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13. ABSTRACT (Maximum 200 words)

The objective of this research is to establish whether the secreted phosphoprotein osteopontin (OPN) plays a biological role in the progression of breast carcinoma cells, and to determine the nature of this role, by asking if cell properties and genes associated with malignancy are regulated by OPN. This work makes use of three established mammary epithelial cell lines (21T series) derived from the same patient. 21PT cells are immortal but nontumorigenic in the nude mouse; 21NT are weakly tumorigenic, but non-metastatic; and 21MT-1 are tumorigenic, weakly metastatic. In addition, MDA-MB-435 cells are included, representative of a highly malignant, metastatic cell line. We have found that OPN mRNA and protein expression is associated with degree of malignancy in this progression series. Further, we have shown that the more malignant members of this series also bind better to OPN in cell adhesion assays. 21T series and MDA-MB-435 cells have all demonstrated directed migration towards exogenous OPN, and this was associated with increased expression of met (HGF receptor) mRNA and increased sensitivity to HGF. We have found that cell adhesion and migration of 21T series and MDA-MB-435 cells are mediated via cell surface integrins. Interestingly, MDA-MB-435 cells show $\alpha v\beta 3$ integrin-dependent cell adhesion and migration, whereas 21T series cells exclusively use $\alpha v\beta 5$ and $\beta 1$ integrins. In cell invasion assays, MDA-MB-435 cells showed a more pronounced response to exogenous OPN than 21PT or 21NT cells. However, invasiveness of 21PT and 21NT cells was upregulated by transfection with a high-expression OPN-containing vector, and this was accompanied by increased urokinase expression. Preliminary results of in $vivo$ assays have indicated that although increased OPN expression was not associated with increased tumor-take, some OPN-transfected 21NT cells have acquired metastatic potential. These studies implicate OPN in the malignancy of human breast cancer, and suggest potential mechanisms to include integin-dependent cell adhesion and migration phenomena, as well as induction of cellular invasiveness.

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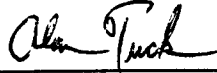
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Research involving animals was conducted under the regulations and governance of the Canadian Council on Animal care (C.C.A.C.), the regulatory agency governing all use of animals for research in Canada. The animal protocol used has been approved by the University of Western Ontario Council on Animal Care (Protocol #95211-9). All hazardous material employed in this research were stored, handled, and disposed of according to all applicable federal, provincial and local regulations. Ontario Regulation 67/93, made under the Occupational Health and Safety Act for Health Care and Residential Facilities, governs the use and disposal of hazardous materials. The London Regional Cancer Centre and Victoria Hospital Research Institute (now London Health Sciences Centre, Research) are in compliance with this act. We also comply with the stipulation of the University of Western Ontario Biohazardous Agents Registry, which covers biohazards and resarch using recombinant DNA technology.

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TABLE OF CONTENTS

THE FUNCTIONAL ROLE OF OSTEOPONTIN IN THE MALIGNANCY OF HUMAN BREAST CANCER

I. GENERAL OBJECTIVE

The goal of this research is to determine the functional role of osteopontin (OPN), a secreted, integrin-binding phosphoprotein, in the malignancy of human breast cancer. Recent evidence detailed below (including a number of clinical studies) strongly implicates OPN in breast cancer. In spite of mounting clinical evidence that increased OPN is associated with worse prognosis in breast cancer patients, the biological role(s) of OPN in progression to malignancy are poorly understood. This information will be important to obtain, as potential utility would lie not only in the interpretation of prognostic information obtained by determination of OPN levels (either plasma or primary tumour), but potentially also in the development of treatment strategies based on blocking the effects of OPN in inducing malignant behaviour of breast carcinoma cells.

Over the past three years, Dr. Tuck has been a member of a multidisciplinary breast cancer research group, based at the London Regional Cancer Centre/London Health Sciences Centre. This team has obtained significant preliminary clinical data to support the overall hypothesis that OPN, as detected in blood and tumours of breast cancer patients, plays an important role in breast cancer (see below). Study of the functional role of OPN in human breast cancer has only just begun. As a new investigator, Dr. Tuck has defined a 3-year research plan to achieve this goal, based on the work here described. The U.S. Army B.C.R.P. "Career Development" Award allows for sufficient protected research time to undertake this project.

II.   BACKGROUND

OPN is a secreted phosphoprotein which is expressed in a regulated fashion by a limited number of normal cells and tissues (eg. developing bone, lactating mammary gland, kidney, activated T-cells and macrophages, smooth muscle cells [reviews 1,2]). OPN has also been identified by several groups as a tumour-associated protein (review 3), and its expression has been found to be increased in ras-transformed fibroblasts (4-6), and during multistage carcinogenesis in mouse skin (5). Furthermore, down-regulation of OPN expression in ras-transformed fibroblasts has been shown to decrease their tumorigenicity (7,8), adding weight to the concept that OPN can contribute functionally to the malignancy of cells that express it.

In a study of a limited number of patients, Senger reported 4-10X elevated blood OPN levels from 10 of 13 patients with disseminated carcinomas (9), relative to low serum levels in 13 patients with other conditions. Included in this small study was serum from a single breast cancer patient, which showed elevated levels of OPN. No clinical information was provided on any of these patients, and there was no attempt to link OPN plasma levels with severity of disease or prognosis. Since that time, there have been three reports of increased osteopontin expression in human breast cancer (10-12). None of these, however, has examined for an association between level of OPN expression and outcome.

Work performed by our group has shown that elevated plasma OPN (as determined by an antigen capture assay) is associated with the presence of metastatic disease in breast cancer (13). In addition, assessment of OPN levels by immunohistochemistry in primary tumours of lymph node negative patients (in a retrospective study) has shown that increased expression of OPN by the malignant cells (as opposed to the tumor infiltrating macrophages) is associated with poor

clinical outcome (14).

Preliminary studies involving function of OPN

Despite the accumulation of recent clinical evidence for a role for OPN in breast cancer, experimental analyses of the biological function of the molecule have only begun. A role for OPN in cell adhesion phenomena has been proposed, and there is evidence that this may be mediated in part by binding of an internal GRGDS (gly-arg-gly-asp-ser) amino acid sequence to a cell surface integrin (αvβ3) (15,16). Work by our group (17-20) has shown that interfering with this RGD sequence of OPN blocks its ability to bind to cells, including human breast cancer cells (19,20). OPN may also bind to other non-integrin cell surface receptors (21). There is evidence that binding of OPN to cell surface receptors may in turn induce a signal transduction pathway via integrin-dependent or independent pathways (22, review 2).

Little is known about how OPN binding may in turn influence behaviour of mammary carcinoma cells. We have shown (23) that three murine mammary carcinoma cell lines (D2HAN series) express levels of OPN RNA considerably higher than levels in virgin mouse mammary gland. We have also found that the metastatic human mammary adenocarcinoma cell line, MDA-MB-435, expresses high levels of OPN, binds to OPN in a cell adhesion assay, and moves towards OPN in a cell migration assay (18-20). Furthermore, both the adhesive and chemotactic properties of OPN may be blocked by RGD peptides or by generation of RGD-deficient mutants (20). Although these studies provide evidence that RGD-mediated events are important in OPN-induced cell adhesion and chemotaxis of some mammary carcinoma cells, the significance of these events to the malignancy of these cells has yet to be determined. Exploration of the functional role(s)

of osteopontin in the malignancy of breast carcinoma cells is thus the focus of the work presented here.

21T series cell lines as a model of breast cancer progression

Band et al. (24) generated a series of breast epithelial cell lines from the same patient, diagnosed with infiltrating and intraductal mammary carcinoma. Permission has been received from Dr. Band to use these cells for the proposed research. These cell lines have been characterized for tumorigenicity and metastatic ability in nude mice. 21PT is immortal, but non-tumorigenic; 21NT is tumorigenic, non-metastatic; and 21MT-1 is metastatic. This series of cell lines has been recently used to demonstrate tumor cell-specific loss of p53 protein in human breast tumor progression (25).

The clinical and experimental data described above provide compelling support for the premise that OPN expression may play a role in the biology of breast carcinoma. What is needed now is an understanding of whether OPN can directly influence the malignancy of breast carcinoma cells, at what stage during progression, and in what manner. The 21T series of cell lines provide a useful model system for the study of influences of OPN on breast epithelial cells at different stages of progression. The present study makes use of this cell culture model to address the questions raised above directly, in a controlled and testable fashion.

III.   HYPOTHESIS/PURPOSE

The hypothesis to be tested is that the secreted phosphoprotein OPN plays a biological role in the progression of breast carcinoma cells, and that this role involves the modulation of

measurable cell properties and alterations of "malignancy-associated" gene expression. The purpose of this research is thus to: **1) establish whether OPN plays a biological role in the progression of breast carcinoma cells, and 2) determine the nature of this role in terms of which cell properties and "malignancy-associated" genes may be regulated by OPN.**

IV.   TECHNICAL OBJECTIVES

1.   To determine whether OPN expression is associated with malignancy of established human breast epithelial cell lines.

2.   To determine whether up-regulation of OPN expression changes the malignant properties of these cells.

3.   To examine how OPN affects other measurable cell properties associated with malignancy.

OBJECTIVE 1: To determine whether OPN expression is associated with malignancy of established human breast epithelial cell lines.

Much of the work related to this objective had been completed at the time of last year's annual report, and was described therein. The Western analysis of cell lysates is new information, which is presented here in light of what we have reported previously. The *in vivo* experiments

in nude mice have been combined with those from Task 2e, and so are described under **Objective 2.**

METHODS

Cell lines and culture

The 21T series cell lines (21PT, 21NT, 21MT-1) were obtained as a kind gift of Dr. Vimla Band (Dana Farber Cancer Institute). These cells are maintained in culture in α-MEM supplemented with 10% FCS, 2mM L-glutamine (all from GIBCO-BRL/Life Technologies, Grand Island, NY), insulin (1 μg/ml), epidermal growth factor [EGF] (12.5 ng/ml), hydrocortisone (2.8 μM), 10 mM HEPES, 1mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μg/ml gentamycin (all from Sigma Chemical, St. Louis, MO) (αHE medium). MDA-MB-435 cells were obtained as a kind gift of Dr. Janet Price (MD Anderson Cancer Center, Houston TX), and are grown in α MEM with 10% FCS (both from GIBCO-BRL/Life Technologies).

RNA (and DNA) isolation and Northern blot analysis of RNA

Cell pellets were mechanically homogenized (Polytron PT 1200, Brinkmannn 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. The DNA containing fraction was also kept, purified and stored as per the manufacturer's protocol. RNA (10 μg/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 with denatured, oligolabelled [^{32}P]-dCTP cDNA probes, using an oligolabelling kit

(Pharmacia, Baie d'Urfe, PQ) according to the procedure provided by the manufacturer, and as previously described (26,27). RNA levels were quantified by densitometry (Phosphorimager SI, Molecular Dynamics, Sunnyvale, CA).

The OPN probe used was the full-length (1493 bp) human OPN c-DNA EcoRI cassette of plasmid OP-10 (14,28). Even loading of lanes was assured by probing blots with a human 18s rRNA probe (p100D9; a kind gift from Dr. D. Denhardt, Rutgers University, NJ) and by examination of 18s and 28s ribosomal bands on the ethidium bromide stained gel.

Western blotting of cell lysates and conditioned media

Cell lysates were prepared from cell cultures (grown to 70-80% confluency on 100mm dishes) by washing each culture dish twice with cold phosphate buffered saline, followed by the addition of 500 μl cold lysis buffer (20mM Hepes pH 7.2, 5mM $MgCl_2$, 1% NP-40, 1mM DDT, 2mM PMSF, 4 μg/ml leupeptin, 4 μg/ml aprotinin). Each cell lysate was scraped from the dish, pipetted up and down to complete lysis, and spun at 16,000xg for 10 minutes to remove insoluble material. Each supernatant was collected and total protein concentration determined by Peterson's modification of the standard Lowry assay (30). 20 μg of total protein from each cell lysate was used for SDS-PAGE gel electrophoresis and immunoblotting as described below.

Conditioned media were prepared by plating cells at 5×10^5 cells/100 mm plate in regular growth medium and incubating overnight (18 hrs) at 37°C, 5% CO_2. 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 24 hrs at 37°C, 5% CO_2. Following the incubation period, the conditioned

medium from each plate was collected, and the cell debris spun out. The supernatant was concentrated by ultrafiltration in Centricon-30 mini-concentrators as per the manufacturer's protocol (Amicon Inc., Beverly, MA). Each corresponding plate was trypsinized, and a cell count performed, to allow appropriate correction in loading for cell equivalents.

Protein gel electrophoresis was done by standard SDS-PAGE methods (29), and immunoblotting by the enhanced chemiluminescence system (Amersham Canada, Oakville, ON). Cell lysates or conditioned media were fractionated on a denaturing SDS-PAGE gel (8% for cell lysates, 12% for conditioned media), electrophoretically transferred to nylon membrane using a semi-dry system (Millipore Canada, Mississauga, ON), and detected with biotinylated monoclonal antibody mAb53 (0.2 μg/ml) (17), followed by streptavidin-horseradish peroxidase conjugate (Jackson Immunological Laboratories). The enhanced chemiluminescence detection system (Amersham Corp.) was used to detect immune-reactive bands. Film exposure time was 20 s. Molecular mass markers used were biotinylated protein standards (Bio-Rad Laboratories, Hercules, CA).

Tumorigenicity and "spontaneous" and "experimental" metastasis assay in nude mice

Tumorigenicity and "spontaneous" and "experimental" metastasis assay for control 21PT and 21NT-derived cells, as well as MT-1 and MDA-MB-435 cells are performed in concert with Task 2e, involving comparison with OPN-transfected/upregulated 21PT and 21NT cells, and are thus described together under **Objective 2.**

RESULTS

Results of Northern analysis have shown that all three parental 21T series cell lines express

relatively low levels of OPN mRNA, compared to highly malignant, metastatic MDA-MB-435

cells (Figure 1). Assay of conditioned medium by Western analysis has shown that the 21T series

cell lines also secrete lower levels of OPN protein than MDA-MB-435 cells, with the highest

levels of expression in the 21T series seen in the weakly metastatic MT-1 cells. The predominant

form of secreted OPN seen in the 21T series cell lines was of high molecular weight ($^-$97kD),

most likely representing a conjugated (perhaps by transglutaminase) form (Figure 2a). We have

recently extended this analysis to include cell lysates (i.e. cell-associated OPN). Examination of

cell lysates by Western analysis (Figure 2b) showed that the major intracellular form of OPN

present in all four cell lines was of 66 kDa MW. Again, highest levels of OPN protein were

found in the metastatic (21MT-1 and MDA-MB-435) cells.

