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# Cell migration promoted by a potent GRGDS-containing thrombin-cleavage fragment of osteopontin

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**Abstract**

Osteopontin (OPN) is a secreted adhesive glycoprotein with a gly-arg-gly-asp-ser (GRGDS) cell binding domain. Several independent studies have suggested that OPN functions in tumor growth and metastasis, and one likely possibility is that OPN facilitates tumor invasion by promoting tumor cell migration. Consistent with this hypothesis, immobilized OPN promoted concentration-dependent tumor cell migration (i.e., haptotaxis) in modified Boyden chambers. In particular, cleavage of OPN by thrombin, which likely occurs in the tumor microenvironment, resulted in enhancement of OPNs haptotactic activity; and assays performed with purified preparations of the two individual OPN thrombin-cleavage fragments demonstrated that all detectable activity was associated with the GRGDS-containing fragment. In contrast to the activity of both OPN and its GRGDS-containing fragment in promoting haptotaxis, neither of these proteins in solution promoted chemotaxis, indicating that each must be immobilized to promote cell migration. In haptotaxis assays, antibody LM609 to integrin $\alpha_v\beta_3$ blocked > 80% cell migration towards the GRGDS-containing OPN fragment, implicating $\alpha_v\beta_3$ as its principal functional receptor. In comparison with equimolar quantities of other adhesive proteins, the GRGDS-containing OPN thrombin-cleavage fragment was not only > 2-fold more effective than intact OPN at promoting haptotaxis, but also > 8-fold and > 6-fold more effective than fibrinogen and vitronectin, respectively, indicating that this OPN fragment is highly active relative to other $\alpha_v\beta_3$ ligands.

*Keywords:* Haptotaxis; Chemotaxis; $\alpha_v\beta_3$ Integrin; Fibrinogen; Vitronectin

## 1. Introduction

Tumor cell migration leads to tumor invasion and ultimately to the development of tumor metastases. Because cell migration is dependent on adhesive interactions with the extracellular matrix, matrix components at the tumor/host interface could be important determinants of the rate of tumor cell migration into adjacent host tissue. In particular, ligands for the cell surface integrin $\alpha_v\beta_3$ may be especially significant in this process. For example, increased expression of $\alpha_v\beta_3$ correlates with melanoma vertical growth phase and metastasis [1–3] and with aggressive glioblastoma [4]. Moreover, melanoma cells selected for low expression of $\alpha_v\beta_3$ were less tumorigenic than controls; and when $\alpha_v\beta_3$ expression was restored by stable transfection, tumorigenicity increased [5].

Abbreviations: BSA, bovine serum albumin; OPN, osteopontin.

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One $\alpha_v\beta_3$ ligand which participates in adhesive interactions with tumor cells at the tumor/host interface is osteopontin (OPN), a secreted glycoprotein with a glycine-arginine-glycine-aspartate-serine (GRGDS) cell binding domain ([6]; reviewed in Ref. [7]). OPN is substantially overexpressed in a variety of experimental models of malignancy, and OPN expression correlates with the metastatic potential of transformed mouse cell lines [8–10]. Moreover, tumor cells transfected for expression of OPN antisense RNA exhibit a reduced ability to form tumors in vivo [11,12]. Analyses of human cancers have indicated that OPN is also highly overexpressed in a large variety of carcinomas, principally by host macrophages at the invasive edge of carcinomas arising in the breast, lung and gastrointestinal tract, and by tumor cells and macrophages in carcinomas of the kidney and endometrium [13]. Consistent with overexpression of OPN in tumors, the concentrations of OPN in the blood plasma of patients with metastatic cancer are substantially elevated in comparison with the low levels that normally circulate [14].

OPN promotes attachment and spreading of a variety of cell types including tumor cells [15–17] primarily through interactions with $\alpha_v\beta_3$ [18,19]; however, interactions with $\alpha_v\beta_1$ and $\alpha_v\beta_5$ also contribute [20,21]. Although the native form of OPN is active in cell attachment assays, thrombin-cleavage of OPN results in substantial enhancement of its adhesive activity [19]. This cleavage occurs within six amino acid residues of the GRGDS sequence [9,22], raising the interesting possibility that thrombin-cleavage further activates OPN by allowing greater accessibility of the GRGDS domain to cell surface receptors. Moreover, thrombin cleaves the OPN present in blood plasma during the natural course of blood coagulation, suggesting that thrombin-cleavage of OPN occurs in vivo wherever OPN is present and the blood coagulation pathway is activated [14]. In particular, such cleavage likely occurs in tumors where the presence of active thrombin is indicated by the deposition of cross-linked fibrin (reviewed in Refs. [23,24]).

Thus the collective evidence indicates that OPN is an important element of tumor extracellular matrix and that this protein functions in cell adhesion at the tumor/host interface where it could influence tumor invasion. Moreover, enhancement of OPNs adhesive properties by thrombin-cleavage suggested that thrombin may be important in regulating OPN function in cell migration. Therefore, to investigate OPN activity in promoting tumor cell migration and its regulation by thrombin-cleavage, we tested OPN, unfractionated thrombin-cleaved OPN, and purified preparations of the two individual OPN thrombin-cleavage fragments in quantitative cell migration assays. In particular, these studies identified the N-terminal GRGDS-containing OPN thrombin-cleavage fragment as an $\alpha_v\beta_3$ ligand which, in comparison with intact OPN, fibrinogen, and vitronectin, is highly active in promoting tumor cell migration.

