Report

Osteopontin(OPN)-induced increase in human mammary epithelial cell invasiveness is urokinase (uPA)-dependent

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Summary

We have recently shown that either exogenous or endogenous, transfected OPN induces both uPA expression and increased invasiveness of 21PT (non-tumorigenic) and 21NT (tumorigenic) human mammary epithelial cells. Here we asked whether uPA contributes functionally to the increased invasiveness of these cells. The most invasive OPN-transfected cells were assessed for migration through Matrigel in transwell assays, in the presence or absence of various blocking antibodies and uPA inhibitors. Antibodies to both uPA and uPA receptor (uPAR) were shown to significantly inhibit cell invasion, as did the uPA inhibitors (plasminogen activator inhibitor-1 [PAI-1], p-aminobenzamidine [PABN], aprotinin, and amiloride). Both anti-uPA and anti-uPAR antibodies inhibited invasion to a level comparable to that of the control vector transfected cells. In contrast, non-specific IgG showed no anti-invasive effect. Cell migration experiments performed with the parental cell lines in the presence or absence of anti-uPA or anti-uPAR antibodies showed that uPA is also required for migratory responsiveness to exogenous OPN. These data thus provide direct evidence that OPN-induced invasion and migration of these cells requires uPA.

Introduction

Osteopontin (OPN) is a secreted, integrin-binding glycoprophosphoprotein which has been associated with malignancy of breast cancer and other tumors. Our clinical studies have shown an association between level of OPN in the primary tumor [1] or blood [2] and outcome in patients with breast cancer. In cell culture experiments, we have recently shown that either exogenous, or endogenous, transfected OPN induces both urokinase-type plasminogen activator (uPA) expression and increased invasiveness of 21PT (non-tumorigenic) and 21NT (tumorigenic) human mammary epithelial cells [3].

uPA is a serine protease, which when bound to its cell surface receptor, urokinase-type plasminogen activator receptor (uPAR), is capable of activating a variety of other proteases (either directly or indirectly by activating plasminogen) (e.g., pro-MMP-1, -2, -3, -9, -14) [4–6]. This, in turn, results in digestion of various components of the ECM (reviewed in [6]), as

well as activation of certain growth factors (reviewed in [6, 7]). uPA expression and activity has been associated with invasiveness of a variety of cancer cell lines, and clinically with poor prognosis of patients with breast cancer and other malignancies (reviewed in [7]).

Here we have asked whether uPA contributes functionally to the invasiveness of mammary epithelial cells transfected to overexpress OPN. OPN-transfected 21T series cells were tested in invasion assays performed in the presence or absence of either blocking antibodies (to uPA or uPAR), or natural or chemical inhibitors of urokinase, in order to determine whether uPA/uPAR is necessary for their invasiveness. We found that anti-uPA and uPAR antibodies and uPA inhibitors all effectively blocked invasiveness of the OPN transfectants. As others have shown that integrin-mediated cell migration of different mammary epithelial cell lines may be uPA dependent [8, 9], we also examined the effect of anti-uPA and uPAR antibodies on responsiveness of the parental (non-

transfected) 21PT and 21NT cell lines to exogenous OPN in cell migration assays. We found that both antibodies inhibited OPN-induced cell migration as well, indicating that a component of the uPA-dependency of OPN-induced cell invasion in these cells is due to effect on cell migration.

Materials and methods

Cell lines and culture

The 21T series cell lines (21PT, 21NT) were obtained as a kind gift of Dr. Vimla Band (Dana Farber Cancer Institute) [10]. Osteopontin and control vector (pcDNA3) transfectants of these cell lines were as described previously [3]. Transfectant cell lines are defined as follows: PT/Ci and NT/Ci: pooled populations of 21PT (non-tumorigenic) and 21NT (tumorigenic, non-metastatic) cells transfected with the neo-containing control vector (pcDNA3) only, PT/OPaiiPool and PT/OPaiiC12: pooled and cloned population (respectively) of OPN-transfected 21PT cells expressing the highest levels of OPN, NT/OPbiPool and NT/OPaiiC4: pooled and cloned population (respectively) of OPN-transfected 21NT cells expressing the highest levels of OPN [3]. The transfectant cell lines were maintained in culture in α -MEM supplemented with 10% FCS, 2 mM L-glutamine (all from GIBCO-BRL/Life Technologies, Grand Island, NY), insulin (1 μ g/ml), epidermal growth factor [EGF] (12.5 ng/ml), hydrocortisone (2.8 μ M), 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μ g/ml gentamycin (all from Sigma Chemical, St. Louis, MO) (α HE medium), and G418 (200 μ g/ml [active], GIBCO-BRL/Life Technologies).