In order to directly compare tumorigenicity and "spontaneous" and "experimental"

metastatic ability of the control 21T series cell lines with OPN-transfected cells and the metastatic

MDA-MB-435 cells (positive controls), nude mouse injections were delayed until stable

transfectants of the 21T series cells were obtained and characterized for OPN expression, such that

those expressing the highest levels of OPN could be compared against those transfected with the

vector-control. Results of these experiments are presented under **Objective 2.**

DISCUSSION

Baseline expression of OPN by the 21T series of cell lines in culture has been examined

at the RNA and protein level, in comparison with MDA-MB-435 cells. Interestingly, the 21T

series of cell lines, all of which have been shown to be much less malignant than MDA-MB-435 cells when assayed in nude mice, show much lower levels of basal OPN RNA and protein expression. Furthermore, those of the 21T series that are more malignant (21MT-1 cells > 21NT > 21PT) appear by Western analysis of conditioned medium to secrete higher levels of OPN protein. This also would be consistent with the hypothesis that OPN may be associated with malignancy in this series of cell lines. The fact that the predominant form of OPN secreted by the 21T series is of high molecular weight is also interesting, and would be consistent with the observations of Beninati et al (31) that transglutaminase catalyzed covalent cross-linking of OPN may occur either to itself to form high molecular weight OPN aggregates, or to fibronectin (and perhaps other extracellular matrix components) to form OPN/fibronectin (or other ECM component) complexes. The relative biological activity of these complexes has not yet been shown in comparison with monomeric OPN. Also of note is that EGF, a growth factor that is essential to the continued propagation of the 21T series cell lines in culture (24), and which is thus included in the culture medium for routine culture, has been shown to itself induce increased cell surface transglutaminase activity in some cell types (32,33), which might at least in part account for the predominance of a high molecular weight (presumably conjugated) form in conditioned medium from the 21T series cell lines in comparison with MDA-MB-435 cells, which are normally cultured in the absence of exogenously added EGF. In other cell types (chondrocytes), EGF is known to increase the phosphorylation status of OPN (34). If this were the case in breast epithelial cells as well, this would be another potential mechanism for post-translational modification to a higher molecular weight form. Finally, the relatively low basal levels of expression of OPN in the 21T series cell lines (21PT and 21NT in particular) makes these cell

lines good candidates for assessment of the effects of upregulation of OPN expression on malignancy.

OBJECTIVE 2: **To determine whether up-regulation of OPN expression changes the malignant properties of these cells.**

The initial generation and screening of transfectants was described in last year's annual report. This information is again outlined here, along with new results of Western analysis of conditioned media from these cell lines, as background for the presentation of results from the first series of *in vivo* assays in the nude mouse, which were pending at the time of last year's annual report.

METHODS

Transfections

An expression vector for use in transfection has been generated by cloning the full-length human OPN cDNA (EcoRI fragment of plasmid OP-10, [28]) into plasmid pcDNA3 (Invitrogen Corp., San Diego, CA) at the multiple cloning site between the strong, constitutive CMV immediate early gene enhancer-promoter and the (bovine) growth hormone polyadenylation and transcriptional termination signal sequences (between Not I and Apa I sites). This plasmid also contains the neomycin resistance gene, allowing for selection of stable transfectants in G418-containing medium. The control plasmid used for "vector-only" transfections consisted of the unmodified pcDNA3 plasmid. Transfections were performed using the LIPOFECTIN reagent and

the procedure described by the manufacturer (Gibco-BRL/Life Technologies), using 2μg plasmid DNA for every 100μl of OPTI-MEM I in Solution A, and 10μl LIPOFECTIN reagent for every 100μl of OPTI-MEM I in Solution B. Following a 48 hr recovery period, transfected cells were subcultured into αHE medium containing 200 μg/ml (active) G418 (Gibco-BRL/Life Technologies) in order to select out stable transfectants. Plates were incubated until discrete colonies had developed, at which time both pooled populations and cloned transfectants were isolated for expansion in culture and further analysis. Conditioned medium was prepared for each transfectant population (essentially as described above, but without the need for Centricon concentration) for initial screening by ELISA assay for OPN expression. As the basal level of OPN protein expression by the 21T series parental lines in general was quite low in comparison to that expressed by the transfectants, this proved to be the most efficient method of screening for integration **and expression** of the transfected OPN-containing plasmid DNA (as opposed to Southern analysis of all transfectants). Those transfectant pooled and cloned populations expressing the highest levels of OPN were then chosen for expansion, preparation of RNA, DNA, and cytosolic protein, and eventual injection into nude mice (as above).

ELISA for OPN protein expression by transfected cell populations

Initial screening of transfectants for OPN protein expression was performed by ELISA of conditioned medium, essentially as described previously for plasma (13,35).   Briefly, this is a capture ELISA based on high affinity mouse monoclonal (17) and rabbit polyclonal antibodies developed against a recombinant human OPN-GST fusion protein (GST-hOPN) (19) that recognizes native human OPN. Maxisorp immunoplates (Life Technologies, Burlington, Ontario,

Canada) were coated with mouse monoclonal anti-OPN antibody mAb53 (100 μl/well, 10μg/ml), then blocked with 1% BSA in ST buffer (0.15M NaCl, 0.01M Tris pH 8.0) with 0.05% Tween 20 (Bio-Rad, Mississauga, Ontario, Canada). The wells were extensively washed with the ST-Tween 20 buffer prior to loading 100 μl of conditioned medium at various dilutions in ST-Tween 20 buffer +1% BSA. The samples were incubated for two hours at 4°C for the primary antigen capture step. Sequential incubations at 37°C of 100μl followed by washing were performed with (a) rabbit anti-OPN antibodies (0.8 μg/ml); (b) biotinylated goat anti-rabbit IgG (1:2000 dilution, Jackson Immunological Laboratories, Inc., West Grove, PA). After washing, streptavidin conjugated alkaline phosphatase (1:2000, Jackson Immunological Laboratories Inc.) was added for 30 minutes at 37°C. The wells were washed with buffer and 100μl of p-nitrophenyl phosphate (1 mg/ml in 100 mM Tris pH 9.5, 100mM NaCl and 5 mM MgCl$_2$) was added and the signal was allowed to develop at room temperature over 4-6 minutes. The reaction was stopped with 50 μl of 0.2 M Na$_2$EDTA (pH 8.0). A Bio-Rad plate reader was used to quantify the color signal. Recombinant GST-hOPN fusion protein (19) was used as standard, and background estimated by comparison against equivalent amounts of BSA protein. Internal controls of samples of known OPN concentration were used to normalize OPN values obtained from independent assays.

Tumorigenicity and "spontaneous" and "experimental" metastasis assay in nude mice

For tumorigenicity and "spontaneous" metastasis assay, 10^7 cells are injected into a mammary fat pad (first inguinal) of female nude mice at 6 weeks of age. Mice are observed weekly, and are kept for up to 12 months. At the time of sacrifice, a necropsy is performed, including examination of the injection site in the mammary fat pad and a survey for gross

metastases. The mammary fat pad (including primary tumor if present) and associated draining lymph node are dissected out, fixed and processed for light microscopy (routine H&E). Organs (including lungs, heart, liver, spleen, kidneys, brain) are fixed and examined for the presence of metastatic disease. Any other tissues suspicious for metastatic involvement are fixed and processed for light microscopy (routine H&E).

For "experimental" metastasis assay, 10^6 cells are injected intravenously into the tail vein of female nude mice at 6 weeks of age. These mice are kept for up to 12 months, at which time they are euthanized and examined for presence of metastatic disease. Necropsy is performed as described above, except that the mammary fat pad dissection is not performed. Lungs of all mice are embedded in toto and processed and sectioned for light microscopy by routine H&E.

RESULTS

The OPN-containing expression vector, prepared as described above, was used in parallel with control experiments using the unmodified parental plasmid (pcDNA3) for LIPOFECTIN transfections (as described). Successful transfection of 21PT and 21NT cells was achieved, both with the OPN-containing construct and the unmodified pcDNA3 plasmid. Four pooled populations of G418-resistant OPN-transfected PT cells were obtained, designated PT/OPai, PT/OPaii, PT/OPbi, and PT/OPbii. These consisted of combined harvesting of approximately 20 colonies, 25 colonies, 20 colonies, and 20 colonies respectively. Two pools of G418-resistant vector-only control transfectants of 21PT cells were also obtained, designated PT/Ci and PT/Cii. These pools originated from combined harvesting of approximately 70 colonies and 50 colonies respectively. Similarly, four pools of G418-resistant OPN-transfected 21NT cells were obtained,

designated NT/OPai, NT/OPaii, NT/OPbi, and NT/OPbii. These consisted of combined harvesting of approximately 45 colonies, 25 colonies, 20 colonies, and 20 colonies respectively. G418-resistant control transfectants of 21NT cells were obtained and designated as NT/Ci and NT/Cii. These consisted of combined harvesting of approximately 20 colonies and 30 colonies respectively. From each of these pools, individual cloned populations were also isolated (7-12 per pool). Despite multiple attempts, transfections of 21MT-1 cells with either the OPN-containing plasmid or the vector control consistently resulted in only small, indistinct colonies which could not be successfully expanded. Combined harvesting of whole plates resulted in cell populations that gradually died off in culture. Thus, although initial low level expansion of transfectants was achieved, these transfectants were unstable and could not be continuously propogated for further analysis.

OPN expression by the transfected cells was assessed firstly by assay of conditioned media by ELISA. Those OPN-transfected pools and clones of 21PT and 21NT cells expressing the highest levels of secreted OPN (by ELISA) are shown in Figure 3a, along with representative vector-only transfected controls. Four OPN-transfected cell populations were thus chosen for further study; for 21PT cells, these are: pooled population PT/OPaii and clone 12 derived from Pool PT/OPaii, designated PT/OPaiiC12; for 21NT cells these are: pooled population NT/OPbi and clone 4 derived from pool NT/OPaii, designated NT/OPaiiC4. Both pooled and cloned vector-control transfectant populations consistently showed levels of OPN expression by ELISA that were barely above background (BSA-only control), such that pools PT/Ci and NT/Ci were arbitrarily chosen for further comparative analysis.

Conditioned media from these transfectant populations were then also examined by

Western analysis, as shown in Figure 3b. The OPN-transfected cells (PT/OPaiiPool; PT/OPaiiC12; NT/OPbiPool; NT/OPaiiC4) were found to secrete two major forms of OPN, migrating at about 66 kDa and 97 kDa, while vector-transfected controls (PT/Ci; NT/Ci) secreted low levels of OPN, predominantly of about 97 kDa.

On Feb. 19 and 20, 1997, 30 nude mice were injected i.v. by tail vein (5 mice each group) with: PT/Ci (control), PT/OPaiiPool (OPN transfected), NT/Ci (control), NT/OPbiPool (OPN transfected), MT-1 cells (untransfected), and MDA-MB-435 cells. On Feb 26 and 28, 1997, the same series of cell lines (minus the MDA cells) were injected into the mammary fat pads of groups of 5 nude mice (total of 25 nude mice) for tumorigenicity and "spontaneous" metastasis assay. Due to the early onset of signs of metastatic disease in the MDA-MB-435 tail-vein injected mice, it was necessary to sacrifice these mice at 3 months post-injection. Mice injected with 21T series-derived cell lines (either by mammary fat pad or tail vein) were kept for up to 12 months.

Results for this initial series of injections are shown in Tables 1 and 2. In mammary fat pad-injected mice (Table 1), all 5 mice injected with 21MT-1 (MT-1) cells formed tumors. Two of these five showed metastases to regional lymph node or distant sites. Of the 21NT transfectants, both the control (NT/Ci) and OPN-transfectant (NT/OPbiPool) cells tested formed tumors in 1/5 mice injected. Interestingly, in comparison with the tumor which formed in the mouse injected with the NT/Ci cells, the tumor that formed in the mouse injected with the NT/OPbiPool cells was much larger, with an invasive (as opposed to *in situ*) morphology and much larger areas of tumor necrosis. Further, this mouse showed evidence of metastases to subpleural lymphatics. Metastases from 21NT cells have not been previously documented (24), and none were found in the control group of mice described here. Of the 21PT transfectants, none

of the control (PT/Ci) or OPN-transfectant (PT/OPaiiPool) cells injected into the mammary fat pad formed primary tumors, and none showed evidence of metastasis. In "experimental" metastasis (tail vein injection) experiments (Table 2), 4/5 mice injected with positive control MDA-MB-435 cells showed evidence of metastasis. All four showed macroscopic metastases by three months after injection. Sites of metastasis included lungs, pleura, spleen, liver, brain, GI tract, pelvis, and para-aortic lymph nodes. Of mice injected (via tail vein) with MT-1 cells, 1/5 showed microscopic metastases, involved organs including lungs, spleen and liver. Although 0/5 mice injected (via tail vein) with NT/Ci cells showed metastases, 2/5 injected with NT/OPbiPool cells (OPN-transfected) showed evidence of microscopic lung metastases. This thus supplies further evidence that some OPN-transfected 21NT cells have acquired metastatic potential. In contrast, none of the mice injected with either PT/Ci or PT/OPaiiPool cells showed evidence of gross or microscopic metastases.

In order to expand the data base for the *in vivo* experiments, a second series of injections was performed in February and March of this year. Over February 24 to March 3, 1998, 41 nude mice were injected into the mammary fat pad as follows: 5 mice each with PT/Ci, PT/OPaiiPool, PT/OPaiiC12, NT/Ci, NT/OPbiPool, NT/OPaiiC4, and MT-1; 6 mice with MDA-MB-435 cells. Results of this series of injections are pending at the time of this report.

DISCUSSION

The OPN-containing expression vector was effective in obtaining stable transfectants of both 21PT and 21NT cells. A number of pooled and cloned OPN-transfectant populations (of both 21PT and 21NT) were established, and screening of these transfectants by ELISA has

allowed us to select out those expressing the highest levels of OPN protein for further study. High levels of OPN expression were confirmed in these transfectants by Northern analysis for OPN mRNA. Stable control (vector-only) transfectant populations of 21PT and 21NT were also obtained, and consistently express comparatively low levels of OPN protein and RNA.

Although, for technical reasons outined in last year's annual report, we have not been able to obtain stable transfectants of 21MT-1 cells, this will not significantly affect our ability to address the objectives of this project. It was agreed upon review of last year's report that it would be appropriate to use these cells as a positive control for *in vivo* and gene expression assays (as described in Tasks 1a and 3a). We have proceeded to assess the ability (or lack thereof) of OPN to confer tumorigenicity on non-tumorigenic cells (21PT), and the ability of OPN to confer metastatic ability on tumorigenic, non-metastatic cells (21NT) (see below). In addition, we will, and have been able to (see below) assess the ability of OPN to modify measureable cell properties associated with malignancy. We have continued to assess 21MT-1 cells as described in Task 3a, and will use these cells as a basis for comparison, representing a weakly metastatic cell line expressing low to intermediate basal levels of OPN. In addition, we will continue to use MDA-MB-435 cells as a positive control, representing a highly metastatic breast epithelial cell line expressing high levels of OPN.