## 2. Materials and methods

### *2.1. Purification of OPN and OPN thrombin-cleavage fragments*

Human OPN was purified from breast milk as described previously [19], and purified OPN was cleaved with purified thrombin (Sigma Chemicals, St. Louis, MO) to yield two polypeptide fragments of similar size [22]. Cleaved OPN (2 mg) was diluted with 15 volumes of buffer A (0.02 M Tris pH 8.0, 0.01 M NaCl) and applied to a DEAE 5PW Protein-Pak high pressure liquid chromatography column (Millipore/Waters Associates, Milford, MA) at 1 ml/min. After loading, the column was washed with buffer A and bound fragments eluted with the following series of linear gradients at a flow rate of 1 ml/min: time zero, 100% buffer A; $t = 25$ min, 75% buffer A, 25% buffer B (0.02 M Tris pH 8.0, 1.0 M NaCl); $t = 75$ min, 50% buffer A, 50% buffer B; $t = 100$ min, 100% buffer B. One-ml fractions were collected and aliquots were subjected to immunoblotting with two different peptide antisera, one of which was raised to a peptide sequence uniquely present in the N-terminal thrombin-cleavage fragment of human OPN and the other which was raised to a peptide representing a unique sequence present in the C-terminal fragment (see below for description of antibodies). Fractions containing the N-terminal or C-terminal fragments were initially identified by staining with these antibodies. The C-terminal fragment eluted first as a peak centered at fractions #35 and 36 and the N-terminal fragment eluted second as

a peak centered at fractions #36-39. Despite the partial overlap of the elution profiles, individually purified fragment preparations were obtained from the earlier and later portions of the C-terminal fragment and N-terminal fragment elution peaks, respectively. The identity and purity of both fragment preparations were established independently with N-terminal amino acid sequence analyses (residues #1-5) and SDS PAGE [25]. The N-terminal fragment did not stain significantly with Coomassie blue, but this fragment was visualized readily with a copper stain procedure (Bio-Rad Laboratories, Hercules, CA).

### 2.2. Cell lines and cell migration assays

Cell lines were obtained from the American Type Culture Collection (Rockville, MD). They were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. Prior to assay, cells were harvested with brief trypsinization (0.05% trypsin, 0.5 mM EDTA), in Hanks' balanced salt solution without divalent cations. Cells then were washed twice with serum-free DMEM and resuspended in serum-free DMEM containing 10 mg/ml bovine serum albumin (BSA, Sigma Chemicals).

Cell migration was assayed with 8-micron pore size Transwell migration chambers (Costar Corp., Cambridge, MA) [26,27]. For haptotaxis assays, the undersides of membranes were coated at room temperature with OPN, OPN thrombin-cleavage fragments, fibrinogen, or vitronectin, as indicated. OPN and OPN fragments were purified as described above. Human vitronectin was purchased from Gibco-BRL/Life Technologies (Gaithersburg, MD) and Collaborative Biomedical Products (Bedford, MA). Vitronectin from both sources gave equivalent results. Human fibrinogen was purchased from Calbiochem (La Jolla, CA) and any contaminating fibronectin was removed by adsorption to gelatin coupled to cross-linked agarose beads (Sigma Chemical, St. Louis, MD) [28]. After 60 min, the protein coating solutions were removed and remaining protein binding sites on the undersides of the membranes were blocked by incubation with a solution of 100 mg/ml BSA at room temperature for 60 min; membranes next were washed with DMEM. To quantitate binding of the different proteins to the membranes, aliquots of each were radiolabeled with [$^{125}$I] using the iodobead method (Pierce Chemicals, Rockford, IL) and binding efficiencies calculated as the ratio of bound dpm to total dpm applied.

In assays designed to compare native OPN with thrombin-cleaved OPN, the undersides of membranes were coated sequentially with OPN and BSA; where indicated, the bound OPN then was cleaved in situ by incubating the underside of each membrane with 1 unit of thrombin. As determined with polyacrylamide gel electrophoresis and autoradiography of radiolabeled OPN tracer extracted from such membranes, thrombin-cleavage was ~60% complete with this method. After incubation with thrombin, membranes were washed extensively with DMEM. No thrombin was detected after washing as determined with the Kabi S2238 chromogenic substrate assay which detects thrombin at concentrations as low as 10 pM (Kabi Pharmacia Diagnostics, Piscataway, NJ). However, as an additional control, we included in several experiments the irreversible thrombin inhibitor D-phe-L-pro-L-arg-$CH_2Cl$ (PPACK, Calbiochem). Thus for membranes treated with 1 unit of thrombin we subsequently added 2 μg of PPACK which, as determined with the Kabi substrate assay, was a 25-fold excess of inhibitor.

After membranes were prepared as outlined above, $1 \times 10^5$ cells were added to the upper chambers in serum-free DMEM containing 10 mg/ml BSA. Cell migration was allowed to proceed at 37°C in a standard tissue culture incubator for the indicated intervals of time; cells then were removed from the upper side of the membrane with a cotton swab, and cells that migrated to the undersides were stained with 0.2% crystal violet in 2% ethanol followed by a brief rinse in distilled water. Dried membranes were cut out and mounted on glass slides in immersion oil. At least 10 random high power fields from each of triplicate membranes were counted for each experimental condition. No cell migration was observed when membrane filters were coated with BSA alone, and in no cases did we observe cells in the lower chamber which had traversed the membranes but did not remain attached.

To investigate chemotaxis promoted by soluble OPN and soluble OPN thrombin-cleavage fragments, membranes were prepared by coating undersides with 100 μg/ml gelatin (Sigma Chemical) followed by 100 mg/ml BSA to block remaining protein binding

sites. Soluble OPN or OPN fragment was added to the lower chambers at the indicated concentrations together with 10 mg/ml BSA. Under these conditions, OPN and OPN fragments in the lower chambers were prevented from binding to the membranes so that chemoattractant activity, as opposed to haptotactic activity, could be assayed.

