Dr H Nagase, University of Kansas Med. Ctr., U.S.A.) was added to cell suspension approximately 30 min before plating on to Matrigel-coated filters [32, 33].

Evaluation of in vitro *proliferation and invasion parameters*

Cell proliferation. The % cell number variation was calcualted as

$$[(C_1 - C_0)/C_0] \times 100,$$

where C_1 and C_0 are the cell numbers in response to treatment and in the parallel control assay, respectively.

Cell invasion. The % cell number variation was calculated as

$$[(C_1 - C_0)/C_0] \times 100,$$

where C_1 and C_0 are the numbers of cells penetrating Matrigel in response to a chemoattractant and in the parallel control assay, respectively; the invasion index (I.I.) and relative invasion index (R.I.I.) were calculated by the following formulae:

$$\text{I.I.} = C_{MG+}/C_{MG-} \times 100$$

where C_{MG+} and C_{MG-} are the numbers of migrated cells in parallel chemoinvasion and chemotaxis tests, respectively;

$$\text{R.I.I.} = \text{I.I.}_1/\text{I.I.}_0$$

where $\text{I.I.}_1/\text{I.I.}_0$ are the invasion indexes versus a chemoattractant and in the parallel control assay, respectively. R.I.I. values <1, $= 1$ and >1 were indicative of invasion-restraining, -ineffective and -promoting action of the chemoattractant tested [31, 32].

RESULTS

Identification of uPA in cell CM

Samples of cell CM were submitted to dot-blot and immunoassayed with 5B4 MAb in order to assess whether uPA was secreted in the extracellular milieu. As shown in Figure 1, the reaction was clearly positive indicating that uPA transcript, whose constitutive expression by 8701-BC cells had been previously demonstrated by reverse-transcriptase-linked polymerase chain reaction (RT-PCR) technique [23], is actually translated and the product released. We therefore quantified the uPA content in cell CM by "indirect" (i.e. plasminogen-mediated) determination of uPA activity. We used a relatively long incubation period of the chromogenic mixture (30 min) to allow for activation of pro-uPA, if present, by the plasmin formed, thus estimating the total uPA activity. Results from triplicate assay gave an estimation of 26 ± 3 mU uPA/ml of CM (mean ± S.E.M.).

Identification of uPAR in cell extracts

In order to determine whether 8701-BC cells could potentialy recognise uPA and its N-terminal fragment, we searched for uPAR in cell extracts. Acidification and subsequent neutralisation was performed on an 8701-BC cell monolayer to uncouple uPAR from potentially-bound endogenous ligand. Figure 2 shows the cross-linking of 8701-BC cell preparation with ^{125}I uPA-ATF indicating that the receptor is present (lane 4) and that the binding is specific since it can be displaced by excess unlabelled uPA-ATF (lane 5). It is noteworthy that the M_r of the positive control (from LB6 clone 19 cells, lane 2) is lower than that of 8701-BC cell uPAR (approx. 65 kDa), possibly due to the minor degree of glycosylation of the former as previously reported for other cell lines [34].

Growth behaviour of 8701-BC cells

Figure 3 shows that native uPA had a dose-dependent growth-promoting effect (up to +86 ± 14% versus control) at the concentration tested, but equimolar amounts of its N-terminal peptide reduced cell proliferation by −23 ± 7% versus control. The promoting effect of uPA was reversed by pre-absorption with anti-uPA 5B4 MAb at a 1:100 dilution (data not shown). When cells were grown in a medium supplemented with either 5B4 or R3 blocking MAbs at a 1:100 dilution, both MAbs restrained cell proliferation by 22 ± 5% and 12 ± 6% (average of triplicate assay ± S.E.M.; not shown), respectively.