Studies involving the *in vivo* growth characteristics of these cell lines are ongoing. The initial series of "spontaneous" and "experimental" metastasis assays in nude mice showed results for the 21T series controls consistent with those expected from the work of Band et al. (24). None of the 21PT/Ci injected mice (mammary fat pad or tail vein) showed evidence of primary tumor formation or metastasis. None of the 21NT/Ci injected mice (mammary fat pad or tail vein)

showed evidence of metastasis, and only 1/5 formed a primary tumor at the site of injection in those injected into the mammary fat pad. In contrast, results for MT-1 cells showed that all of the mice injected into the mammary fat pad formed a primary tumour, and that 2/5 mice injected into the mammary fat pad and 1/5 injected via the tail vein formed distant metastases. No dectable alteration in growth in nude mice has yet been found for OPN-transfected 21PT cells (PT/OPaiiPool) (i.e. remain non-tumorigenic, non-metastatic). However, the OPN-transfected 21NT cells (NT/OPbiPool) have shown evidence of distant metastases in 1/5 mice injected into the mammary fat pad and 2/5 mice injected via tail vein. Interestingly, although there is as of yet no evidence of increased tumor-take of OPN-transfected 21NT cells (1/5 mfp injected mice formed a primary tumor), the tumor that did form was larger, with more necrosis and a more invasive morphology, and was associated with metastases to subpleural lymphatics. In contrast, there is no evidence to date of metastasis from tumors of the parental 21NT cells (24), and none were detected in our NT/Ci controls.

In order to get a more quantitative impression of the affect of OPN on the malignant behavior of 21NT cells, as well as to clarify whether there is indeed no detectable alteration in *in vivo* growth behavior of OPN-transfected 21PT cells, another series of nude mouse injections has been performed. In addition to the pooled OPN-transfectant populations described above, this series includes the OPN-transfected clone of each of 21PT and 21NT cells which has been found (by ELISA and Western analysis) to express the highest level of OPN protein. Results of this series of injections are still pending at the time of this report.

OBJECTIVE 3: To examine how OPN affects other measurable cell properties associated with malignancy.

Results presented in last year's annual report showed evidence for involvement of OPN in cell adhesion, migration and invasion. The cell adhesion phenomenon was found to be RGD-dependent, implicating the involvement of cell surface integrins. The more malignant cell lines of the progression series were found to adhere better to OPN. The cell migration affect was found to be directed (i.e. chemo/haptotactic) and also RGD-dependent. Evidence for a synergistic interaction between OPN and growth factors EGF and HGF in inducing migration of 21PT and 21NT cells was also presented. Increased cellular invasion was demonstrated of all parental cell lines in response to exogenous OPN, and of OPN transfectants of 21PT and 21NT cells (PT/OPaiiPool vs. PT/Ci and NT/OPbiPool vs. NT/Ci) overexpressing endogenous OPN. A trend towards increased cell growth in low serum in the presence of exogenous OPN was shown for the 21T series parental cell lines, although the cell growth effect was not proven to be statistically significant.

Over the course of the past year, we have further investigated the cell adhesion and cell migration affects of OPN, focusing on determination of the specific cell surface integrins involved in these phenomena. We have repeated experiments examining the induction of cellular invasiveness by exogenous OPN, with results substantiating those presented in last year's report. We have extended the analysis of cellular invasiveness of OPN-transfectants to include the clones of OPN-transfected 21PT (PT/OPaiiC12) and OPN-transfected 21NT (NT/OPaiiC4) expressing the highest levels of OPN protein. We have further examined the influence of OPN on cell

growth in low serum, focusing on specific time points determined by the preliminary experiments outlined in last year's report. Experiments to examine cell growth ability in soft agar have been performed, comparing OPN- vs. control vector-transfected 21PT and 21NT cells with 21MT-1 and MDA-MB-435 cells. Finally, expression of a number of "malignancy" associated genes has been examined in OPN-transfected cells vs. controls, and in parental cell lines treated in culture with exogenous hrOPN.

METHODS

Cell growth in low serum

For comparison of cell growth under conditions of low serum in the presence or absence of OPN, cells were plated on 24 well plates at 5×10^3 cells/well in αHE with 0.5% FBS. Four wells were seeded per time point per cell line, either in the presence or absence of hrOPN (50 μg/ml). Cells were allowed to grow for the specified time period, then trypsinized and counted.

Cell adhesion

Cell adhesion assays were performed essentially as described previously (17-20). Briefly, wells of 96-well polystyrene plates were precoated by overnight incubation at 4°C with a given concentration of either human recombinant (hr)OPN (19), RGD deletion mutant hrOPN (mutOPN) (20), fibronectin (Collaborative Research, Bedford, MA), or BSA (heat-inactivated fraction V; Sigma Chemical Co, St. Louis, MO). Exponentially growing cells were trypsinized and treated with soyabean trypsin inhibitor, washed twice and resuspended in attachment medium (αHE plus 2 mg/ml BSA), and then added to plates at a concentration of 4×10^4 cells/well. Cells

were allowed to adhere at 37°C for specified times (range of 30 mins to 4 hours). Nonadherent cells were removed by rinsing twice in phosphate-buffered saline and adherent cells were fixed with 1% gluteraldehyde, stained with hematoxylin, and counted under a microscope.

In blocking experiments, cells were pretreated by suspension for 20 minutes in the attachment medium with the specified concentration of blocking antibody, then added to plates and incubated, rinsed, fixed, stained and examined as described above. Blocking antibodies used were as follows: anti-OPN monoclonal antibody 53 (mAb53) (17), anti-αvß3 integrin monoclonal antibody (LM609) (Calbiochem/Cedarlane Laboratories, Hornby, Ontario), anti-ß1 integrin monoclonal antibody (Gibco-BRL/Life Technologies), anti-αvß5 integrin monoclonal antibody (Gibco-BRL/Life Technologies), or normal mouse IgG (negative control) (Calbiochem/Cedarlane Laboratories).

Cell migration

Cell migration assays were performed essentially as described previously (20), using 24-well transwell chambers with polycarbonate filters of 8 μm pore size (Costar, Cambridge, MA). Gelatin (Sigma) was applied at 6 μg/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 800 μl of αHE plus 0.1% BSA, with or without test proteins. Cells (5 X 10^4) were added to each upper well in αHE medium with 0.1% BSA and incubated at 37°C; the time of incubation (5hrs) for this series of cell lines was based on preliminary experiments in which optimal time for achieving countable numbers of all cell lines to be examined was determined. At the end of the specified incubation time, the cells that had migrated to the undersurface of the filters were fixed in place

with gluteraldehyde 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 wet Q-tips. The lower

surfaces of the filters were examined microscopically under 100X magnification and representative

areas were counted to determine the number of cells that had migrated through the filters.

For 21PT, 21NT, and MDA-MB-435 cells, in order to assess for the integrin dependence

of the cell migration response, experiments were conducted in the presence or absence of each of

the same set of antibodies described above (added to the lower chamber at specified

concentration). Finally, the migration responses of 21PT and 21NT cells to EGF (12.5 ng/ml)

and human hepatocyte growth factor (HGF, 10 ng/ml, Sigma) were also assessed, by performing

the migration assay in α medium with hydrocortisone and 0.1% BSA, in either the presence or

absence of each growth factor. The ability of hrOPN to effect cell migration either alone or in

combination with EGF or HGF was also assessed by performing these assays in the presence or

absence of 50 μg/ml hrOPN.

Cell invasion

In vitro invasiveness through Matrigel was assayed as described previously (27), 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) per filter. The Matrigel

concentration was determined by preliminary experiments using MDA-MB-435 cells and

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 X 10⁴ cells per well in serum-free

αHE medium containing 0.1% BSA. The lower chamber was filled with serum-free culture

medium with 0.1% BSA and either 5 μg fibronectin (for assays involving transfectants) or 100

μg hrOPN (for assays of parental (non-transfected) cell lines). Plates were incubated for 72 hours

in a 5% CO_2 incubator at 37°C. Following incubation, the upper wells were removed and

inverted, fixed with 1% gluteraldehyde 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 X100 magnification.

All cell adhesion, migration and invasion assays were performed in triplicate. Statistical

differences between groups were assessed using the Mann-Whitney test, *t*-test, or ANOVA, using

SigmaStat (Jandel Scientific, San Rafael, CA) statistical software.

Expression of "malignancy-associated" genes in 21T series cells, baseline vs. OPN-induced

Near-confluent (85-90%) cell cultures of 21PT, 21NT, 21MT-1 and MDA-MB-435 cells

were incubated overnight (18 hours) in serum-free αHE medium (with 0.1% BSA) in either the

presence or absence of 50 μg/ml hrOPN. Cells were then trypsinized, counted, and washed in

PBS. Total cellular RNA was then prepared by the Trizol procedure. Similarly, total cellular

RNA was prepared from 21T series cell lines stably transfected with either the OPN-containing

vector (PT/OPaiiPool, PT/OPaiiC12, NT/OPbiPool, NT/OPaiiC4) or control vector (PT/CiPool,

NT/CiPool). RNA (10 μg/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, oligolabelled [^{32}P]-dCTP cDNA probes (labelled using a kit provided by Pharmacia, Baie d'Urfe, PQ), or with 5'[γ^{32}P]-ATP end-labelled oligomers (labelled using a kit provided by Oncogene Science, Manhasset NY), according to the procedures provided by the manufacturers, and as previously described (26,27).

Northern blots were consecutively stripped and reprobed with the series of probes described below. In each case, the previous probe is removed by washing 6-8X in boiling 0.1X SSC/0.1%SDS. The set of probes include: OPN (^{32}P oligolabelled full-length (1493 bp) human OPN c-DNA EcoRI cassette of plasmid OP-10 [14,28]), EGF receptor (EGFR) (^{32}P oligolabelled 1.65 kb EcoRI/ClaI cassette of plasmid hEGF-R (human EGF-R in Bluescript KS'[36]), hepatocyte growth factor (HGF) (^{32}P oligolabelled 540 bp BamHI-XhoI fragment of human HGF cDNA [37]), HGF receptor (HGFR) (800bp EcoRI-EcoRV fragment of the human *met* c-DNA [38]), MMP-9 (92 kDa Type IV collagenase) (1046 bp insert from plasmid p92MO1) (gift of Dr. W.G. Stetler-Stevenson), MMP-2 (72 kDa Type IV collagenase) (1117 bp insert from plasmid p3Ha) (39), cathepsin B (1.6 kb KpnI insert from plasmid pLC343) (gift of Dr. B. Sloane) (40), cathepsin D (2.0 kb insert from plasmid pM13mp10), (gift of Dr. H. Rochefort) (41), cathepsin L (800 bp insert from plasmid pHCL800.1) (gift of Dr. D.T. Denhardt) (42), urokinase-type plasminogen activator (40mer antisense oligonucleotide derived from the translated sequences of exon 4) (Calbiochem/Cederlane Laboratories) (43), urokinase-type plasminogen activator receptor (45mer antisense oligonucleotide probe to the first 15 amino acids [not including the signal peptide]) (44). Even loading of lanes was confirmed by probing blots with a human 18s rRNA probe (p100D9; a kind gift from Dr. D.T. Denhardt).

RESULTS

Results from experiments presented in last year's annual report showed a trend towards improved *in vitro* growth of 21PT and 21NT under conditions of low serum when hrOPN was added to the medium. In order to determine whether this effect is statistically significant, the experiment was repeated with increased numbers of samples at time points which showed the greatest separation on the growth curves previously obtained (days 6 and 14). The results are shown in Figure 4. No significant increase was seen in cell growth in the presence of hrOPN (50 μg/ml) for any of the parental cell lines (21PT, 21NT, 21MT-1 or MDA-MB-435). We have thus not been able to demonstrate a significant affect of OPN on cell growth in low serum conditions for any of the cell lines tested. These experiments need to be repeated, however, using higher concentrations of hrOPN, in order to more definitively exclude the possibility of an OPN affect.

Cell adhesion studies have shown that all of the 21T series cell lines bind to OPN, although with lesser avidity than MDA-MB-435 cells (Figure 5). Preliminary experiments using varying concentrations of hrOPN at a fixed time point showed optimal binding in the range of 10-50 μg/ml. At 10 μg/ml hrOPN and a 2 hour incubation time, cell attachment was seen in the following hierarchy: MDA-MB-435 > 21MT-1 > 21NT > 21PT. One-way ANOVA (Student-Newman-Keuls method) showed all of these differences to be significant at the $p < 0.05$ level. The rank order for cell adhesion ability to OPN thus corresponded to rank order of malignancy of each of these cell lines. Use of the RGD deletion mutant hrOPN protein in this assay resulted in only background levels of binding, indicating that the cell adhesion seen with intact hrOPN is integrin-dependent (Figure 5). We have further explored the nature of the cell surface integrin-mediated binding to hrOPN, using blocking antibodies to several integrins known to bind OPN in other

systems (αvß1, αvß3, αvß5) (Figures 6,7). We have found that only anti-αvß5 and anti-ß1 antibodies block binding of 21PT and 21NT to hrOPN. Anti-αvß3 antibody, in concentrations up to 25 μg/ml, showed no significant inhibition of cell binding of 21T series cells to hrOPN. In contrast, MDA-MB-435 cells showed cell binding to hrOPN that was inhibitable by all three anti-integrin antibodies (anti-αvß3, anti-αvß5 and anti-ß1). The blocking of MDA-MB-435 cell adhesion to hrOPN by anti-αvß3 antibody was significant at 15 μg/ml. These results would suggest that, in contrast to the highly malignant MDA-MB-435 cells, 21PT and 21NT cells either do not express αvß3 integrins, or express but do not use these integrins for cell binding to hrOPN.

Migration assays performed last year have shown a specific, directed, RGD-dependent response of all of the cell lines tested to hrOPN, with the greatest response seen in the MDA-MB-435 cells (results shown in 1997 annual report). The nature of the integrin-dependent, hrOPN-induced cell migration has been examined, focusing on the 21PT, 21NT, and MDA-MB-435 cell lines (Figures 8-10). Blocking experiments using the same anti-integrin antibodies described above have indicated that although MDA-MB-435 cells have been shown (above) to bind to αvß5 and ß1 integrins in addition to αvß3 (Figure 7), only αvß3 integrins appear to be important in hrOPN-induced cell migration of these cells (Figure 10). In contrast, 21PT and 21NT cells bind to hrOPN via αvß5 and ß1-dependent mechanisms (Figure 6), and show a migration response which is also αvß5 and ß1-dependent (Figures 8,9). Anti-αvß3 antibody, at concentrations up to 25-30 μg/ml, showed no significant influence on either cell adhesion or cell migration of 21PT or 21NT in response to hrOPN (Figures 6,8). This would again suggest that either 21PT and 21NT cells do not express αvß3 integrin, or it is not used in the cell migration response (or in cell adhesion) to hrOPN. Together, the cell adhesion and migration results thus indicate that while

cells at earlier stages of progression may bind to and migrate in response to OPN, their migration response is dependent upon different cell surface integrins than the highly malignant MDA-MB-435 cells. In addition to a migration response to hrOPN, we have also shown that 21PT and 21NT cells migrate in response to EGF and HGF (Figures 11,12). Interestingly, hrOPN was found to augment the migration response of both 21PT and 21NT cells to either EGF or HGF.