### 2.3. Antibodies to osteopontin and integrins

Affinity-purified rabbit IgG to a 20 amino acid synthetic peptide representing the GRGDS region of human OPN was prepared as described previously [19]. The peptide antigen used to derive this antibody had the following sequence:

VPTVDTYD*GRGDS*VVYGLR (C), with cysteine added for conjugation to keyhole limpet hemocyanin. This sequence is present in the N-terminal thrombin-cleavage fragment, and antibody derived from immunization with this peptide bound to the N-terminal but not the C-terminal fragment. Antibody to the C-terminal thrombin-cleavage fragment of human OPN was prepared by immunizing rabbits with the following peptide, also conjugated to keyhole limpet hemocyanin: (C) DPKSKEEDKHLKFRISHE. As expected, this antibody bound the C-terminal but not the N-terminal thrombin-cleavage fragment, and it also bound intact OPN. A similar antibody to a rat OPN synthetic peptide has been described previously [29]. Control rabbit IgG was purified with protein A Sepharose (Pharmacia).

$\alpha_v\beta_3$ Monoclonal antibody LM 609 ascites [30] was kindly provided by Dr. David Cheresh, Scripps Clinic and Research Foundation, La Jolla, CA. Mouse monoclonal anti-$\beta_1$ integrin (P4C10) and anti-$\alpha_v\beta_5$ integrin (P1F6) ascites were purchased from Gibco-BRL/Life Technologies. Each monoclonal antibody was purified with the Affigel protein A MAPS II kit (Bio-Rad Laboratories). Control mouse IgG was purchased from Sigma and re-purified according to the same procedure.

## 3. Results

To assay OPN activity in promoting tumor cell migration, cell migration assays were performed with modified Boyden chambers (Transwell motility chambers, Costar Corp.) in which the undersides of the membranes were coated with graded concentrations of OPN. Such assays measure haptotaxis, i.e., cell migration in a gradient of bound ligand [26], and as shown in Fig. 1, OPN promoted haptotaxis of human T24 bladder carcinoma cells and MDA-MB-435s breast carcinoma cells. Membranes coated with increasing concentrations of OPN were correspondingly more active in promoting cell migration, up to a coating concentration of ~ 10 μg/ml, at which maximal activity was observed.

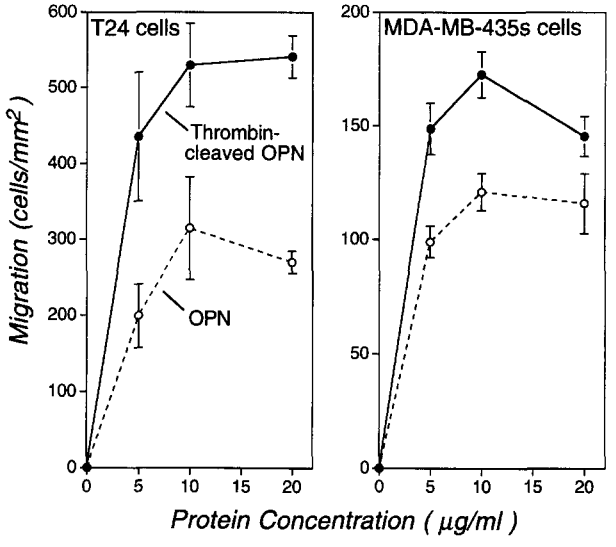


Fig. 1. Tumor cell haptotaxis through filters coated with increasing concentrations of OPN (dashed lines) in comparison with migration through filters coated with OPN which was subsequently cleaved by thrombin in situ (solid lines) (see Section 2). After coating with OPN, all filters were incubated with 100 mg/ml BSA to block remaining protein binding sites; and following incubation with thrombin, filters were washed extensively to eliminate all detectable thrombin (see text). Assays were terminated at 5 h; each point represents the mean ± S.D. of triplicates.

In parallel, haptotaxis assays with thrombin-cleaved OPN were performed to test the consequences of cleavage of OPN by thrombin for directed tumor cell migration. For these experiments, OPN was first bound to the membranes and then cleaved by thrombin in situ as described in Section 2. Upon cleavage, OPN was more active at promoting tumor cell haptotaxis and this increase in functional activity was evident at all protein concentrations tested (Fig. 1). Moreover, in similar experiments designed to follow the time course of tumor cell haptotaxis over

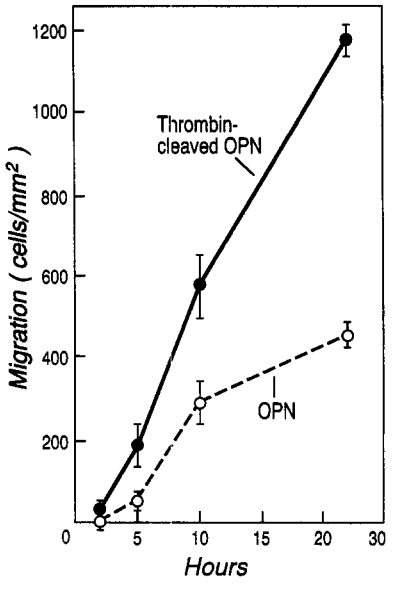


Fig. 2. Time course of T24 cell haptotaxis through filters coated with 20 μg/ml OPN (dashed line) in comparison with identically coated filters which were subsequently incubated with thrombin to cleave OPN in situ (solid line). Each point represents the mean ± S.D. from triplicate assays.

24 h, thrombin-cleaved OPN promoted a greater rate of tumor cell migration throughout this interval (Fig. 2). Significantly, the enhanced activity of thrombin-cleaved OPN relative to intact OPN illustrated in Figs. 1 and 2 is an underestimate because thrombin-cleavage of filter-bound OPN was ~60% efficient (see Section 2).