RNA isolation and northern blot analysis

Cell pellets from subconfluent monolayers were mechanically homogenized (Polytron PT 1200, Brinkman Instruments [Canada] Ltd., Mississauga, ON) and RNA extracted using TRIzol Reagent (Canadian Life Technologies Inc., Burlington, ON), according to the protocol supplied by the manufacturer. RNA (10 μ g/lane) was run on a 1.1% agarose gel with 6.8% formaldehyde, and capillary-transferred to GeneScreen Plus filters (DuPont Canada Inc., Mississauga, ON). Blots were probed either with denatured, oligolabeled [32 P]-dCTP cDNA probes (labeled using a kit provided by Pharmacia, Baie d'Urfe, PQ),

or with 5' [γ - 32 P]-ATP end-labeled oligomers (labeled using a kit provided by Oncogene Science, Manhasset NY), according to the procedures provided by the manufacturers, and as previously described [11, 12].

The OPN probe used was the full-length (1493 bp) human OPN cDNA *Eco*RI cassette of plasmid OP-10 [13]. The probe for human urokinase-type plasminogen activator was a 40mer antisense oligonucleotide derived from the translated sequences of exon 4 (Calbiochem/Cederlane Laboratories, Hornby, Ontario, Cat#ON333) [14]. The urokinase-type plasminogen activator receptor probe was a 45mer antisense oligonucleotide to the first 15 amino acids (not including the signal peptide) [15]. Even loading of lanes was confirmed by probing blots with a human 18S rRNA probe (p100D9, a kind gift from Dr. D.T. Denhardt).

Enzymogram analysis of uPA in conditioned medium

Conditioned media were prepared by plating cells at 5×10^5 cells/100 mm plate in regular growth medium and incubating overnight (18 h) at 37°C, 5% CO₂. Medium was then removed, and plates were washed X1 with warm, sterile PBS, and X2 with serum-free OPTI-MEM (GIBCO-BRL/Life Technologies). Serum-free OPTI-MEM was then added at 3 mls/100 mm plate, and plates were incubated for 48 h at 37°C, 5% CO₂. Following the incubation period, the conditioned medium from each plate was collected, and the cell debris spun out. The corresponding plate was trypsinized, and a cell count performed, to allow appropriate correction in loading for cell equivalents.

Aliquots of conditioned medium were adjusted for cell number and diluted 1/50 for enzymogram analysis. The samples were separated by 11% SDS-PAGE, the gel impregnated with 0.1% casein and 10 μ g/ml plasminogen as previously described [16]. The gel was then incubated in 2.5% Triton-X 100 for 2 h, followed by incubation in 50 mM Tris (pH 8.3) and 0.1 M glycine for 18 h at 37°C. The gel was fixed in 15% acetic acid and stained with 0.25% Coomassie blue. Following destaining, plasminogen-dependent proteolysis was detected as a white band on a dark background.

Cell invasion assay

In vitro invasiveness through Matrigel was assayed as described previously [12], using 24-well transwell