As described in our 1997 annual report, assays performed to examine the ability of the parental 21T series cell lines, and MDA-MB-435 cells to invade through basement membrane (Matrigel) in response to exogenous (hr)OPN, showed an inductive effect of OPN on all four cell lines. At the time of last year's report, we had also examined the invasiveness of the OPN-transfected pool of 21PT (PT/OPaiiPool) and 21NT (NT/OPbiPool) cells expressing the highest levels of OPN, in comparison with control-vector transfectants of each (PT/Ci, NT/Ci). We have since extended these experiments to include the OPN-transfected clone of 21PT (PT/OPaiiC12) and 21NT (NT/OPaiiC4) cells expressing highest levels of OPN (Figure 13, c.f. Figure 3). Using a 35 μg/well membrane of Matrigel and a 72 hour incubation, 21NT cells transfected with the control vector (NT/Ci) showed a basal level of invasion which was significantly greater than that of control vector-transfected 21PT (PT/Ci) cells. Interestingly, both pooled and cloned cell populations of OPN-transfected 21PT and 21NT cells (PT/OPaiiPool, PT/OPaiiC12; NT/OPbiPool, NT/OPaiiC4 respectively) showed significantly increased invasiveness over that of the respective control cell population ($p < 0.05$ for all using Student's t-test). The cellular invasiveness of NT/OPaiiC4 cells was found to approach that of the highly metastatic MDA-MB-435 control cell line.

Studies to assess expression of certain "malignancy-associated" genes in 21PT, 21NT,

21MT-1 and MDA-MB-435 cells induced by overnight (18 hour) incubation with 50 μg/ml hrOPN (vs. uninduced) have been performed. Results of Northern analysis for expression of a number of growth factor/receptor and other "malignancy-associated" genes are shown in Figure 14. One of the most interesting findings is the apparent induction of HGF receptor (HGFR, met) mRNA expression by incubation with hrOPN. The 8.0 kb band, which represents the full-length human HGFR (met) transcript, was seen to be strongly induced in 21PT, 21NT, and MDA-MB-435 cells. Although induction of HGFR mRNA was not seen for 21MT-1, these cells were found to show quite high basal levels of expression. In parallel with this, there was a suggestion of a slight increase in HGF mRNA expression of 21PT and 21NT cells after incubation with hrOPN as well. In contrast, no change in EGF receptor (EGFR) expression was seen. Although we have not yet examined EGF expression, this growth factor is an essential component of the cell culture medium for continuous propogation of the 21T series cells.

Of the secreted proteases examined, increased mRNA for all three cathepsins (B,D,L) was seen in 21PT and 21NT cells after 18 hr incubation with hrOPN. 21MT-1 cells showed upregulation of Cathepsin B, and MDA-MB-435 cells slight upregulation of Cathepsin L. At this time point (18 hrs), with this concentration of hrOPN (50 μg/ml), no significant increase in uPA, MMP-2 or MMP-9 was detected in any of the cell lines.

mRNA levels for the same set of secreted proteases described above was also examined in OPN-transfectant (PT/OPaiiPool, PT/OPaiiC12; NT/OPbiPool, NT/OPaiiC4) and control vector transfectant (PT/Ci; NT/Ci) cell populations of 21PT and 21NT cells vs. MDA-MB-435 cells (Figure 15). As a reference, the blot has also been probed for OPN, showing high levels of OPN mRNA in the OPN-transfectants and in MDA-MB-435 cells, in keeping with the protein

data. A number of the secreted proteases showed differences in expression between the control 21PT/Ci (non-tumorigenic) and 21NT/Ci (tumorigenic) cells. Cathepsins B, D and L, and MMP-9, all showed higher level expression in 21NT/Ci than 21PT/Ci. In contrast, both cell lines expressed MMP-2 at similar levels. However, none of the cathepsins or metalloproteinases studied showed a consistent relationship of level of expression with that of OPN.

Of all the secreted proteases examined in the transfectant cell lines, only uPA showed a close association of expression with that of OPN. Increased uPA mRNA was seen in all of the OPN-transfectants (of both 21PT and 21NT), at a level commensurate with the level of OPN. Similarly, MDA-MB-435 cells expressed high levels of both OPN and uPA mRNA. Level of OPN and uPA mRNA expression also showed association with invasive capacity in the transwell assay (cf. Figure 13) (i.e. transfected cells expressing high levels of OPN also express high levels of uPA and invade better through Matrigel in transwell assays). uPA receptor (uPAR) expression was also examined, but was found to be expressed at comparable levels in control and OPN-transfected cells.

DISCUSSION

Although a trend towards improved cell growth in low serum-containing medium was seen for 21T series cells in the presence of 50 μg/ml hrOPN, this has not proved to be statistically significant (at day 6 or day 14 time points) upon replicate sampling. Similary, no statistically significant affect of 50 μg/ml hrOPN on cell growth in low serum has been found for MDA-MB-435 cells. However, as we have recent results from other work with human vascular endothelial cells (HUVEC) to suggest that higher concentrations of hrOPN (in the range of 150 μg/ml or

greater) do increase cell growth in low serum of HUVEC cells (unpublished observations), we will repeat the experiments with the mammary epithelial cell lines using concentrations of hrOPN in this range.

Cell adhesion studies have shown a range of cell binding to OPN, with MDA-MB-435>21MT-1>21NT>21PT (10 μg/ml OPN, 2 hour incubation, Figure 5). The rank order for cell adhesion to OPN thus corresponds to the rank order of malignancy of each of these cell lines. This could be due to increased receptor numbers, or increased affinity of receptors for OPN in the more malignant cell lines. Use of the RGD deletion mutant hrOPN protein in this assay resulted in only background levels of cell binding, indicating that the cell adhesion seen with intact hrOPN is integrin dependent (Figure 5). Previous studies examining the adhesion of other cell types (eg. osteoclasts, osteosarcoma cells, smooth muscle cells, endothelial cells) to OPN have indicated the involvement of integrin receptors αvβ3, αvβ1 and αvβ5 (2,45,46), and in some instances non-integrin receptors such as CD44 (21,47). In our studies, as only background binding is seen when RGD deletion mutant hrOPN is used in the adhesion assays, we are not able to detect a significant RGD-independent (i.e. non-integrin) binding of any of the four cell lines tested to hrOPN. This does not however exclude the possibility that cells may show CD44-dependent binding to native (post-translationally modified) OPN, and this is a possibility that we are now beginning to address. With respect to the integrin-dependent binding, we have shown that the non-metastatic 21T series cell lines (21PT and 21NT) differ from metastatic MDA-MB-435 cells in that 21PT and 21NT show only αvβ5 and β1 integrin-dependent cell adhesion, whereas MDA-MB-435 cells bind to αvβ3 in addition to αvβ5 and β1 integrins. This finding suggests the possibility that the ability to bind extracellular matrix components such as OPN via

αvß3 integrins may be important in some aspect of breast cancer metastasis, a concept that is further supported by cell migration experiments described below.

Integrin-dependence of the cell migration response to hrOPN has also been examined, focusing on 21PT, 21NT, and MDA-MB-435 cell lines (Figures 8-10). Interestingly, although MDA-MB-435 cells bind to hrOPN via αvß3, αvß5 and ß1 integrins (as described above), blocking experiments indicate that only αvß3 appears to be important in hrOPN-induced cell migration of MDA-MB-435 cells. In contrast, 21PT and 21NT cells show αvß5 and ß1-dependent, αvß3-independent migration in response to hrOPN. This would suggest that the 21T series cell lines examined may show a different expression pattern and spacial organization/availability of cell surface integrins than MDA-MB-435 cells, or that signal transduction pathway couplings involved in the cell migration response differ for these integrins between cell types. Consistent with these findings is the recent report of Wong *et al.* (48) that MDA-MB-435 cells express substantial levels of αvß3 integrin, whereas less malignant MDA-MB-231 cells and MCF-7 cells do not, and that MDA-MB-435 cells in turn adhere better to and migrate better in response to vitronectin (another αv integrin-binding molecule). In another recent study, Senger and Perruzzi (49) have shown that MDA-MB-435s cells (and human T24 bladder carcinoma cells) migrate in response to a GRGDS-containing thrombin-cleavage fragment of osteopontin, and that this migration is primarily αvß3 integrin-dependent. We have yet to examine whether or not the 21T series cell lines (which appear not to express αvß3 integrin) may respond in a similar fashion to thrombin-cleaved OPN. In addition, reports that OPN may bind to non-integrin receptors such as CD44 (21,47), suggest the need for investigating a possible role for CD44 binding in the response of mammary epithelial cells to OPN as well. Although we have

no evidence that non-integrin dependent binding of mammary epithelial cells occurs to hrOPN, cell adhesion experiments need to be repeated using native (post-translationally modified) OPN. If these cells are found to bind to native OPN via CD44, cell migration experiments in the presence or absence of blocking antibody to CD44 would then be performed as described above for the investigation of integrin involvement, in order to determine if CD44 also may be involved in the induction of cell migration by OPN.

Cell migration studies performed in this work also show evidence for a synergistic interaction between OPN and growth factors EGF and HGF in inducing the migration of 21PT and 21NT cells (Figures 11,12). Similar synergistic interactions beween growth factors and other integrin-binding proteins have been recently reported, and potential pathways for "cross-talk" between signal transduction pathways activated by these two different (but perhaps convergent) routes have been suggested (reviews in 50-54). Interestingly, we have now evidence from Northern analysis that one manner in which OPN may increase responsiveness of breast epithelial cells to HGF is via upregulation of HGFR (met) mRNA expression (Figure 15). Further work is now underway, in collaboration with Dr. Bruce Elliott's lab (Kingston, Ontario, Canada) to establish by quantitative RT-PCR whether HGF expression is significantly induced by OPN as well. Also in collaboration with Dr. Elliott's lab, we are exploring whether activation of HGFR (met) kinase is seen upon incubation with hrOPN. This work will thus continue to examine specific signal transduction components/pathways involved in OPN induction of cell migration, with the emphasis on potential for "cross-talk" with pathways which have been described for EGF and HGF.

Data for cell invasiveness of OPN-transfectants has now been extended to include the

highest OPN-expressing clones (PT/OPaiiC12, NT/OPaiiC4), as well as the pooled transfectants

(PT/OPaiiPool, NT/OPbiPool). These OPN-transfected 21PT and 21NT cells consistently showed

increased invasiveness over the pooled populations transfected with the control vector (PT/Ci,

NT/Ci) (Figure 13). The OPN-transfected 21NT clone expressing the highest levels of OPN

(NT/OPaiiC4) in fact showed a degree of invasiveness approaching that of the highly malignant

MDA-MB-435 cells. In general, level of OPN expression was found to be closely associated with

degree of cellular invasiveness in the transwell assay (cf Figure 15).

In the OPN-transfectants, cellular invasiveness and OPN expression were further found

to be closely associated with uPA mRNA expression (Figures 13, 15). We are presently in the

process of determining how this translates to uPA protein expression and functional activity. uPA

in turn is known to activate a variety of proteases (either directly or indirectly by activating

plasminogen) (eg. pro-MMP-1, -2, -3, -9, -14) (55-57) which are capable of digesting various

components of the ECM (eg. fibronectin, tenascin, laminin, proteoglycans) (reviewed in 57), as

well as activating certain growth factors (eg. HGF, TGF-ß, bFGF) (reviewed in 57, 58), some

of which may also be involved in cell migration and invasion (eg. HGF). Thus, through

triggering of a proteolytic cascade, with the ensuing modification of ECM components including

OPN, invading cells expressing uPA are capable not only of clearing a path of migration, but of

continuously modifying their environment. In addition, it has been found that uPA/uPAR

complexes bind vitronectin (which in turn binds to the same αv integrins as osteopontin), and that

uPAR may itself physically associate with certain integrins, such that some have suggested that

uPA may stimulate cell migration and invasion by non-proteolytic mechanisms as well (eg. by

modulating adhesion interactions at focal contacts, or by triggering signal transduction pathways

involved in the motility response) (58,59).

The finding that human breast epithelial cells upregulated for OPN expression are more invasive and express elevated levels of uPA lends functional significance to clinical evidence that high level expression of both OPN and uPA (and uPAR) have been shown to be associated with poor prognosis in breast cancer (eg. 13, 14, 58 [review]). It would appear from our work that the ability of breast epithelial cells to show increased invasiveness in response to OPN (with associated increased uPA expression) may be present even at early stages of progression (non-tumorigenic, non-metastatic 21PT cells), such that the availability of OPN in the microenvironment, or relative affinity for OPN may be critical. In this light, the ability of a tumor cell to synthesize its own OPN would be expected to afford a distinct selective advantage, allowing for increased migratory ability and invasive growth, presumably with increased propensity for metastasis.

In contrast to the findings in cells constituitively expressing high levels of endogenous OPN, 21T series and MDA-MB-435 cells induced for 18h in culture with 50 μg/ml of exogenous hrOPN showed a different pattern of protease mRNA expression. 21PT and 21NT cells showed increased expression of cathepsins B,D, and L in response to hrOPN. 21MT-1 cells showed upregulation of cathepsin B only, and MDA-MB-435 cells slight upregulation of cathepsin L only. At this time point (18 hours), with this concentration of hrOPN (50 μg/ml), no significant increase in uPA mRNA expression was detected in any of the cell lines. This may be due to different effects of short-term vs. long-term exposure to high levels of OPN, or to differences in cell response to hrOPN vs. native (endogenous) OPN. Time course experiments are presently underway to assess the prior possiblity. The latter possibility may in turn be assessed by

comparing the pattern of protease gene expression upon incubating with native vs. recombinant OPN at specific time points. Collectively, these studies are providing new insight into the different mechanisms by which OPN may induce invasive behavior of human mammary epithelial cells.

RECOMMENDATIONS

No new recommendations need be made at the time of this annual report. At the time of the 1997 annual report, it was recommended that the time course for the *in vivo* testing in nude mice be extended over the full three years of the award (primarily due to the long incubation times required for tumorigenesis from 21T series cells to occur). This was deemed acceptable to the reviewer, and we have proceeded with this in mind. Results from the first series of nude mouse injections are presented in this report, and are suggestive of an influence of transfected OPN on malignancy of 21NT-derived cells. Another series of injected mice is presently being monitored, which will allow for more quantitative assessment of the affect of OPN on malignancy of the 21NT-derived transfectants, and will help rule out any significant affect of transfected OPN on *in vivo* growth of 21PT cells. As we have now verified the status of the parental cells lines (21PT: immortal, non-tumorigenic; 21NT: tumorigenic, non-metastatic; 21MT-1: tumorigenic, weakly metastatic), Task 1 of the statement of work has been completed, and for Task 2, only the completion of the *in vivo* characterization of transfectants is still pending.