Several control experiments were performed to establish that the enhanced activity of OPN following cleavage by thrombin was a direct consequence of structural changes in OPN mediated by the enzymatic activity of thrombin and that it was not due to differences in efficiencies of protein binding to the membranes or to thrombin contamination of the membranes. First, as determined with [$^{125}$I]-labeled tracers, intact and thrombin-cleaved OPNs bound to the migration chamber membranes with equal efficiency. Second, as determined with sensitive thrombin substrate assays (see Section 2), no thrombin remained after washing the membrane filters that had been treated with thrombin to cleave bound OPN in situ. Furthermore, and as shown in Fig. 3, post-treatment of membranes with a molar excess of PPACK, which is a highly specific and irreversible thrombin inhibitor [31], was without effect. In contrast, pre-treatment of thrombin with PPACK blocked the enhancement, demonstrating that the enzymatic activity of thrombin was required. Because PPACK is a specific thrombin inhibitor, these experiments also established that enhancement was not attributable to any contaminating proteases which might be present. Finally, and also shown in Fig. 3, membranes coated with thrombin at the concentration used for cleavage of bound OPN did not promote any cell migration.

Thrombin cleaves an arginyl-serine peptide bond in OPN thus generating two fragments of very similar size, one of which contains the GRGDS sequence just six residues from its carboxyterminus and another which lacks a GRGDS sequence [9,22]. To purify the individual fragments for functional characterization, we developed a purification scheme employing anion-exchange high pressure liquid chromatography. Identification of column fractions consisting of purified N-terminal or C-terminal fragment was facilitated by antibodies specific for each (see Section 2), and confirmation was independently obtained with

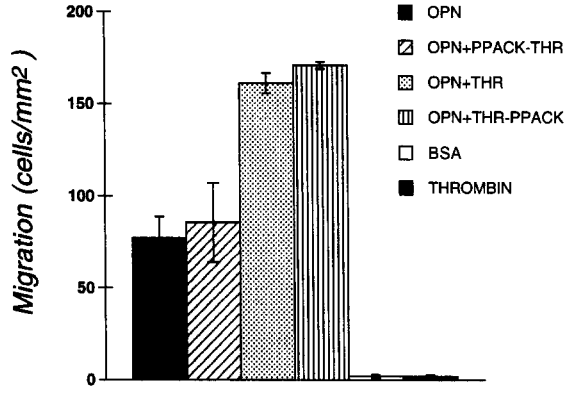


Fig. 3. T24 cell haptotaxis. The undersides of migration chamber filters were coated with OPN (10 μg/ml) followed by BSA (100 mg/ml) to block remaining protein binding sites. Subsequently, some filters were incubated with thrombin (OPN + THR) to cleave OPN in situ, others were incubated with thrombin and then with a molar excess of the thrombin inhibitor PPACK (OPN + THR-PPACK), and others were incubated with thrombin together with PPACK (OPN + PPACK-THR) (see Section 2). Migration assays were terminated after 5 h; each bar represents the mean ± S.D. from triplicates. Note that inclusion of PPACK in the thrombin incubation prevented enhancement of OPN migration promoting activity but that filters treated with thrombin followed by PPACK promoted the same increase in haptotaxis observed on identical filters not treated with PPACK. Also, filters coated with thrombin (THR) followed by BSA did not promote any haptotaxis nor did filters coated with BSA alone.

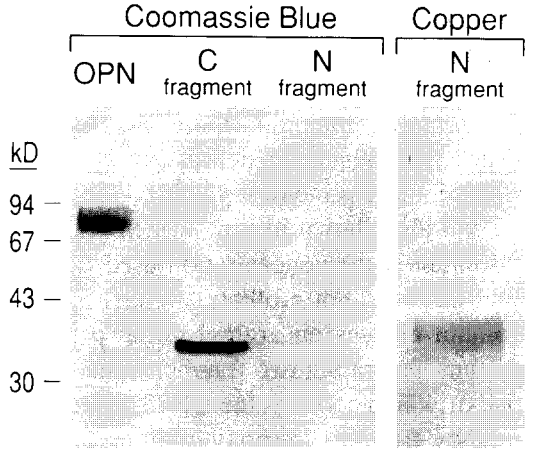


Fig. 4. SDS PAGE with 8.5% (w/v) acrylamide of intact OPN (10 μg) and the two fragments generated from OPN by thrombin cleavage (10 μg each). In contrast to intact OPN and the C-terminal fragment, the N-terminal GRGDS-containing fragment did not stain significantly with Coomassie blue R-250, but it was visible with a copper staining procedure (see text). Molecular weight standards included rabbit phosphorylase b (94 kDa), bovine serum albumin (67 kDa), chick ovalbumin (43 kDa), and bovine carbonic anhydrase (30 kDa).

N-terminal sequence analysis of purified protein. As shown in Fig. 4, SDS PAGE indicated that the two purified fragments have very similar electrophoretic mobilities, as expected, corresponding to $M_r$ ~ 35000. The C-terminal fragment stained readily with Coomassie blue, but, in contrast, the N-terminal fragment did not bind this dye significantly. Instead, the N-terminal fragment was visualized with a copper staining procedure [32].