Invasive behaviour of 8701-*BC cells*

The results obtained indicate that 100 ng/ml (i.e. approximately 1.8 nM) was the optimal uPA concentration in both BSA–RPMI medium and CM to achieve chemoinvasive stimulation of cells, with 200 ng/ml or lower concentrations being respectively weaker and inefficient in eliciting cell invasion *in vitro* (Figure 4). Conversely, incubation with uPA-ATF both in BSA–RPMI medium and in CM resulted in a marked and dose-dependent inhibition of cell invasion, which reached the plateau at a concentration of 1.8 nM (Figure 5). We also evaluated cell R.I.I., a parameter which, taking into account the changes in both cell chemotactic and chemoinvasive behaviour, can be used to extrapolate the real modulation

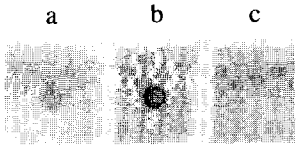

Figure 1. Dot blot assay of 8701-BC CM (a), control uPA purchased from Sigma (b) and irrelevant antigen (c) with anti-human uPA MAb 5B4. The positivity of the reaction indicates that uPA is released by 8701-BC cells in their CM.

Figure 2. Crosslinking of human uPA-ATF to 8701-BC cells. Lane 1: negative control, ^{125}I-uPA-ATF only; lane 2, positive control, ^{125}I-uPA-ATF and a pool of media from mouse LB6 clone 19 cells; lane 3: molecular weight standards (^{14}C Rainbow markers, Amersham), the bands correspond to 200, 97.4, 69, 46, 30, 21.5 and 14.3 kDa; lane 4, ^{125}I-uPA-ATF and lysate of 8701-BC cells; lane 5, same as lane 4 in the presence of 100 nM unlabelled uPA-ATF. The results show that 8701-BC cells express on their surface a receptor which is recognised by iodinated uPA-ATF, and that the crosslinking is specific. SDS-PAGE 12.5%.

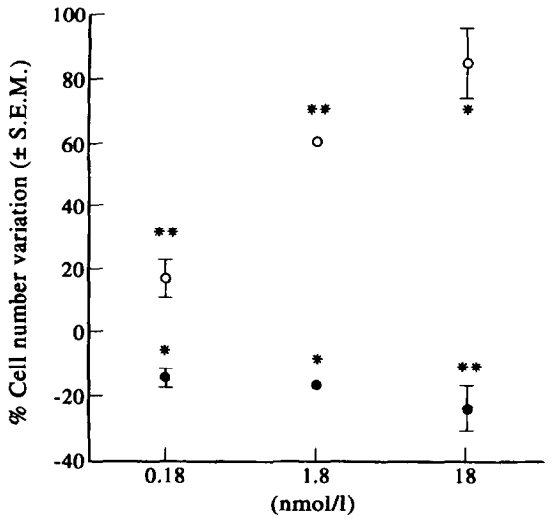

Figure 3. Growth response of 8701-BC cells to the exogenous addition of uPA (○) and uPA-ATF (●). The results indicate that, whilst uPA exerts a dose-dependent growth-promoting effect, equimolar amounts of its N-terminal fragment do not induce proliferation and even slightly restrain it. Five different sets of experiments were performed; vertical bars indicate the standard error of the mean (S.E.M.). *$P < 0.05$, **$P < 0.01$.