Also as agreed was appropriate at the time of last year's annual review, as we have been unsuccessful in repeated attempts at stably transfecting 21MT-1 cells (for reasons outlined in last year's report), and as the ability to obtain transfectants of OPN will not affect our ability to

address the objectives of this project, we have kept 21MT-1 cells (along with MDA-MB-435 cells), as a basis for comparison and positive control in *in vivo* and gene expression assays. The recommendation that studies regarding the effects of transfected OPN be focused on the 21PT and 21NT cells was accepted at the time of last year's annual report.

We would like to continue to examine the cell surface receptors involved in the induced cell migration effect of OPN, as was deemed appropriate at the time of last year's review. As mentioned in the text of this year's report, in addition to information presented here on integrin-dependence of OPN-induced migration, we would like to extend the analysis to possible CD44 binding of native OPN as well. In addition, as we have found exciting potential for collaborative interaction between OPN and HGF-induced pathways in migration of these cells, we would like to continue to expand our knowledge of the mechanisms of this interaction as well, in work to be done in continuing collabortation with Dr. Bruce Elliott's laboratory.

CONCLUSIONS

This project is proceeding as outlined in the statement of work, with good progress. Manuscripts related to this work are included in the Appendix section of this report. Results from this work have also been presented at the U.S. Army BCP "Era of Hope" meeting in Washington D.C. (Nov. 1997) and the San Antonio Breast Cancer Symposium (Dec. 1997), as well as locally (Ontario Association of Pathologists meeting, June 1998, London, Ontario, Canada). A further abstract has been accepted for poster presentation at the Metastasis Research Society meeting in San Diego, Oct. 1998.

Work to this point has revealed a number of potential biological functions for OPN in the malignancy of human breast cancer. Using the model system of the 21T series cell lines, along with the highly metastatic MDA-MB-435 cells, we have shown that the more malignant cells of this series not only secrete higher levels of OPN protein, but bind better to OPN in cell adhesion assays (i.e. MDA-MB-435 > MT-1 > 21NT > 21PT). The latter would suggest either higher numbers of appropriate cell surface receptors for OPN on the more malignant cells, or a higher affinity/availability of those present for OPN binding. We have identified an integrin-dependence of this OPN binding, and identified the specific integrins involved. Interestingly, we have found that the non-metastatic 21PT and 21NT cells make use of only $\alpha v \beta 5$ and $\beta 1$ receptors, whereas metastatic MDA-MB-435 cells in addition use $\alpha v \beta 3$, a finding which appears to be important in cell migration phenomena as well, as described below.

In addition, we have shown (in data presented in the 1997 annual report) that all of the breast epithelial cell lines tested (21PT, 21NT, 21MT-1, and MDA-MB-435 cells) respond to OPN by increased cell migration and increased invasion through Matrigel (basement membrane).

In more detailed work focusing on 21PT and 21NT cells (in comparison with MDA-MB-435 cells), we have found that cell migration induced by OPN is integrin-dependent. Whereas $\alpha v \beta 5$ and $\beta 1$ are important in migration of 21PT and 21NT cells, only $\alpha v \beta 3$ is found to be involved in migration of MDA-MB-435 cells, suggesting that the expression and/or availability of these integrins, or relative coupling with signal tranduction pathways involved in cell migration may differ in cells at different stages of progression.

A synergistic relationship between OPN and EGF and HGF in inducing cell motility has also been identified through this work. Northern analysis has shown induction of HGFR (met) mRNA by incubation with OPN, and have suggested possible upregulation of HGF expression as well. In collaboration with Dr. Elliott's lab, these findings are being verified by quantitative PCR, and any changes in amount or activity of HGFR (met) protein are also being assessed.

The increased cell invasiveness (through Matrigel) in response to OPN was seen both using exogenous hrOPN, and by assessment of pools of transfected (21PT and 21NT) cells stably expressing high levels of endogenous OPN. This may be due to some combination of OPN effect on cell motility, as well as on properties related to the ability to breakdown and pass through a basement membrane barrier. Studies to examine the mRNA expression of various secreted proteases have shown different patterns of expression in cells transiently induced with exogenous hrOPN than in cells constitutively overexpressing endogenous (transfected) OPN. Cells induced by transient (18 hour) exposure to exogenous hrOPN showed increased mRNA expression of various cathepsins (B,D, and L for 21PT and 21NT cells; cathepsin B for MT-1; cathepsin L for MDA-MB-435). In contrast, constitutive overexpression of endogenous (transfected) OPN resulted in increased mRNA expression of uPA only. These findings suggest that expression of

various different proteases involved in cell invasiveness may indeed be altered by OPN, and that different patterns of protease induction may be seen with transient vs. prolonged OPN effect, or with different forms (recombinant, unmodified vs. native, post-translationally modified) of OPN. These different possibilities will be further tested by time course experiments and transient induction experiments using native OPN.

This work is thus providing us with candidate invasiveness-associated genes which may be regulated by OPN. The evidence for upregulation of uPA mRNA in the presence of constitutive high levels of endogenous (transfected) OPN is particularly compelling, as the level of OPN expression, uPA expression, and invasiveness of the transfected cells appear to be quite closely associated. We would like to extend these studies to assessment of the amount and activity of uPA protein present in OPN-transfected vs. untransfected cells, to further strengthen this association.

Studies involving the *in vivo* growth characteristics of these cell lines are ongoing. The initial series of "spontaneous" and "experimental" metastasis assays in nude mice showed no primary tumor formation, and no metastases in either the control vector transfected or OPN-transfected pool of 21PT cells. Hence, to this point, we have found no evidence for induction of tumorigenicity or metastatic ability of 21PT cells by OPN. Although no increase in tumor-take has yet been found for the OPN-transfected pool of 21NT cells, these cells showed metastases in both "spontaneous" and "experimental" metastasis assays, whereas, as expected the control vector transfected 21NT cells did not. A second series of nude mouse injections are presently being monitored, which will extend these results and also include the OPN-transfected clone of each of 21PT and 21NT cells expressing the highest levels of OPN protein.

Interestingly, one pattern that appears to be forming in this work is that breast epithelial cells actually are capable of responding to OPN even from early stages of tumor progression (transformed, non-tumorigenic [i.e. 21PT]). At this early stage, the cells are already capable of responding to either exogenous or endogenous OPN by increased directed cell movement, and even by increased invasiveness through basement membrane in response to OPN. (In the case of 21PT cells, however, we have not yet been able to show that the increased *in vitro* motility and invasiveness induced by OPN translates to increased malignancy *in vivo*.) Differences between cells at various stages of progression may lie more in their **relative** ability to bind OPN (reflected in their degree of adhesiveness), and perhaps also in their relative ability to synthesize their own OPN (i.e. degree of autonomy for OPN expression). This concept fits well with our finding in lymph node negative breast cancer patients that it is the amount of OPN protein present in the tumor cells themselves (as opposed to within tumor infiltrating inflammatory cells), that predicts survival (14). In addition, the specific findings of OPN-induced expression of HGFR (met) and certain secreted proteases (cathepsins, uPA) are now providing more specific targets for the analysis of the manner in which OPN may influence cell motility and invasiveness.

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Table 1: "Spontaneous" Metastasis Assay in Nude Mice Following Mammary Fat Pad Injection

Cell Type	# of mice with 1° tumor/#of mice in group	Maximum tumor area	# of mice with lymph node metastasis	# of mice with distant metastasis
PT/Ci	0/5	NA	0/5	0/5
PT/OPaiiPool	0/5	NA	0/5	0/5
NT/Ci	1/5	4 mm²	0/5	0/5
NT/OPbiPool	1/5	25 mm²	1/5	1/5
MT-1	5/5	mean = 112 mm²	2/5	2/5

NA = Not Applicable

Table 2: "Experimental" Metastasis Assay in Nude Mice
          Following Tail Vein Injection

Cell Type	# of mice with metastasis/# of mice in group	Site of metastasis
PT/Ci	0/5	NA
PT/OPaiiPool	0/5	NA
NT/Ci	0/5	NA
NT/OPbiPool	2/5 (micro)	lungs
MT-1	1/5 (micro)	lungs, spleen, liver
MDA-MB-435	4/5 (macro)	lungs, pleura, spleen, liver, brain, GI tract, pelvis, para-aortic lymph nodes

NA = Not Applicable

FIGURE 1

Northern analysis of OPN mRNA expression by 21T series human mammary epithelial cell lines (21PT, 21NT, 21MT-1) vs. MDA-MB-435 (highly metastatic human breast carcinoma) cells. Total RNA (10 μg/lane) was separated, blotted, and probed as described in Methods. The OPN mRNA message size was 1.8 kb (upper panel). Equivalent RNA loading and integrity were verified by assessment of 18s rRNA bands (2.1 kb, lower panel).

Figure 1

FIGURE 2

Western blot analysis of OPN protein in a) conditioned media and b) cell lysates of 21T series cells vs. MDA-MB-435 cells. Protein was fractionated in SDS-PAGE and detected with mAb53 as described in Methods. In a), serum-free culture supernatants were concentrated by microfiltration prior to fractionation in 12% SDS-PAGE. The equivalent of 8-10 μg of total secreted protein was loaded after correction for cell equivalents based on cell counts at the time of collection of conditioned media (see Methods). In b), 20 μg of total cell lysate was loaded per lane, followed by fractionation in 8% SDS-PAGE. Molecular mass markers used were biotinylated protein standards (BioRad).

FIGURE 2

a

116 kDa
97.4 kDa
66.0 kDa

21PT   21NT   21MT-1   MDA-MB-435

b

66.0 kDa
45.0 kDa

21PT   21NT   21MT-1   MDA-MB-435

FIGURE 3

Relative level of OPN protein expression of 21PT and 21NT OPN-transfected cells vs. control vector-transfected cells, as determined by ELISA (a) and Western analysis (b) of conditioned media. ELISA assay and Western blotting were performed as outlined in Methods and in Figure 2. Cell lines are as follows: 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 levels of OPN protein); PT/OPaiiC12: clone 12 of OPN-transfected 21PT pool aii (clone expressing highest levels 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 levels of OPN protein); NT/OPaiiC4: clone 4 of OPN-transfected 21NT pool aii (clone expressing highest levels of OPN protein). Conditioned media from MDA-MB-435 cells is included in (b) as a positive control and basis for comparison.

FIGURE 3

a

b

FIGURE 4

Growth in low serum (0.5% FBS)-containing αHE medium of 21PT (PT), 21NT (NT), 21MT-1 (MT) and MDA-MB-435 (MDA) cells in either the absence (0) or presence (OP) of 50 μg/ml hrOPN. Cells were plated on 24-well plates at 5 X 10^3 cells/well. Four wells were trypsinized and counted per time point at each of days 6 and 14. The *bar graphs* represent the mean number of cells per well and the error bars represent the standard error of the mean. No significant improvement in cell growth in low serum-containing medium was seen in the presence of hrOPN (50 μg/ml) for any of the cell lines tested. (N.B. The apparent decrease in cell growth of 21PT cells in the presence of hrOPN is not a consistent finding.)

FIGURE 4

CELL GROWTH IN LOW SERUM MEDIUM (0.5 % FBS)
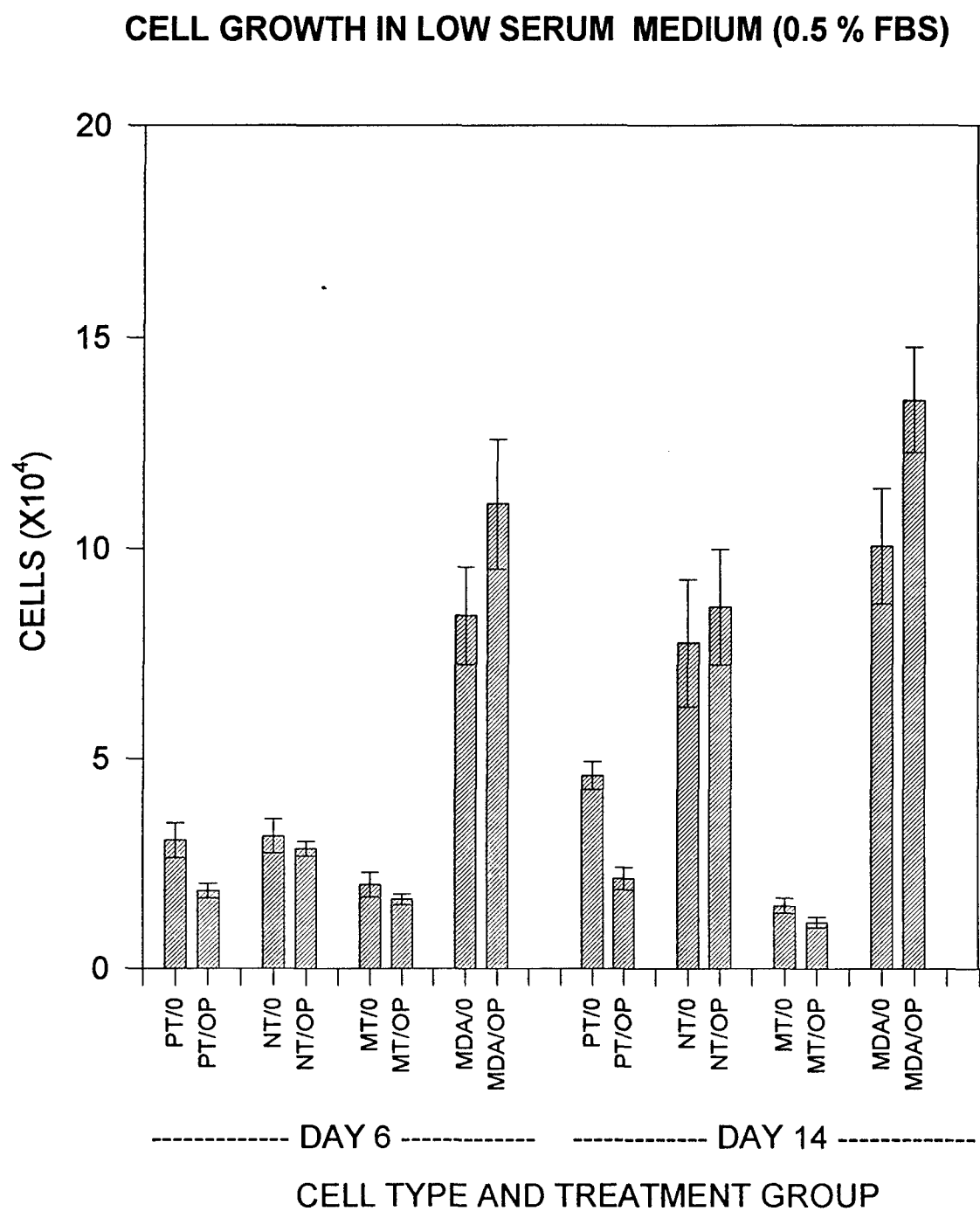
FIGURE 5

Adhesion of cells to coated surfaces. 21PT (PT), 21NT (NT), 21MT-1 (MT), and MDA-MB-435 (MDA) cells were added to 96-well plates that had been coated with hrOPN (OP), RGD deletion mutant hrOPN (mutOP), or BSA only (0), all at 10 μg/ml. Cells were allowed to adhere for 2 hours, and plates were washed, fixed, stained and counted as described in Methods. Attached cells per high power field were counted (field area 0.13 mm^2). The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean.