Next, the purified OPN thrombin-cleavage fragments were subjected to analyses in haptotaxis assays. Data presented in Fig. 5 (top panel) relate the comparative cell migration promoting activities of membranes coated with increasingly concentrated solutions of intact OPN and each of the two purified fragments. These data indicate that no detectable activity is associated with the C-terminal fragment and that the N-terminal fragment, similar to unfractionated thrombin-cleaved OPN, is more active than intact OPN. To control for any differences in efficiencies of binding to the membranes, we labeled intact OPN and both fragments with [$^{125}$I] and determined binding efficiencies at each of the concentrations employed. For example, at a protein concentration of 10 μg/ml, binding efficiencies were as follows: intact OPN (13.8%), C-terminal fragment (12.6%), N-terminal fragment (11.2%). Next, the cell migration data were plotted as a function of picomoles ligand bound to the membranes, taking into account the minor differences in binding efficiencies as well as the differences in molecular weight (~ 70000 for OPN; ~ 35000 for each fragment). As shown in Fig. 5 (bottom panel), these experiments indicated that the N-terminal fragment is nearly 3-fold more active than intact OPN on a molar basis.

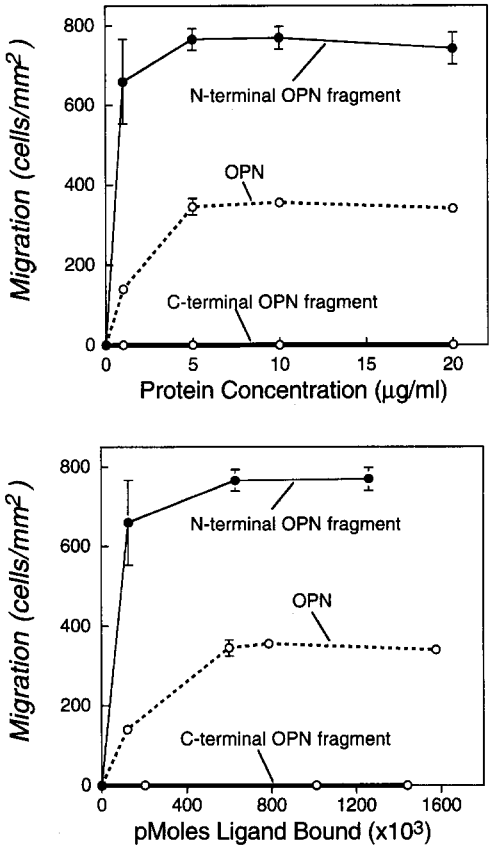


Fig. 5. Top panel: T24 cell haptotaxis (5 h) promoted by membranes coated with increasing quantities of intact OPN, and the two individual fragments generated from OPN by thrombin-cleavage. In contrast to the N-terminal GRGDS-containing fragment, no activity was found in association with the C-terminal fragment. Bottom panel: Haptotaxis, as in top panel but expressed as a function of picomoles ligand bound to the membrane. Efficiencies of ligand binding were determined with [$^{125}$I]-labeled tracers (see text). Data are presented as the mean ± S.D. from triplicate assays; absence of error bars indicates that S.D. was less than the diameter of the circles used to represent the data points.

Absence of detectable activity associated with the C-terminal fragment was consistent with findings made in independent experiments performed with affinity-purified rabbit antibody raised to a 20 amino acid synthetic peptide containing the GRGDS region of human OPN (see Section 2). This antibody, which binds the N-terminal fragment but not the C-terminal fragment, blocked > 90% of T24 cell migration and > 85% MDA-MB-435s cell migration towards filters coated with 20 μg/ml unfractionated thrombin-cleaved OPN. Control antibody at the same concentration (10 μg/ml) had no effect. In addition, we performed experiments to test the possibility that the C-terminal fragment interferes with haptotaxis towards the active N-terminal fragment. However, membranes coated with solutions of 10 μg/ml N-terminal fragment together with 10 μg/ml C-terminal fragment promoted cell migration identically to membranes coated with 10 μg/ml N-terminal fragment alone, indicating that the C-terminal fragment lacks inhibitory activity.

To test the involvement of $\alpha_v\beta_3$ in haptotaxis towards the purified N-terminal thrombin-cleavage fragment of OPN, we performed cell migration assays, as in Fig. 5, but in the presence of 10 μg/ml $\alpha_v\beta_3$ antibody LM609 [30]. This antibody inhibited > 90% T24 haptotaxis and > 80% MDA-MB-435s haptotaxis on filters coated with 20 μg/ml of the N-terminal OPN fragment; and neither control antibody nor $\alpha_v\beta_5$ P1F6 antibody had any effect, indicating that $\alpha_v\beta_3$ is the principal receptor on these cells which functions in cell migration towards this OPN fragment. In control experiments, $\alpha_v\beta_3$ antibody LM609 did not inhibit migration of either of these cell lines towards filters coated with laminin, establishing the specificity of LM609 antibody inhibition of cell migration towards the N-terminal OPN fragment. For both cell lines, $\alpha_v\beta_3$ antibody LM609 also inhibited nearly all (~ 95%) haptotaxis towards intact OPN.

Cell migration assays also were performed to investigate whether the N-terminal OPN fragment was not only more active than intact OPN but also more active than other $\alpha_v\beta_3$ ligands. As shown in Fig. 6A, membranes coated with increasingly concentrated solutions of the N-terminal OPN fragment promoted greater cell migration than membranes coated with corresponding concentrations of fibrinogen. To com-