chambers with polycarbonate filters of 8 μm pore size (Costar, Cambridge, MA), coated with 35 μg Matrigel (Collaborative Research Inc., Bedford, MA) containing 50 $\mu\text{g}/\text{ml}$ plasminogen (Sigma). The Matrigel concentration was determined by preliminary experiments using representative OPN-transfected 21T series cell lines. Matrigel was diluted to the desired final concentration with cold, sterile, distilled water, applied to the filters, dried overnight in a tissue culture hood, and reconstituted the following morning with serum-free αHE medium. Cells for the assay were trypsinized and seeded to the upper chamber at 5×10^4 cells per well in serum-free αHE medium containing 0.1% BSA, with or without the specified antibody or inhibitor. Studies examining the effects of blocking antibodies used 200 $\mu\text{g}/\text{ml}$ mouse anti-human uPA (American Diagnostica, Montreal, Quebec, Canada), 200 $\mu\text{g}/\text{ml}$ mouse anti-human uPAR (American Diagnostica), or 200 $\mu\text{g}/\text{ml}$ non-specific mouse IgG (Cedarlane). Studies examining the effects of uPA inhibitors used 50 $\mu\text{g}/\text{ml}$ human recombinant plasminogen activator inhibitor-1 (PAI-1) (American Diagnostica), 2 mM p-amino-benzamidine (Sigma), 200 $\mu\text{g}/\text{ml}$ aprotinin (Sigma), or 200 μM amiloride (Sigma). The concentration of inhibitor used was determined by preliminary experiments to achieve maximum effect with minimal toxicity ($>90\%$ cell survival in viability assay). The lower chamber was filled with serum-free culture medium with 0.1% BSA and 10 $\mu\text{g}/\text{ml}$ fibronectin. Plates were incubated for 72 h in a 5% CO_2 incubator at 37°C . Following incubation, the upper wells were removed, fixed with 1% glutaraldehyde in phosphate-buffered saline, stained with hematoxylin, dipped briefly in 1% ammonium hydroxide, and washed with water. The cells and Matrigel were then wiped off the upper surface of each filter with a cotton swab. After air-drying, cells from various areas of the lower surface of the filters were counted under $\times 100$ magnification.

Cell migration assay

Cell migration assays were performed essentially as described previously [17], using 24-well transwell chambers with polycarbonate filters of 8 μm pore size (Costar). Gelatin (Sigma) was applied at 6 $\mu\text{g}/\text{filter}$ and air dried. The gelatin was rehydrated with 100 μl of serum-free αHE medium at room temperature for 90 min. Lower wells contained 600 μl of αHE plus 0.1% BSA, with or without human recombinant OPN (50 $\mu\text{g}/\text{ml}$) [18]. Previous control experiments have

shown that the GST portion alone has no influence on migration of these cells. Cells (5×10^4) were added to each upper well in αHE medium with 0.1% BSA and incubated for 5 h at 37°C . At the end of the incubation time, the cells that had migrated to the undersurface of the filters were fixed in place with glutaraldehyde and stained with hematoxylin. Cells that had not migrated and were attached to the upper surface of the filters were removed from the filters with a cotton swab. The lower surfaces of the filters were examined microscopically under $100\times$ magnification and representative areas were counted to determine the number of cells that had migrated through the filters. For experiments with blocking antibodies, cells were pre-incubated for 20 min at room temperature with medium containing either 200 $\mu\text{g}/\text{ml}$ mouse anti-human uPA (American Diagnostica) or 200 $\mu\text{g}/\text{ml}$ anti-uPAR (American Diagnostica).

All cell migration and invasion assays were performed in triplicate. Statistical differences between groups were assessed using Student's *t*-test, with SigmaStat (Jandel Scientific, San Rafael, CA) statistical software.

Results

Relative levels of OPN mRNA and protein (as determined by northern and western analysis respectively) expressed by OPN versus control vector transfectants of 21PT and 21NT cells are shown in Table 1. Both OPN transfected 21PT (PT/OPaiiPool, PT/OPaiiC12) and 21NT (NT/OPbiPool, NT/OPaiiC4) cells showed increased invasiveness through Matrigel compared to controls (PT/Ci, NT/Ci), in proportion to their respective level of OPN expression (Table 1). Northern analysis (Figure 1(a)) further indicated that although uPAR mRNA levels did not show significant differences between the cell lines, uPA mRNA expression was closely related to the level of OPN expression, and cellular invasiveness (cf Table 1). Similarly, enzymogram analysis of conditioned media showed the same rank order of the cells, with the highest uPA activity detected in the highest OPN-expressing cells (Figure 1(b), Table 1). Hence, OPN expression was found to be tightly correlated with cellular invasiveness and uPA expression and activity.