FIGURE 5

ADHESION ASSAY AT 2 HOURS
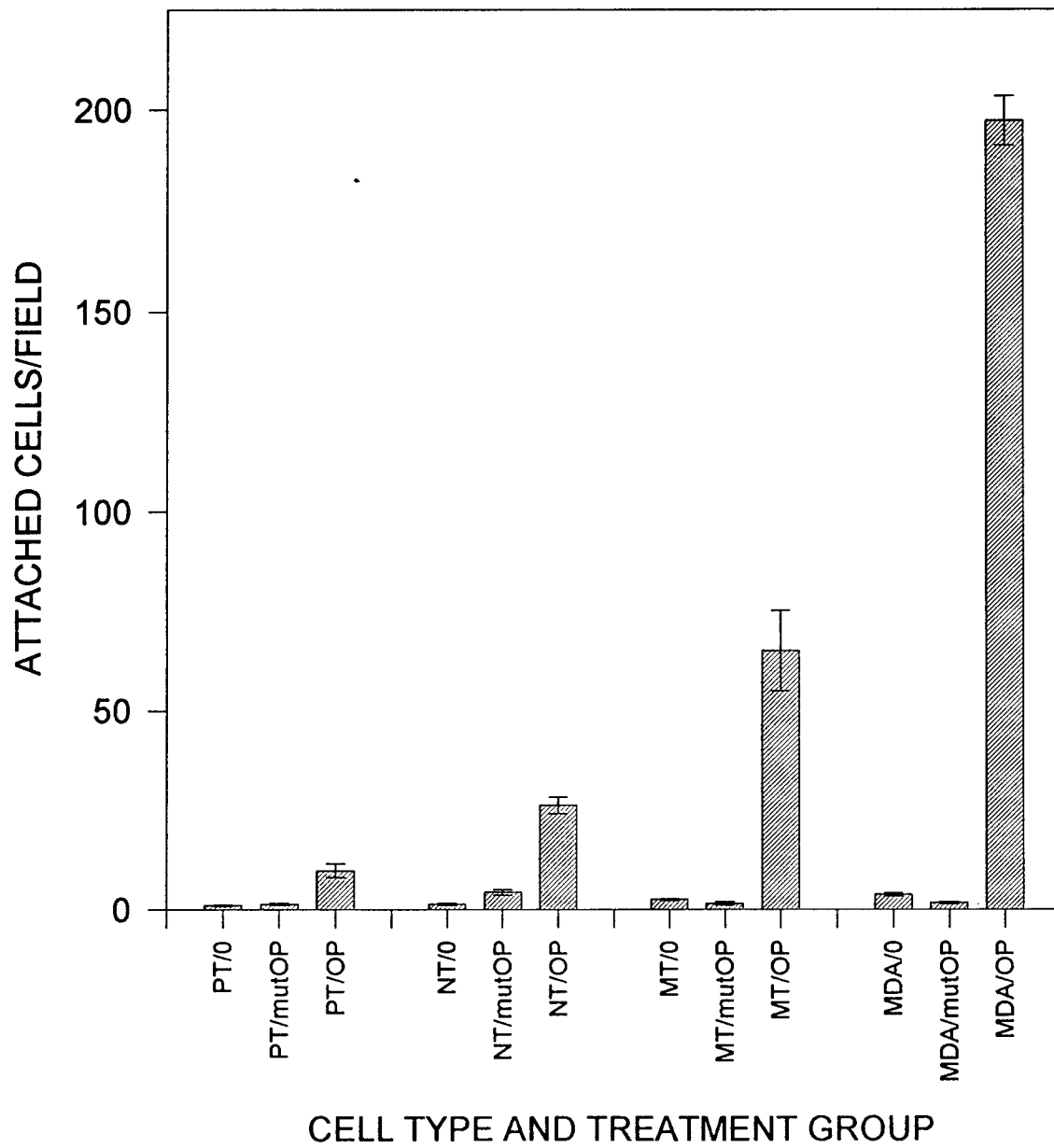
FIGURE 6

Integrin-dependence of 21PT (PT) and 21NT (NT) cell adhesion to hrOPN. Cells were added to wells that had been previously coated with either 10 μg/ml BSA only (0) or 10 μg/ml hrOPN (OP). In the specified samples, either non-specific mouse IgG (25 μg/ml) (nsIgG), or anti-β1 integrin (15 μg/ml) (b1ab), anti-αvβ3 integrin (25 μg/ml) (avb3ab), or anti-αvβ5 integrin (15 μg/ml) (avb5ab) blocking antibodies were added to the suspended cells in attachment medium and incubated for 20 minutes prior to adding the cells to the plate. Cells were allowed to adhere for 2 hours, and plates were washed, fixed, stained and counted as described in Methods. Attached cells per high power field were counted (field area 0.13 mm^2). The *bar graphs* represent the mean of four or five counts form each of three separate wells and the error bars represent the standard error of the mean.

FIGURE 6


ADHESION ASSAY AT 2 HOURS

CELL TYPE AND TREATMENT GROUP
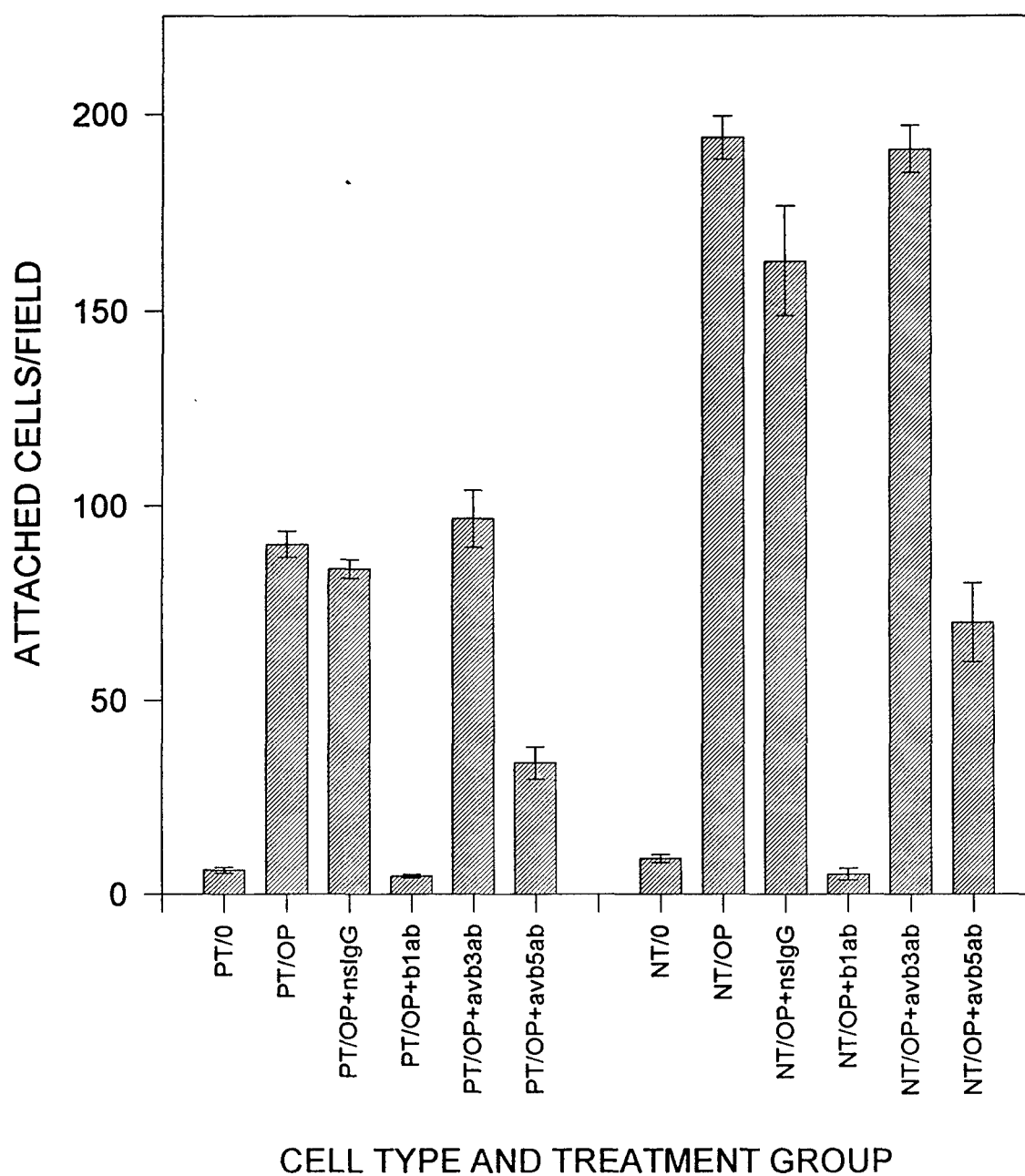
FIGURE 7

Integrin-dependence of MDA-MB-435 (MDA) cell adhesion to hrOPN. Cells were added to wells

that had been previously coated with either 10 μg/ml BSA only (0) or 10 μg/ml hrOPN (OP). In

the specified samples, either non-specific mouse IgG (15 μg/ml) (nsIgG), or anti-β1 integrin (15

μg/ml) (b1ab), anti-αvβ3 integrin (10 μg/ml) (avb3ab), or anti-αvβ5 integrin (15 μg/ml) (avb5ab)

blocking antibodies were added to the suspended cells in attachment medium and incubated for

20 minutes prior to adding the cells to the plate. Cells were allowed to adhere for 2 hours, and

plates were washed, fixed, stained and counted as described in Methods. Attached cells per high

power field were counted (field area 0.13 mm^2). The *bar graphs* represent the mean of four or

five counts form each of three separate wells and the error bars represent the standard error of the

mean.