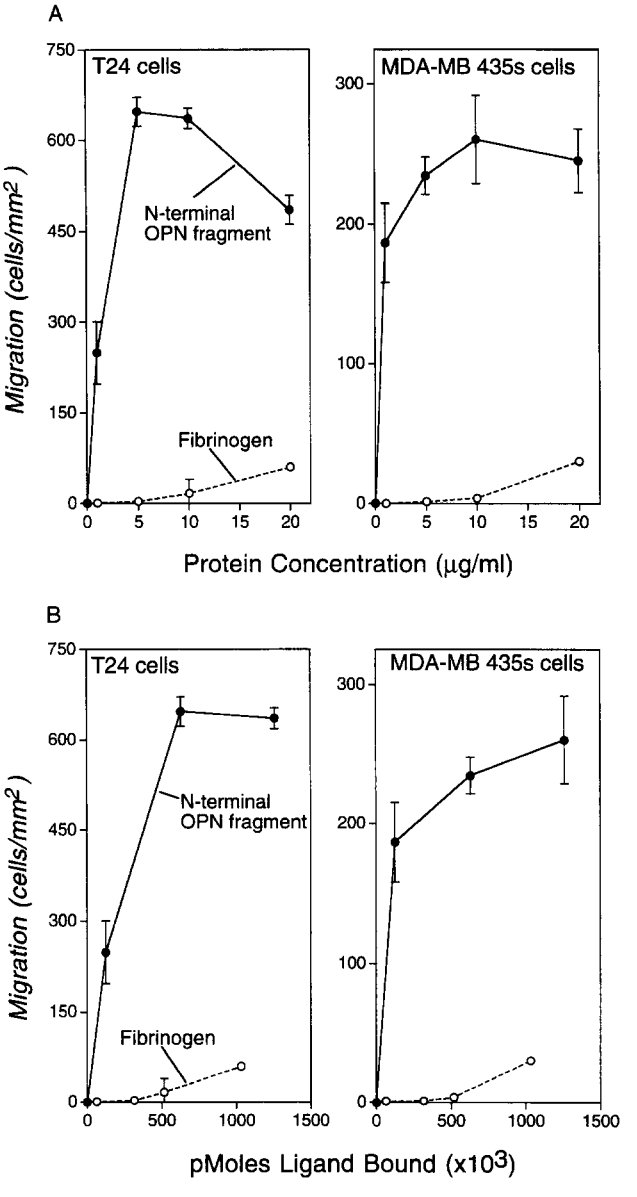


Fig. 6. Panels A: Tumor cell haptotaxis (5 h) promoted by membranes coated with increasing quantities of fibrinogen in comparison with membranes coated with increasing quantities of the N-terminal GRGDS-containing OPN thrombin-cleavage fragment. Each point represents the mean ± S.D. from triplicate assays. Panels B: Data presented in Panels A replotted as a function of picomoles of ligand bound to the membrane, taking into account molecular weights of the ligands (fibrinogen = 341000; N-terminal OPN fragment = 35000) and efficiencies of binding of each to the membrane (e.g., at a concentration of 10 μg/ml 44.3% fibrinogen bound and 11.2% N-terminal OPN fragment bound).

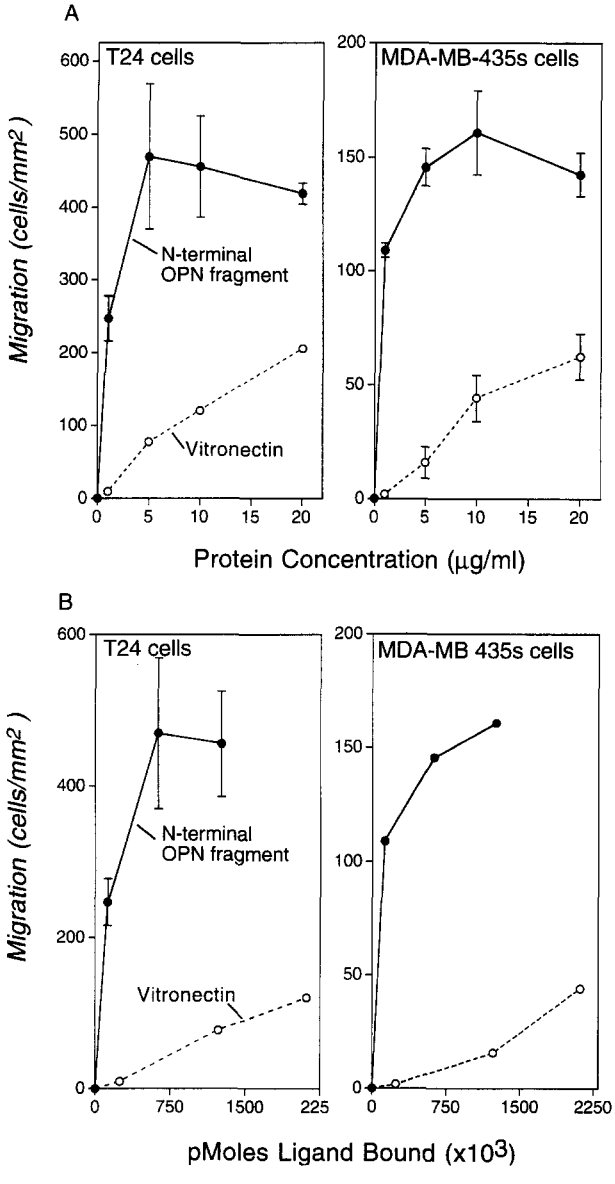


Fig. 7. Panels A: Tumor cell haptotaxis (5 h) promoted by membranes coated with increasing quantitites of vitronectin in comparison with increasing quantitites of the N-terminal OPN thrombin-cleavage fragment. To eliminate possible interactions between these ligands and integrins $\alpha_v\beta_1$ or $\alpha_v\beta_5$, assays were performed in the presence of $\beta_1$ and $\alpha_v\beta_5$ blocking antibodies (see text). However, similar results were obtained in the absence of these antibodies, indicating that $\beta_1$ and $\alpha_v\beta_5$ integrins do not function significantly in the migration of these cell lines towards either ligand. Data points represent the mean ± S.D. of triplicate assays. Panels B: Data presented in Panels A replotted as a function of picomoles ligand bound to the membrane (average $M_r$ vitronectin = 70000; at a concentration of 10 μg/ml, 36.7% vitronectin bound).