We then examined the uPA-dependency of OPN-induced cell invasiveness, focusing initially on the 21NT-derived OPN-transfected cell lines. Invasion through Matrigel of both NT/OPbiPool and

Table 1. Osteopontin expression, urokinase activity and cellular invasion through Matrigel (35 µg) in transwell assay for control (Ci) and osteopontin (OP) transfected 21PT (PT) and 21NT (NT) cells

Cell line*	Relative level of OPN mRNA by northern analysis	Relative level of OPN protein by western analysis of conditioned media	uPA activity of conditioned media by densitometry of enzymogram	Invasion through Matrigel (cells/hpf +/- S.E)
PT/Ci	1.0	1.0	1.9 +/- 0.09	5.42 +/- 1.34
PT/OPaii Pool	10.9	5.3	2.9 +/- 0.07	38.25 +/- 4.63
PT/OPaiiC12	35.5	10.1	3.7 +/- 0.08	56.15 +/- 4.63
NT/Ci	3.5	1.1	2.2 +/- 0.01	14.83 +/- 0.79
NT/OPbi Pool	28.4	4.6	3.6 +/- 0.03	44.80 +/- 1.83
NT/OPaiiC4	34.9	8.0	4.7 +/- 0.16	63.67 +/- 1.86

*Key to cell lines: PT/Ci: pooled population of 21PT cells transfected with the control vector (pcDNA3) only; PT/OPaiiPool: pooled population of 21PT cells transfected with the OPN-expression vector (pool expressing highest level of OPN protein); PT/OPaiiC12: clone 12 of OPN-transfected 21PT pool aii (clone expressing highest level of OPN protein); NT/Ci: pooled population of 21NT cells transfected with the control vector (pcDNA3) only; NT/OPbiPool: pooled population of 21NT cells transfected with the OPN-expression vector (pool expressing highest level of OPN protein); NT/OPaiiC4: clone 4 of OPN-transfected 21NT pool aii (clone expressing highest level of OPN protein). All cells as obtained in reference [3].

NT/OPaiiC4 cells was markedly inhibited (to control NT/Ci transfectant levels) by both anti-uPA and anti-uPAR antibodies, as well as by the natural plasminogen activator inhibitor, PAI-1 (Figure 2). In contrast, no blocking effect was seen with the non-specific IgG (Figure 2). Incubation of the cells in the presence of several chemical inhibitors of uPA activity (p-amino-benzamidine, amiloride, aprotinin) also showed significant inhibition of both OPN-transfected 21NT cell lines (Figure 3). In the case of p-amino-benzamidine and amiloride, inhibition of cell migration was seen to levels below that of the control transfectant (NT/Ci) cell line, whereas that achieved with aprotinin was to levels approximating that of the control. The OPN-transfected 21PT cell lines (PT/OPaiiPool and PT/OPaiiC12) were also tested, and showed the same pattern of inhibition by anti-uPA and anti-uPAR antibodies, by PAI-1, and by the chemical inhibitors p-amino-benzamidine, amiloride and aprotinin (data not shown).

In order to assess whether cell migration responsiveness to uPA/uPAR is a component of cell invasiveness induced by OPN, we also examined the ability of anti-uPA and anti-uPAR antibody to block OPN-induced cell migration of the parental (non-transfected) 21PT and 21NT cell lines (Figure 4). Both cell lines showed significant inhibition (to levels around or below baseline) of OPN-induced cell migration with either anti-uPA or anti-uPAR antibody treatment.