FIGURE 7

ADHESION ASSAY AT 2 HOURS
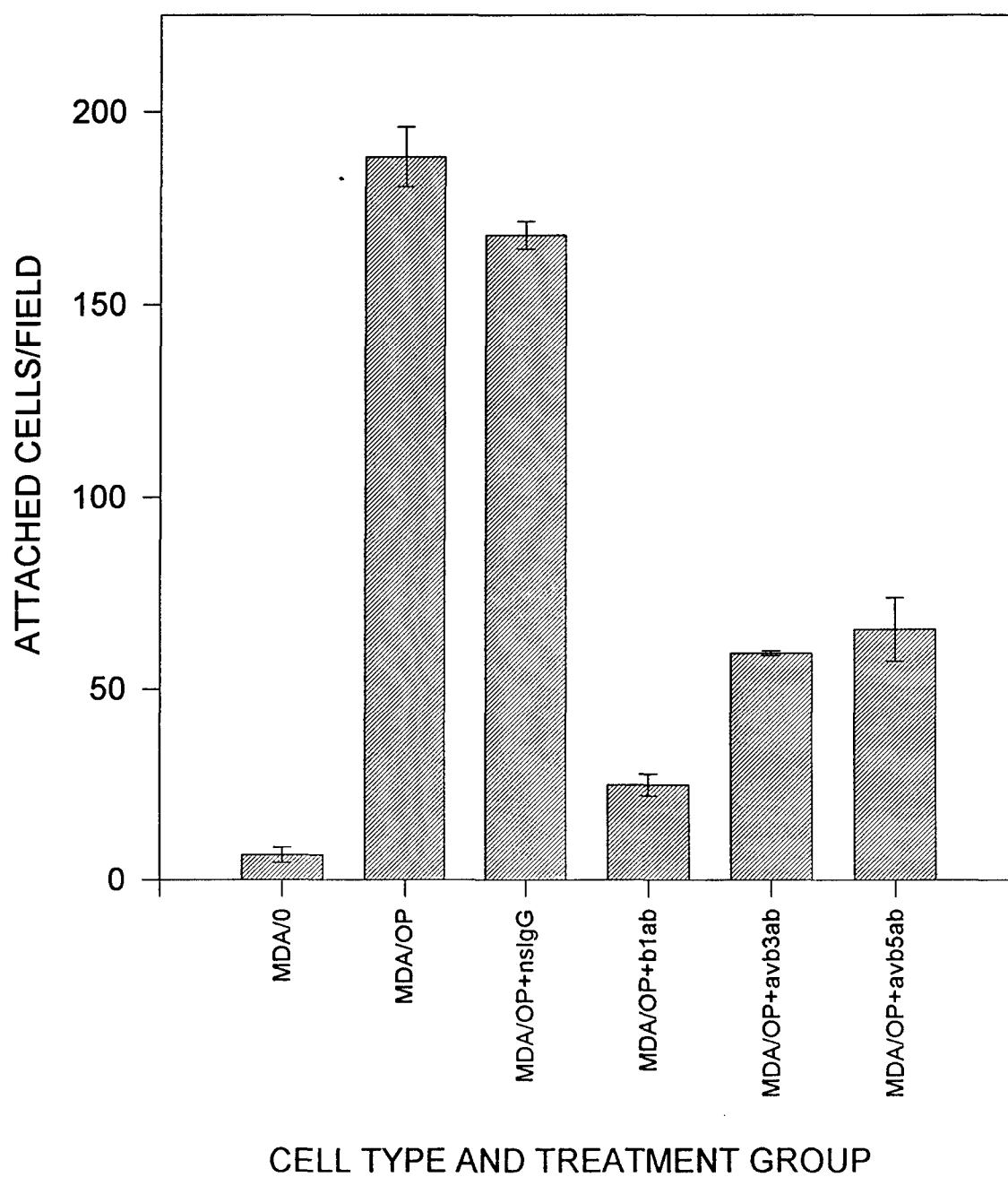
FIGURE 8

Integrin dependence ($\alpha v\beta 3$ vs. $\alpha v\beta 5$) of 21PT (PT) and 21NT (NT) cell migration response to hrOPN. Cells were seeded at 5 X 10^4 cells/well in the upper chamber of 8 μm pore transwells, with the specified lower chamber conditions, and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 15 μg/ml non-specific mouse IgG (OP+nIg); 50 μg/ml hrOPN with 30 μg/ml anti-$\alpha v\beta 3$ integrin blocking antibody (OP+aavb3); or 50 μg/ml hrOPN with 15 μg/ml anti-$\alpha v\beta 5$ integrin blocking antibody (OP+aavb5). The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean.
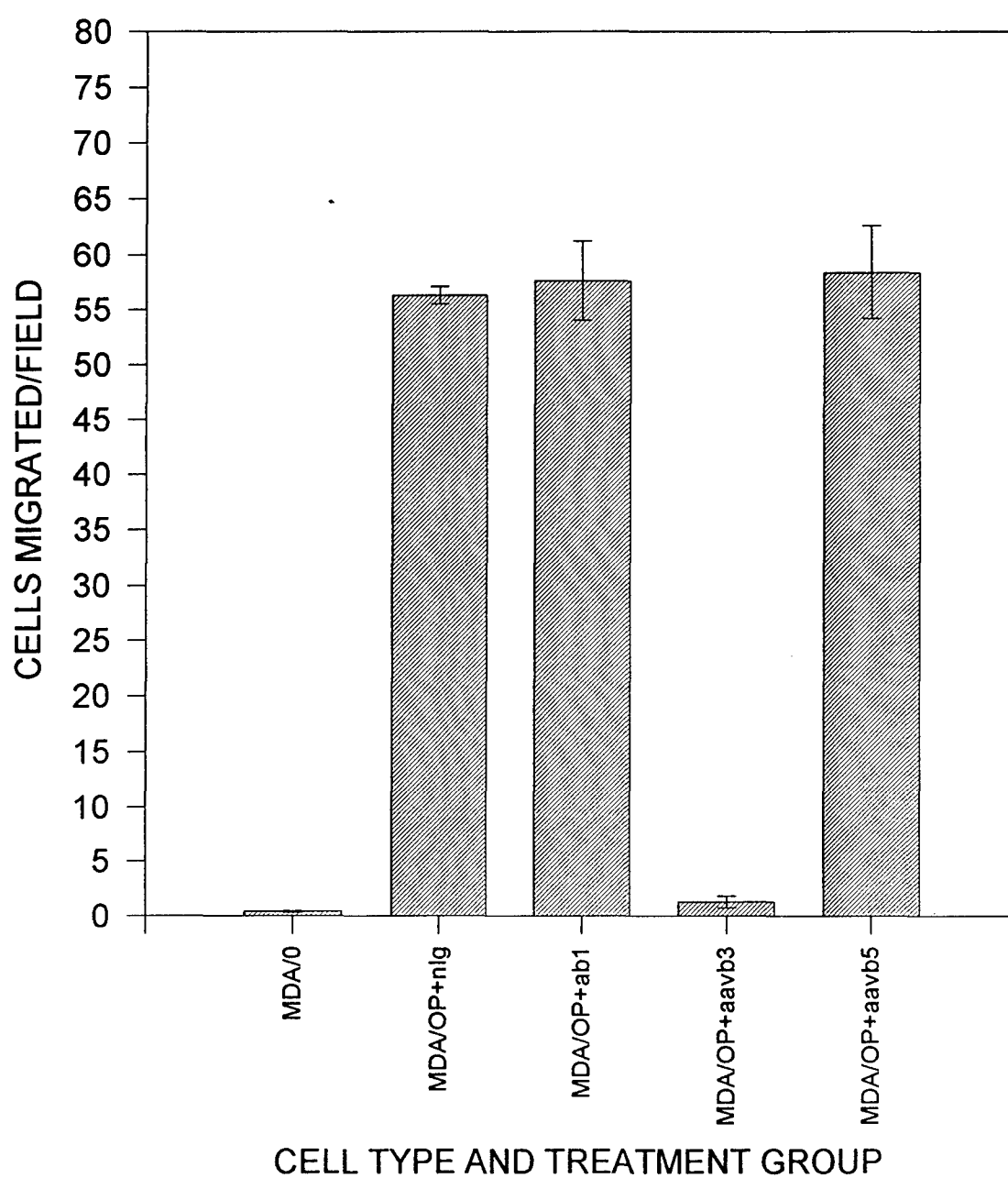
FIGURE 8

MIGRATION ASSAY AT 5HRS
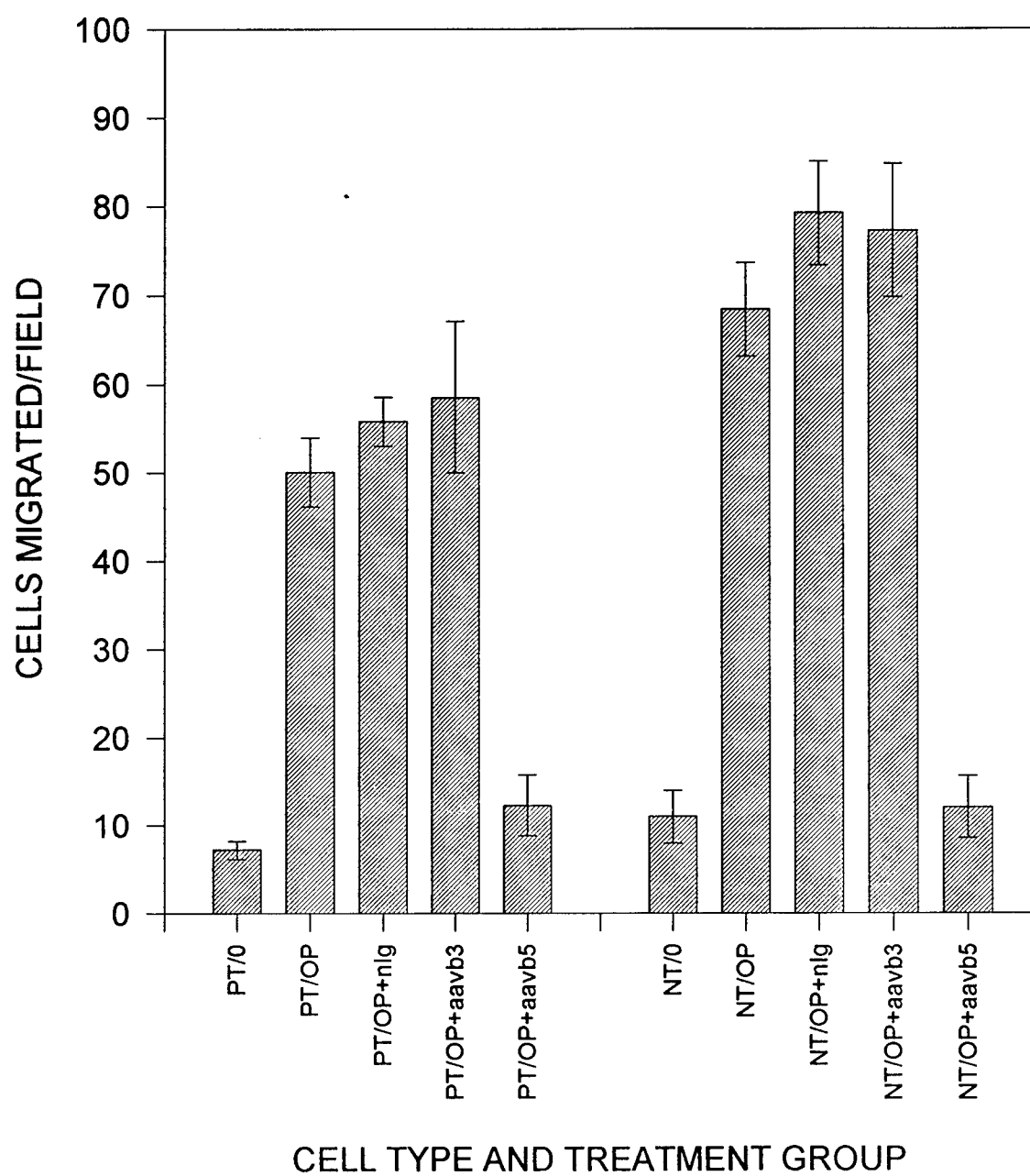
FIGURE 9

Integrin dependence (β1) of 21PT (PT) and 21NT (NT) cell migration response to hrOPN. Cells were seeded at 5 X 10^4 cells/well in the upper chamber of 8 μm pore transwells, with the specified lower chamber conditions, and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 15 μg/ml non-specific mouse IgG (OP+nIg); or 50 μg/ml hrOPN with 15 μg/ml anti-β1 integrin blocking antibody (OP+ab1). The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean.

FIGURE 9

MIGRATION ASSAY AT 5HRS
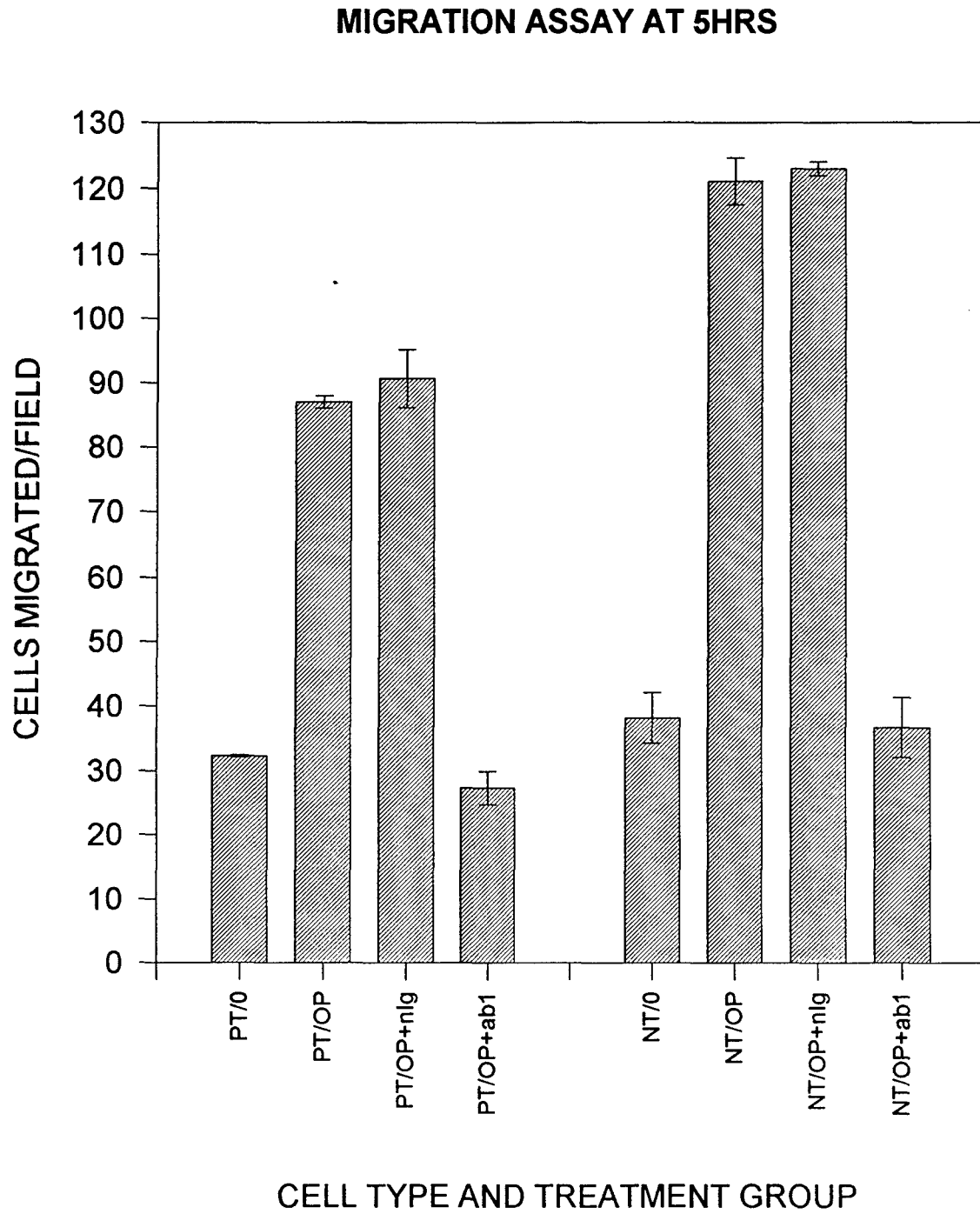
FIGURE 10

Integrin dependence (β1 vs. αvβ3 vs. αvβ5) of MDA-MB-435 (MDA) cell migration response to hrOPN. Cells were seeded at 5 X 10^4 cells/well in the upper chamber of 8 μm pore transwells, with the specified lower chamber conditions, and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. Lower chamber conditions were as follows: 0.1% BSA only (0); 50 μg/ml hrOPN only (OP); 50 μg/ml hrOPN with 25 μg/ml non-specific mouse IgG (OP+nIg); 50 μg/ml OPN with 15 μg/ml anti-β1 integrin blocking antibody (OP+ab1); 50 μg/ml hrOPN with 25 μg/ml anti-αvβ3 integrin blocking antibody (OP+aavb3); or 50 μg/ml hrOPN with 15 μg/ml anti-αvβ5 integrin blocking antibody (OP+aavb5). The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean.
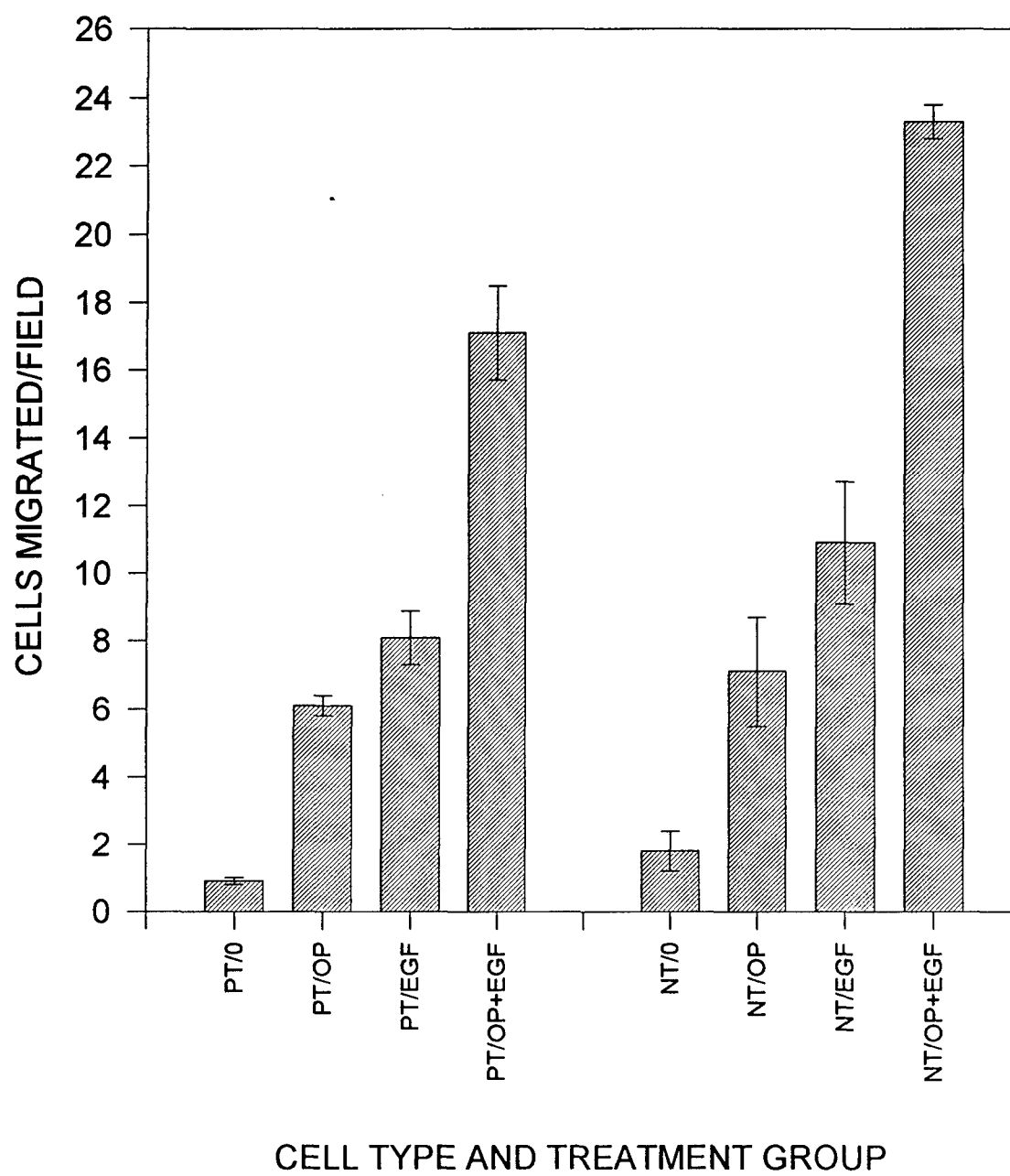
FIGURE 10