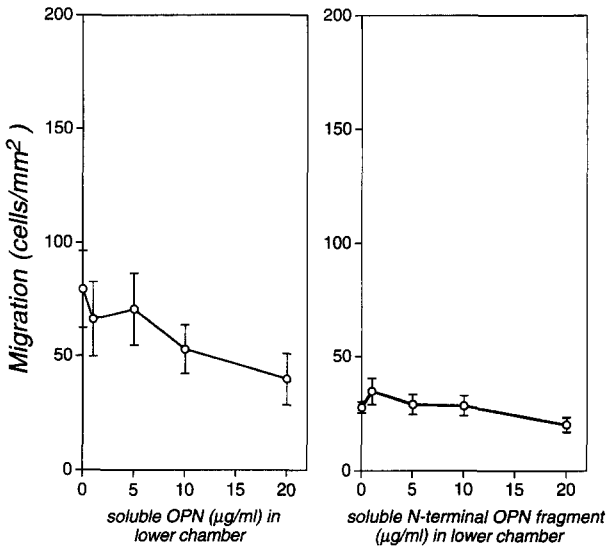


Fig. 8. Assay of soluble OPN and the N-terminal GRGDS-containing OPN thrombin-cleavage fragment for chemoattractant activity. T24 cell migration assays were performed with chambers in which the undersides of the membranes were coated first with gelatin, followed with BSA to block remaining protein sites (see Section 2). To test for chemotaxis, soluble OPN or N-terminal OPN fragment was included in the lower chambers at the concentrations indicated. Assays were terminated after 5 h; data are presented as the mean ± S.D. from triplicate assays. In all cases, cell migration appeared to be independent of soluble OPN concentration in the lower chamber; i.e., there was no evidence of chemoattractant activity.

pare relative activities of the N-terminal OPN fragment and fibrinogen as a function of picomoles ligand bound to the membrane, we replotted the data, taking into account the binding efficiencies of each ligand to the membrane, and found that the N-terminal fragment was greater than 8-fold more active than fibrinogen on a molar basis (Fig. 6B). Similarly, we found that the N-terminal OPN fragment was considerably more active than vitronectin, which is a ligand not only for $\alpha_v\beta_3$ but also for $\alpha_v\beta_1$ [33] and $\alpha_v\beta_5$ [34]. Therefore, to compare the activities of the N-terminal OPN fragment and vitronectin in promoting cell migration, specifically as they relate to $\alpha_v\beta_3$ function in the absence of contributions by $\alpha_v\beta_1$ or $\alpha_v\beta_5$ integrins, cell migration assays were also performed with 10 μg/ml each of a $\beta_1$-blocking monoclonal antibody (P4C10) together with $\alpha_v\beta_5$-blocking monoclonal antibody (P1F6). As shown in Fig. 7, under these conditions the N-terminal OPN fragment

was greater than 6-fold more active than vitronectin on a molar basis.

Finally, assays were designed to test the possibility that OPN or its N-terminal fragment also promote chemotaxis, i.e., cell migration in a gradient of soluble ligand. To distinguish unambiguously the promotion of chemotaxis by soluble OPN from the promotion of haptotaxis by immobilized OPN, it was essential to carry out migration assays under conditions such that OPN included in the lower chambers was prevented from binding to the undersides of the membranes. This required blocking of all protein binding sites on the undersides of membranes prior to assay with a high concentration of BSA (see Section 2). However, because blocking of protein binding sites with BSA alone would preclude cell migration due to elimination of all cell attachment sites on the undersides of membranes, we tested soluble OPN for promotion of chemotaxis in experiments in which the undersides of membranes were coated first with an adhesive ligand (gelatin) followed by blocking of the remaining protein binding sites with BSA. As shown in Fig. 8, cells migrated through these membranes; however, neither soluble OPN nor soluble N-terminal OPN fragment enhanced migration, indicating the absence of chemoattractant activity. Moreover, we found no evidence of chemoattractant activity associated with the C-terminal OPN fragment. In related experiments we also tested the possibility that soluble ligand in the lower chamber could promote chemotaxis of cells in chambers with membranes to which OPN had been immobilized. For these purposes, the undersides of membranes were coated first with a concentration of OPN (5 μg/ml) which was previously determined to be suboptimal for cell migration (see Fig. 1). Remaining protein binding sites then were blocked with BSA. Chemotaxis assays were performed as in Fig. 8, and we again found no evidence of chemoattractant activity.

## 4. Discussion

Data presented here demonstrated that a gradient of immobilized OPN promotes cell migration, i.e., haptotaxis, and that cell migration is dependent on OPN concentration. In particular, we found that cleavage of OPN by thrombin increased OPN activity in promoting haptotaxis. Enhanced activity of thrombin-cleaved OPN in comparison with intact OPN was observed at all protein concentrations tested; and in experiments designed to study haptotaxis as a function of time, thrombin-cleaved OPN was found to promote a 2- to 3-fold increase in cell migration throughout a 24-h interval. Control experiments established that the observed difference in relative activities of the intact and thrombin-cleaved OPNs was a direct consequence of enzymatic cleavage of OPN by thrombin and that it was not due to binding of thrombin to the membranes. Moreover, membranes coated with thrombin alone did not promote any cell migration.