Discussion

Several clinical studies have indicated a worse prognosis of breast cancer in patients showing elevated expression of either OPN [1, 2] or uPA (review in [7]). One recent study provides evidence that breast cancers metastatic to bone have a tendency to over-express both OPN and uPA, raising the possibility that these proteins interact to contribute to metastasis [19]. Work by ourselves and others have shown that high level expression of OPN by mammary epithelial cells is associated with increased malignancy in terms of cell migration [3, 17, 20–22], invasion [3, 23] and metastatic ability [24, 25]. Recently, we have further shown that a component of the cellular response to OPN is upregulation of uPA expression [3]. In the present study, we have examined specifically whether the uPA/uPAR axis is functionally required for the OPN-induced increase in cellular invasiveness of 21T series mammary epithelial cells. Through antibody blocking and inhibitor experiments, we have shown that OPN-induced invasiveness of these cells is indeed dependent upon uPA/uPAR. In parallel studies with MDA-MB-435 cells, which show an inherent high level expression of OPN, we have seen the same inhibition of cell invasiveness with blocking anti-uPA and anti-uPAR antibodies and uPA inhibitors (Tuck and Chambers, unpublished observations). In addition, using 21PT and 21NT cells, we have found that a component of the uPA/uPAR dependency involves

Figure 1. uPA and uPAR mRNA expression (a) and uPA protease activity (b) of control and OPN-transfectant cell lines. Vector-only transfected controls of 21PT (PT/Ci) and 21NT (NT/Ci) cells are compared with the OPN-transfected pool and clone of 21PT (PT/OPaiiPool; PT/OPaiiC12) and 21NT (NT/OPbiPool; NT/OPaiiC4) cells that expressed the highest levels of OPN. (a) Northern analysis for expression of uPA and uPAR mRNA. Probes were: human urokinase-type plasminogen activator (uPA); urokinase-type plasminogen activator receptor (uPAR); and 18S ribosomal RNA (18S rRNA). (b) Enzymogram assay for uPA protease activity in conditioned media. Enzymogram analysis of conditioned media was performed as described in Materials and methods. Plasminogen-dependent proteolysis is seen as a white band on a dark background. The band is of the expected MW of uPA (55 kDa). Below each band is shown the relative intensity, as determined by densitometry, plus or minus standard deviation on triplicate measurements. All OPN-transfected cell lines were found to exhibit increased amounts of uPA mRNA and enzyme activity relative to the vector-only transfected controls.

cell migration, as blocking antibodies show significant inhibitory effect even in the absence of a basement membrane barrier.

OPN has been shown to bind to the cell surface via a number of different receptors, both integrin [17, 18, 21, 26–30], and non-integrin (i.e., CD44 [31–33]). In 21T mammary epithelial cells, we have found integrins $\alpha v \beta 5$ and $\beta 1$ of particular importance in the cell migration response to OPN [21]. The observation here that this cell migration is also dependent upon uPA/uPAR, suggests the possibility of interaction between activated cell surface integrins and uPA/uPAR for induction of migration by OPN. Consistent with these findings, a collaborative interaction between vitronectin-activated $\alpha v \beta 5$ integrin and uPA-bound uPAR has been reported in induction of migration in other systems [8, 9].

Figure 2. Anti-uPA, anti-uPAR and PAI-1 blocking of NT/OPbiPool (a) and NT/OPaii C4 cell (b) chemoinvasiveness, in comparison with basal level invasiveness of control vector transfectant (NT/Ci). Invasion was assessed in transwells in response to 10 μ g/ml fibronectin in the lower chamber, through 8 μ m pore filters precoated with 35 μ g Matrigel, as described in Materials and methods. 5×10^4 cells/well were seeded to the upper chamber in α HE medium + 0.1% BSA with: 0 = no antibody; nlgG = 200 μ g/ml nonimmune mouse IgG; auPA = 200 μ g/ml mouse anti-human uPA antibody; auPAR = 200 μ g/ml mouse anti-human uPAR antibody; PAI-1 = 60 μ g/ml human recombinant plasminogen activator inhibitor-1. Bars represent the mean of counts from each of three separate wells, error bars are SEM. Anti-uPA and anti-uPAR antibodies showed marked inhibition of invasiveness of both NT/OPbi Pool and NT/OPaii C4 cells ($p = 0.001$ for anti-uPA and anti-uPAR antibody inhibition of NT/OPbiPool; $p = 0.0019$ for anti-uPA and anti-uPAR antibody inhibition of NT/OPaii C4 by Student's *t*-test), whereas no significant inhibition was seen with nonimmune mouse IgG. Significant inhibition of invasiveness of both NT/OPbiPool and NT/OPaii C4 cells was also seen with PAI-1 ($p < 0.001$ by Student's *t*-test).