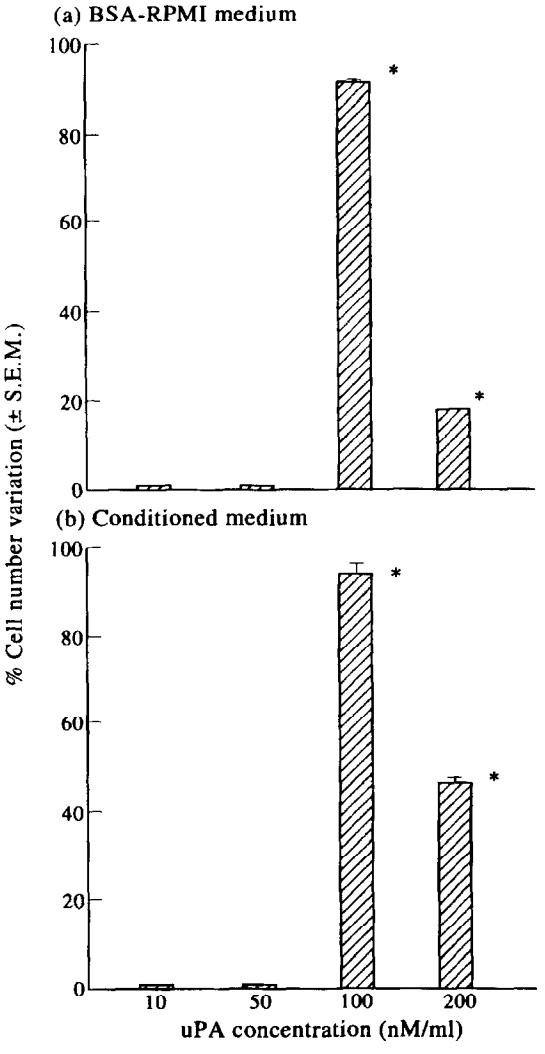

Figure 4. Chemoinvasion response of 8701-BC cells to uPA dissolved in (a) BSA-RPMI medium or (b) CM at a concentration of 10, 50, 100 and 200 ng/ml (corresponding approximately to 0.18, 0.9, 1.8 and 3.7 nM). Peak response was achieved with 100 ng/ml uPA in both media. Experiments made at least in triplicate; vertical bars indicate the standard error of the mean (S.E.M.). *$P < 0.01$.

of invasiveness within the general phenomenon of cell mobilisation [31, 32]. The R.I.I. pattern obtained confirmed, as reported in Table 1, that 100 ng uPA/ml exerted a peak chemoinvasive effect with R.I.I.s of 1.93 and 1.63 in the different media, whilst cell R.I.I. in the presence of uPA-ATF was consistently <1. Pre-absorption of uPA with 5B4 MAb before addition to the medium reversed the biological effect described. In contrast, pre-incubation of 8701-BC cells with TIMP-1 before chemoinvasion assays in the presence of 1.8 nM uPA did not reverse the effect, restraining cell invasion by only 7 ± 2% (average of three different experiments ± S.E.M.; not shown). Moreover, we found that uPA secreted in the CM by 8701-BC cells was able to exert an autocrine promotional effect, since its depletion by treating CM with excess 5B4 MAb resulted in a decrease of cell R.I.I. to 0.88 ± 0.02 (see Table 1).

DISCUSSION

In this paper, we examined whether uPA and its ATF could modulate the phenotype of D.I.C. cells by assaying the proliferation rate and the stimulation of invasion by 8701-BC cell line. This cell line represents an interesting system for the study of the biology of D.I.C., being directly derived from a primary site, and therefore "metastatic selection-exempt", and also retaining most of the characteristics observed in the original neoplasm *ex vivo* (e.g. a marked pleomorphism in cell ultrastructural microanatomy [25]).

The results indicate that 8701-BC cells can be included in the list of those cell lines which concurrently produce uPA and uPAR, thereby having the potential for an autocrine mechanism of uPA- and uPA-ATF-mediated stimulation [35–37]. The results also demonstrated that uPA exerts a specific promotional effect on growth and chemoinvasion of 8701-BC cells, reversible by blocking uPA–uPAR interactions with MAbs, and, more intriguingly, that uPA-ATF, while failing in imparting growth factor-like signals, as demonstrated with other cell types [15, 22], prominently reduces the invasion of the D.I.C. cell line under study through reconstituted basement membranes and slightly restrains cell growth. It is known that the interaction of both native uPA and uPA-ATF is transduced into biochemical events (formation of diacylglycerol [DAG], activation of protein kinase C [PKC]) coupled with chemotactic cell movement [19, 38]. It is also known that only native uPA stimulates cell multiplication [28, 37, 39, 40], although there is evidence to the contrary, at least in one cell line [20]. In terms of a second messenger, native uPA and uPA-ATF do not seem to undergo different transduction pathways [19, 28, 39]. Therefore, it was previously hypothesised that uPA is mitogenic by indirect activation of growth-promoting substrates containing the suitable consensus sequence for the enzyme [28, 39, 40]. It seems reasonable to deduce that the simple interaction between uPA or uPA-ATF and uPAR stimulates cell movement *in vitro*. It seems equally reasonable to infer that *in vivo* and in the chemoinvasion assay the integrity of the catalytic site is required to allow migration of chemotactically-stimulated cells between the biological bar-