Previously, analyses of N-terminal amino acid sequences derived from unfractionated thrombin-cleaved OPNs in comparison with OPN cDNA sequences established that the thrombin-cleavage site is present six amino acid residues carboxyterminal to the GRGDS sequence in both human and rat OPNs [9,22]. Electrophoretic analyses performed under non-denaturing conditions indicated that the two OPN fragments generated by thrombin cleavage do not remain associated [19], however, neither of these fragments had been isolated previously. Therefore, to analyze the activities of each of these two fragments apart from the other, we devised a protocol which yields purified preparations of each of the individual fragments. Next, we tested the purified fragment preparations in haptotaxis assays and found that only the N-terminal fragment had detectable activity and that on a molar basis, the N-terminal fragment was nearly 3-fold more active than intact OPN. Thus, these experiments indicated that the N-terminal, GRGDS-containing fragment is responsible for the enhanced activity associated with unfractionated thrombin-cleaved OPN. Consistent with these findings, antibody raised to a 20 amino acid synthetic peptide containing the GRGDS sequence, which binds the N-terminal OPN thrombin-cleavage fragment, blocked $> 85\%$ cell migration towards unfractionated thrombin-cleaved OPN.

Enzymatic digestion of OPN by thrombin also enhances OPN activity in promoting cell attachment and spreading [19]; however, it is likely that increased activity of thrombin-cleaved OPN in promoting cell migration is of greater significance, particu-

larly in tumors. OPN is overexpressed in a variety of cancers – by tumor cells and invading host macrophages in some tumors, and predominantly by invading host macrophages in others. Such overexpression is especially pronounced at the edge of tumors (i.e., the tumor/stroma interface) and in areas of tumor necrosis [13]. Moreover, fibrin is often found particularly at the tumor periphery [24], indicating that the coagulation pathway is active where OPN is most abundant. The presence of fibrin in association with tumors is a consequence of the hyperpermeability of the tumor-associated vasculature; this hyperpermeability allows for extravasation of fibrinogen and blood clotting factors, resulting in activation of the extrinsic clotting pathway with generation of thrombin and fibrin [23,35]. Consequently, it is highly probable that OPN associated with tumors is cleaved by thrombin, resulting in enhancement of OPN migration promoting activity at the tumor/host interface precisely where tumor invasion proceeds.

Enhancement of OPN haptotactic activity as a consequence of thrombin-cleavage is most probably relevant to cell types in addition to those tested here, and we are currently investigating this possibility. Thus it is also likely that thrombin regulation of OPN function is significant for cell migration in pathologies other than cancer and that such regulation occurs wherever OPN is expressed and the blood coagulation pathway is activated. Probable examples include atherosclerotic lesions [36–38], sites of myocardial infarction [39], glomerulonephritis [40], and a variety of other inflammatory conditions [41].

Previously, others have reported that soluble OPN promotes chemotaxis of vascular smooth muscle cells [18,42]; however, in this study we found no evidence that OPN has chemoattractant activity. Rather, our experiments indicated that OPN must be immobilized to promote cell migration. There are several possible explanations for the apparent discrepancies between our findings and previous reports. For one, vascular smooth muscle cells may respond differently than tumor cells to soluble OPN. Alternatively, and because protocols involving migration assays with vascular smooth muscle cells did not include steps designed to block all protein binding sites on the undersides of membranes prior to assay, soluble OPN included in the lower chambers of such experiments may have bound to the membranes, thus providing a gradient of bound OPN. By contrast, our protocols for chemotaxis assays included blocking of remaining protein sites on the undersides of membranes with a high concentration of BSA (100 mg/ml). With our conditions, soluble OPN in the lower chamber was unable to bind, and in these experiments no chemotaxis was observed.

Our findings that immobilization of OPN is required for promotion of tumor cell migration in vitro suggest that OPN must also be immobilized in vivo for it to participate in promoting of tumor cell invasion. However, there is good evidence that immobilization of OPN does occur particularly through transglutaminase catalyzed covalent cross-linking [43], of either multiple OPN molecules to form high molecular weight OPN aggregates or to fibronectin to form OPN/fibronectin complexes [44]. In addition, high molecular weight OPN complexes have been demonstrated to exist in vivo [16,45]. Interestingly, the amino acid residues present in OPN which participate in tranglutaminase catalyzed cross-linking have be identified in a region near the N-terminus of the native molecule [45], and therefore these sites are also present in the active thrombin-cleavage fragment which contains the GRGDS cell binding region.

Finally, we investigated the involvement of the $\alpha_v\beta_3$ integrin in mediating haptotaxis towards the GRGDS-containing thrombin-cleavage fragment of OPN and the activity of this OPN fragment relative to other $\alpha_v\beta_3$ ligands. We had shown previously with affinity chromatography and cell attachment assays that $\alpha_v\beta_3$ is the principal receptor which binds to native OPN as well as to unfractionated, thrombin-cleaved OPN [19,46]. Consequently, it was not surprising that the LM609 monoclonal antibody to $\alpha_v\beta_3$ nearly abolished T24 bladder carcinoma and MDA-MB-435s breast carcinoma cell migration towards the active OPN fragment as well as towards intact OPN. Of particular significance, however, the GRGDS-containing OPN fragment was on a molar basis $>$ 8-fold more active than fibrinogen, $>$ 6-fold more active than vitronectin, and $>$ 3-fold more active than intact OPN. Collectively, these data indicate that the GRGDS-containing OPN thrombin-cleavage fragment is an $\alpha_v\beta_3$ ligand which is especially potent in promoting tumor cell migration in vitro, thereby predicting that it is also particularly active in promoting tumor cell invasion in vivo.

Moreover, these data illustrate a potentially important mechanism by which activation of the blood coagulation pathway in the vicinity of OPN expression, as it probably occurs in cancer and other pathologies, can lead to substantial enhancement of cell migration.

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