An influence on cell migration thus may be one component of the uPA effect on cell invasiveness induced by OPN. In addition, the uPA/uPAR axis is known to induce a cascade of protease activation

Figure 3. Inhibition of NT/OPbiPool (a) and NT/OPaii C4 cell (b) invasiveness by chemical inhibitors of uPA, in comparison with basal level invasiveness of control vector transfectant (NT/Ci). Transwell invasion assays were performed as described in Materials and methods and Figure 2, with experimental conditions as follows: 0 = no inhibitor; P-aminobenz = 2 mM p-amino-benzamidine; amiloride = 200 μM amiloride; aprotinin = 200 μg/ml aprotinin. Bars represent the mean of counts from each of three separate wells, error bars are SEM. Significant inhibition of cellular invasiveness was seen with all four inhibitors ($p < 0.001$ for all inhibitors, both cell lines, by Student's *t*-test).

Figure 4. Anti-uPA and anti-uPAR antibody blocking of parental 21PT and 21NT cell migration response to exogenous (human recombinant) OPN. Cell migration assays were performed in transwells, as described in Materials and methods, using 50 μg/ml human recombinant OPN as the chemoattractant. Experimental conditions were as follows: PT/0 = 21PT cells with no OPN in lower chamber, no inhibitory antibody; PT/OP = 21PT cells with OPN in lower chamber, no inhibitory antibody; PT/OP + auPA = 21PT cells with OPN in lower chamber, incubated with 200 μg/ml mouse anti-human uPA; PT/OP + auPAR = 21PT cells with OPN in lower chamber, incubated with 200 μg/ml mouse anti-human uPAR; NT/0 = 21NT cells with no OPN in lower chamber, no inhibitory antibody; NT/OP = 21NT cells with OPN in lower chamber, no inhibitory antibody; NT/OP + auPA = 21NT cells with OPN in lower chamber, incubated with 200 μg/ml mouse anti-human uPA; NT/OP + auPAR = 21NT cells with OPN in lower chamber, incubated with 200 μg/ml mouse anti-human uPAR. Bars represent the mean of counts from each of three separate wells, error bars are SEM. Anti-uPA and anti-uPAR antibodies showed marked inhibition of both 21PT and 21NT cell migration ($p < 0.001$ for both antibodies, with both cell lines, by Student's *t*-test).

events which may either directly (by creating spaces through which invading cells may move) or indirectly (by modifying the nature of interactions with extracellular matrix components or growth factors) enhance invasion [4–7]. Thus, in activating uPA, OPN may not only start up the engine needed to drive cell movement, but also the system needed to create the spaces through which to move, and appropriately modify the environment to promote invasion.

The mechanism by which OPN binding to the cell surface results in activation of uPA expression and activity is at present unknown. However, one can postulate that this process may involve the activation of the hepatocyte growth factor (HGF) receptor, Met. Numerous studies with a number of different cell types

have found previously that HGF treatment of cells activates expression of uPA [34–36], which in turn can activate HGF itself [37]. In 21T series cells, we have found that incubation with OPN results in increased expression and tyrosine kinase activity of Met [21], thus providing a potential pathway for uPA activation. If HGF/Met is indeed involved in uPA activation induced by OPN, this may offer a potential therapeutic target to block the effects of OPN in malignancy, in that potent inhibitors of the HGF/Met/uPA system have recently been identified [38].

In conclusion, the finding that cellular invasiveness and migration induced by OPN are dependent upon activation of uPA, provides functional evidence linking a number of studies to date which indicate the significance of both OPN and uPA in clinical and *in vitro* aggressiveness of breast cancer. Furthermore, the fact that uPA activation is functionally required for OPN-induced malignancy also has implications in choice of potential therapeutic targets, as effective strategies are in development for inhibiting pathways specifically involving uPA. Use of these uPA pathway inhibitors thus may be one effective approach to interfering with the malignancy-promoting effects of OPN.

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