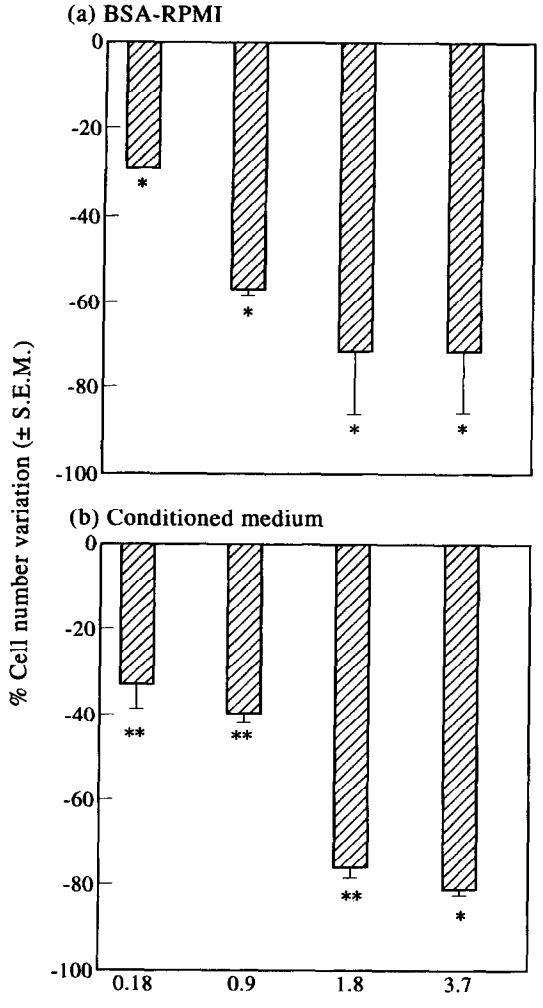

Figure 5. Chemoinvasion response of 8701-BC cells to uPA-ATF dissolved in (a) BSA-RPMI medium or (b) CM. Differently from the intact uPA molecule (see Figure 4), the N-terminal peptide plays a dose-dependent chemoinvasion-inhibiting role which reaches the plateau at 1.8 nM concentration. Experiment made in quadruplicate; vertical bars indicate the standard error of the mean (S.E.M.). *$P < 0.05$, **$P < 0.01$.

Table 1. Relative invasion index (R.I.I.) of 8701-BC cells in the presence of uPA, uPA-ATF and anti-uPA MAb

		No. of assays	R.I.I. (absolute value ± S.E.M.) BSA-RPMI	CM
uPA	(18 pM)	3	0.92 ± 0.02	1.00 ± 0.01
	(90 pM)	3	0.91 ± 0.02	1.00 ± 0.01
	(1.8 nM)	7	1.93 ± 0.06	1.63 ± 0.03
	(1.8 nM-p)*	3	0.90 ± 0.01	0.95 ± 0.01
	(3.7 nM)	7	1.19	1.20 ± 0.03
uPA-ATF	(18 pM)	4	0.85	0.93 ± 0.02
	(90 pM)	4	0.48 ± 0.01	0.85 ± 0.03
	(1.8 nM)	4	0.31 ± 0.01	0.38 ± 0.01
	(3.7 nM)	4	0.31 ± 0.01	0.34 ± 0.01
5B4 MAb	(1:100)	3	—	0.88 ± 0.02

*Pre-absorbed with 5B4 MAb.
S.E.M. <0.01 are not indicated.

riers. Overall, these considerations and the data suggest that in our system: (i) the inhibition of uPA-dependent growth and invasion by uPA-ATF receptor occupancy is not dependent on different signals, but on the competitive impairment of the autocrine loop that provides the receptor with enzymatic activity, able to induce cell multiplication and chemoinvasion of reconstituted basement membrane, and, (ii) uPA may both activate latent extracellular factors which could subsequently exert a mitogenic stimulus, and break the Matrigel barrier, possibly releasing biologically-active substances bound within the gel network [41]. Currently, we have no evidence on endogenous production of plasminogen by 8701-BC cells. Some investigators [5] have described the plasmin-independent action of uPA on various ECM proteins and enzymes, such as the activation of metalloproteinase 2 [14], which is both present in Matrigel preparations [42] and produced by 8701-BC cells (together with metalloproteinases-1, -9 and -10 [27]), but from our inhibition studies with TIMP-1, the contribution of enhanced gelatinase activation to uPA-mediated cell invasion promotion appears unlikely. Further investigation will be needed to clarify the molecular mechanisms underlying this uPA activity.

The biological significance of our result is related to the control of breast carcinoma cell invasion *in vivo* by uPA and its fragments. A number of experiments with animal models have shown that uPA-neutralisation with Abs is able to reduce the frequency and extent of metastasis of human carcinoma cells [e.g [43].]. In addition, Kobayashi and associates [44] have recently reported that human plasmin and leucocyte elastase are able to generate ATF from pro-uPA *in vitro*, and that saturation of ovarian cancer cell uPARs with elastase-derived uPA-ATF inhibits invasion dose-dependently. Our results provide further evidence of the "antimitogenic" and "anti-invasive" role of this uPA domain, by analysing its effect on another neoplastic cell line *in vitro*. Although care must be taken in extrapolating these observations to the *in vivo* situation, it can be hypothesised that a high content of plasmin and elastase around neoplastic cell foci in the affected breast stroma could result in cleavage of endogenously-produced uPA before it binds to the receptor. The ATF generated may thus actively intervene in cell–ECM network of interactions which parallel and underlie cancer progression, and invasion may be alternatively promoted or inhibited, depending on the final balance of enzyme secretion by the tumour, stromal and inflammatory cells and its proteolytic processing. Moreover, taking into consideration our results obtained *in vitro* and those reported by Kobayashi and colleagues [44] with two carcinoma lines of different origin, it appears worth considering the possibility of testing the effect of pharmacologically-active variants [45] of this uPA fragment *in vivo* for the therapy of these (and possibly other) human invasive solid tumours.

1. Danø K, Andreasen PA, Grondal-Hansen J, Kristensen PI, Nielsen LS, Skriver L. Plasminogen activator, tissue degradation and cancer. *Adv Cancer Res* 1985, **44**, 139–166.
2. Mignatti P, Rifkin DB. Biology and biochemistry of proteinases in tumor invasion. *Physiol Rev* 1993, **73**, 161–195.
3. Sappino AP, Busso N, Belin D, Vassalli JD. Increase of urokinase-type plasminogen activator gene expression in human lung and breast carcinomas. *Cancer Res* 1987, **47**, 4043–4046.
4. Ossowski L. In vivo invasion of modified chorioallantoic membrane by tumor cells: the role of surface-bound urokinase. *J Cell Biol* 1988, **107**, 2437–2445.
5. Saksela O, Rifkin DB. Cell-associated plasminogen activation:

regulation and physiological function. *Annu Rev Cell Biol* 1988, **4**, 92–126.
6. Yagel S, Ranjit SP, Jeffrey JJ, Lala PK. Normal nonmetastatic human trophoblast cells share in vitro invasive properties of malignant cells. *J Cell Physiol* 1988, **136**, 455–462.
7. Pereyra-Alfonso S, Solarz G, Bal De Kier Joffè E. Urokinase-type plasminogen activator activity increases during the growth of two murine mammary adenocarcinomas with different metastasizing abilities. *Clin Exp Metastasis* 1992, **10**, 395–401.
8. Schmitt M, Goretzki L, Janicke F, *et al.* Biological and clinical relevance of the urokinase-type plasminogen activator (uPA) in breast cancer. *Biomed Biochim Acta* 1991, **50**, 731–741.
9. Sumiyoshi K, Serizawa K, Urano T, Takada Y, Takada A, Baba S. Plasminogen activator system in human breast cancer. *Int J Cancer* 1992, **50**, 345–348.
10. Bouchet C, Spyratos F, Martin PM, Hacene K, Gentile A, Oglobine J. Prognostic value of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitors PAI-1 and PAI-2 in breast carcinomas. *Br J Cancer* 1994, **69**, 398–405.
11. Danø K, Behrendt N, Brünner N, Ellis V, Ploug M, Pyke C. The urokinase-receptor. Protein structure and role in plasminogen activation and cancer invasion. *Fibrinolysis* 1994, **8** (suppl. 1), 189–203.
12. Needham GK, Sherbet GV, Farndon JR, Harris AL. Binding of urokinase to specific receptors sites on human breast cancer membranes. *Br J Cancer* 1987, **55**, 13–16.
13. Blasi F. Urokinase and urokinase-receptor: a paracrine-autocrine system regulating cell migration and invasiveness. *BioEssays* 1993, **15**, 105–111.
14. Keski-Oja J, Lohi J, Tuuttila A, Tryggvason K, Vartio T. Proteolytic processing of the 72,000-Da type IV collagenase by urokinase plasminogen activator. *Exp Cell Res* 1992, **202**, 471–476.
15. Gudewicz PW, Gilboa N. Human urokinase-type plasminogen activator stimulates chemotaxis of human neutrophils. *Biochem Biophys Res Commun* 1987, **147**, 1176–1181.
16. Fibbi G, Ziche M, Morbidelli L, Magnelli L, Del Rosso M. Interaction of urokinase with specific receptors stimulates mobilization of bovine adrenal capillary endothelial cells. *Exp Cell Res* 1988, **179**, 385–395.
17. Del Rosso M, Fibbi G, Dini G, *et al.* Role of specific membrane receptors in urokinase-dependent migration of human keratinocytes. *J Invest Dermatol* 1990, **94**, 310–316.
18. Del Rosso M, Pedersen N, Fibbi G, *et al.* Selective localization of receptors for urokinase amino-terminal fragments at substratum contact sites of an *in vitro* established line of human epidermal cells. *Exp Cell Res* 1992, **203**, 427–434.
19. Del Rosso M, Anichini E, Pedersen N, *et al.* Urokinase–urokinase receptor interaction: non-mitogenic signal transduction in human epidermal cells. *Biochem Biophys Res Commun* 1993, **190**, 347–352.
20. Rabbani SA, Desjardins J, Bell AW, *et al.* An amino-terminal fragment of urokinase isolated from a prostate cancer cell line (PC-3) is mitogenic for osteoblast-like cells. *Biochem Biophys Res Commun* 1990, **173**, 1058–1064.
21. Rabbani SA, Mazar AP, Bernier SM, *et al.* Structural requirements for the growth factor activity of the amino-terminal domain of urokinase. *J Biol Chem* 1992, **267**, 14151–14156.
22. Odekon LE, Sato Y, Rifkin DB. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. *J Cell Physiol* 1992, **150**, 258–263.
23. Luparello C, Ginty AF, Gallagher JA, Pucci-Minafra I, Minafra S. Transforming growth factor $\beta 1$, $\beta 2$, $\beta 3$, urokinase and parathyroid hormone-related peptide expression by 8701-BC cells and clones. *Differentiation* 1993, **55**, 73–80.
24. Minafra S, Morello V, Glorioso F, *et al.* A new cell line (8701-BC) from primary ductal infiltrating carcinoma of the human breast. *Br J Cancer* 1989, **60**, 185–192.
25. Pucci-Minafra I, Minafra S, Alessandro R, Faccini AM. An ultrastructural evaluation of cell heterogeneity in invasive ductal carcinomas of the human breast. I. An *in vitro* study. *J Submicrosc Cytol Pathol* 1989, **21**, 488–499.
26. Roldan AL, Cubellis MV, Masucci MT, *et al.* Cloning and expression of the receptor for urokinase plasminogen activator, a central molecule in cell surface. *EMBO J* 1990, **9**, 467–474.
27. Alessandro R, Minafra S, Pucci-Minafra I, *et al.* Metalloproteinase and TIMP expression by the human breast carcinoma cell line 8701-BC. *Int J Cancer* 1993, **55**, 250–255.
28. Anichini E, Fibbi G, Pucci M, Caldini M, Chevanne M, Del Rosso M. Production of second messenger following chemotactic and mitogenic urokinase-receptor interaction in human fibroblasts and mouse fibroblasts transfected with human urokinase receptor. *Exp Cell Res* 1994, **213**, 438–448.
29. Ronne E, Behrendt N, Ellis V, *et al.* Cell-induced potentiation of the plasminogen activation system is abolished by a monoclonal antibody that recognizes the NH_2-terminal domain of the urokinase receptor. *FEBS Lett* 1991, **288**, 233–236.
30. Albini A, Iwamoto Y, Kleinman K, *et al.* A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* 1987, **47**, 3239–3245.
31. Pucci-Minafra I, Luparello C, Aquino A, *et al.* OF/LB collagen promotes chemoinvasion of breast cancer cells and directs epithelial cell migration into granulation tissue of experimental dermal wounds. *Int J Oncol* 1995, **6**, 1015–1020.
32. Luparello C, Burtis WJ, Raue F, Birch M, Gallagher JA. Parathyroid hormone-related peptide and 8701-BC cell growth and invasion *in vitro*. *Mol Cell Endocrinol* 1995, **111**, 225–232.
33. Kleiner DE Jr, Stetler-Stevenson WG. Structural biochemistry and activation of matrix metalloproteinases. *Curr Opin Cell Biol* 1993, **5**, 891–897.
34. Behrendt N, Ronne E, Ploug M, *et al.* The human receptor for urokinase plasminogen activator. NH_2-terminal aminoacid sequence and glycosilation variants. *J Biol Chem* 1990, **265**, 6453–6460.
35. Stoppelli MP, Corti A, Soffientini A, Cassani G, Glasi F, Assoian RK. Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. *Proc Natl Acad Sci USA* 1985, **8**, 4939–4943.
36. Stoppelli MP, Tacchetti C, Cubellis MV, *et al.* Autocrine saturation of pro-urokinase receptors on human A431 cells. *Cell* 1986, **45**, 675–684.
37. Kircheier JC, Wojta J, Christ G, Binder BR. Functional inhibition of endogenously-produced urokinase decreases cell proliferation in a human melanoma cell line. *Proc Natl Acad Sci USA* 1989, **86**, 5424–5428.
38. Busso N, Masur Sk, Lazega D, Waxman S, Ossowski L. Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. *J Cell Biol* 1994, **126**, 259–270.
39. De Petro G, Copeta A, Barlati S. Urokinase-type and tissue-type plasminogen activators as growth factors of human fibroblasts. *Exp Cell Res* 1994, **213**, 286–294.
40. He CJ, Rebibou JM, Peraldi MN, Meulders Q, Rondeau E. Growth factor-like effect of urokinase-type plasminogen activator in human renal cells. *Biochem Biophys Res Commun* 1991, **176**, 1408–1416.
41. Vukicevic S, Kleinman HK, Luyten FP, Roberts AB, Roche NS, Reddi AH. Identification of multiple active growth factors in basement membrane matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. *Exp Cell Res* 1992, **202**, 1–8.
42. MacKay AR, Gomez DE, Cottam DW, Rees RC, Nason AM, Thorgeirsson P. Identification of the 72-kDa (MMP-2) and 92 kDa (MMP-9) gelatinase/type IV collagenase in preparations of laminin and matrigel. *BioTechniques* 1993, **15**, 1048–1051.
43. Ossowski L, Reich E. Antibodies to plasminogen activator inhibit tumor metastasis. *Cell* 1983, **35**, 611–619.
44. Kobayashi H, Ohi H, Shinohara H, *et al.* Saturation of tumor cell surface receptors for urokinase-type plasminogen activator by amino-terminal fragment and subsequent effect on reconstituted basement membrane invasion. *Br J Cancer* 1993, **67**, 537–544.
45. Lu H, Yeh P, Guitton JD, *et al.* Blockage of the urokinase receptor on the cell surface: construction and characterization of a hybrid protein consisting of the N-terminal fragment of human urokinase and human albumin. *FEBS Lett* 1994, **356**, 56–59.

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