MIGRATION ASSAY AT 5HRS

FIGURE 11

Cell migration of 21PT and 21NT cells in response to EGF and hrOPN, alone and in combination. Contents of the lower chamber consisted of either: medium without EGF or hrOPN (0); medium with 50 μg/ml hrOPN (OP); medium with 12.5 ng/ml EGF (EGF); or medium with 50 μg/ml hrOPN and 12.5 ng/ml EGF (OP+EGF). Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean. Both 21PT (PT) and 21NT (NT) cells showed significantly increased migration in response to either hrOPN or EGF alone (all show $p < 0.05$ by one-way ANOVA). No significant difference was apparent for either cell line between hrOPN effect alone vs. EGF effect alone. For both 21PT and 21NT cells, the effect of hrOPN in combination with EGF was significantly greater than either agent alone ($p < 0.05$ by one-way ANOVA in all pairwise comparisons), and the combined effect was more than additive (i.e. synergistic).

FIGURE 11

MIGRATION ASSAY AT 5HRS


CELL TYPE AND TREATMENT GROUP

FIGURE 12

Cell migration of 21PT (PT) and 21NT (NT) cells in response to HGF and hrOPN, alone and in combination. Contents of the lower chamber consisted of either: medium (αH, no EGF) without HGF or hrOPN (0); medium with 50 μg/ml hrOPN (OP); medium with 10 ng/ml HGF (HGF); or medium with 50 μg/ml hrOPN and 10 ng/ml HGF (OP+HGF). Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 5 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted as described in Methods. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean. Multiple pairwise comparisons showed all four treatment conditions for both 21PT and 21NT to be significantly different from each other (all show $p < 0.05$ by one-way ANOVA). For both 21PT and 21NT cells, the effect of hrOPN in combination with HGF was more than additive (i.e. synergistic).
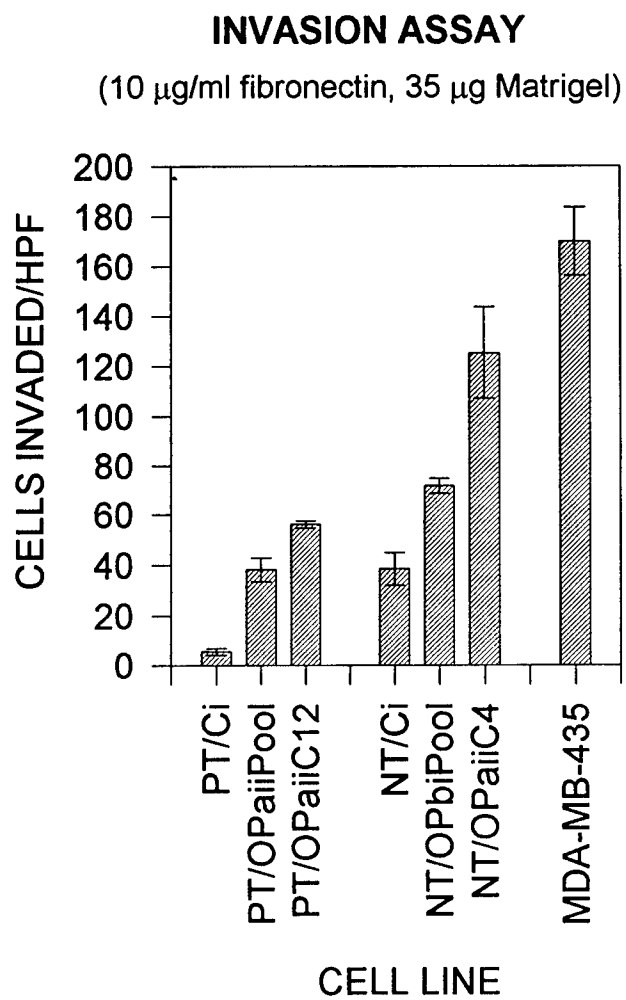
FIGURE 12

MIGRATION ASSAY AT 5HRS
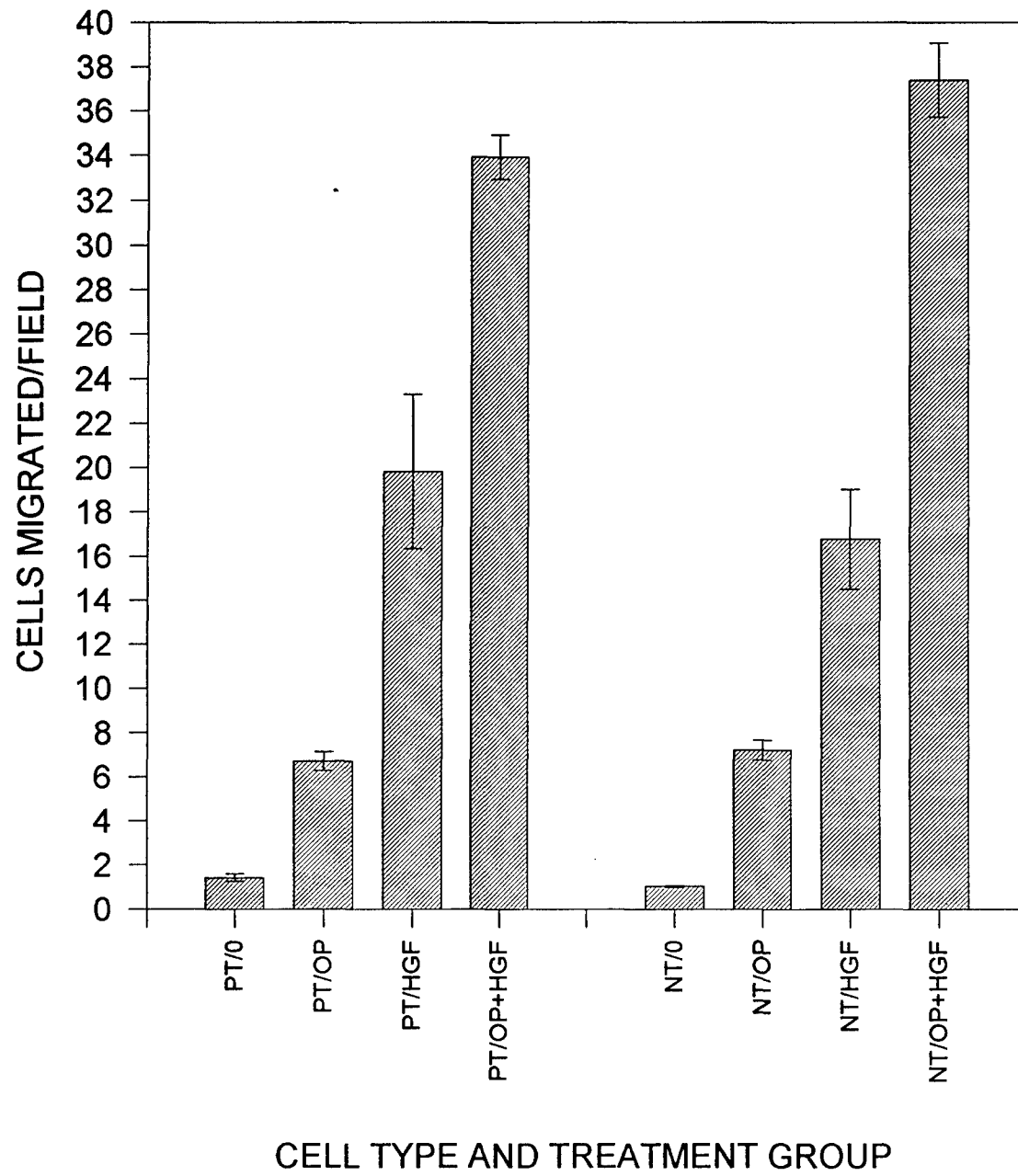
FIGURE 13

In vitro chemoinvasiveness of transfectant cell lines in the transwell assay. 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 expressing the highest levels of OPN (c.f. Figure 3). MDA-MB-435 cells are included as a positive control and basis for comparison. Invasion is in response to 10 μg/ml fibronectin in the lower chamber, through 8 μm pore filters precoated with 35 μg Matrigel, as described in Methods. Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 72 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted. The *bar graphs* represent the mean of counts from each of three separate wells and the error bars represent the standard error of the mean. OPN-transfected 21PT (PT/OPaiiPool, PT/OPaiiC12) and 21NT (NT/OPbiPool, NT/OPaiiC4) showed significantly increased invasiveness over the vector-only transfected controls (PT/Ci, NT/Ci) ($p < 0.05$ for all by Student's *t*-test.

FIGURE 13

INVASION ASSAY

(10 µg/ml fibronectin, 35 µg Matrigel)

FIGURE 14

Northern analysis for expression of mRNA of a number of different "malignancy-associated" genes in cells either induced (+OPN) or not induced (0) by 18 hour incubation with 50 μg/ml hrOPN. Cell lines examined included: 21PT (PT), 21NT (NT), 21MT-1 (MT-1) and MDA-MB-435 (MDA). Probes were prepared as outlined in Methods, and included: human osteopontin (OPN), Epidermal Growth Factor receptor (EGFR), Hepatocyte Growth Factor (HGF) and HGF Receptor (HGFR [met]), urokinase-type Plasminogen Activator (uPA), matrix metalloproteinase 9 (MMP-9; 92 kDa type IV collagenase), matrix metalloproteinase 2 (MMP-2; 72 kDa type IV collagenase), cathepsin B, cathepsin D, cathepsin L, and 18s ribosomal RNA (18s RNA).

FIGURE 14

83

FIGURE 15

Northern analysis of transfectant cell populations described in Figures 3 and 13 (vs. MDA-MB-435 cells) for expression of mRNA of a number of secreted proteases (and urokinase-type plasminogen activator receptor), as well as OPN. Probes were prepared as outlined in Methods, and included: human osteopontin (OPN), human urokinase-type plasminogen activator (uPA), urokinase-type plasminogen activator receptor (uPAR), matrix metalloproteinase 9 (MMP-9; 92 kDa type IV collagenase), matrix metalloproteinase 2 (MMP-2, 72 kDa type IV collagenase), cathepsin B, cathepsin D, cathepsin L, and 18s ribosomal RNA (18s RNA). Only uPA expression showed close association with OPN mRNA and cellular invasiveness (c.f. Figure 13).

FIGURE 15
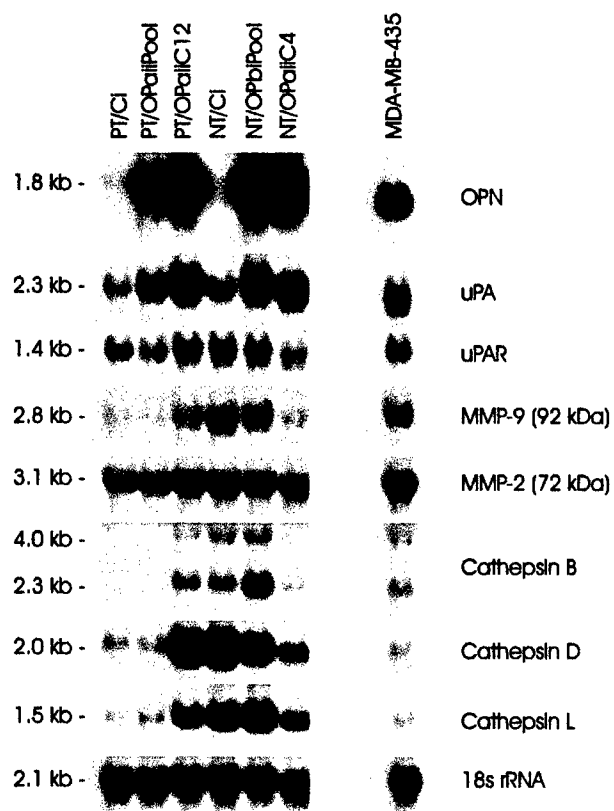
APPENDICES

Elevated Plasma Osteopontin in Metastatic Breast Cancer Associated with Increased Tumor Burden and Decreased Survival[1]

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ABSTRACT

Osteopontin (OPN) is a secreted, integrin-binding phosphoprotein that has been implicated in both normal and pathological processes; qualitative increases in OPN blood levels have been reported in a small number of patients with metastatic tumors of various kinds. We measured plasma OPN levels in 70 women with known metastatic breast carcinoma, 44 patient controls who were on follow-up after completion of adjuvant treatment for early breast cancer, and 35 normal volunteers.

The median plasma OPN of patients with metastatic disease was 142 μg/liter (range, 38–1312 μg/liter) and was significantly different ($P < 0.0001$, Mann Whitney U test) from both control groups (medians, 60 and 47 μg/liter; ranges, 15–117 and 22–122 μg/liter). Furthermore, we found that increasing plasma OPN is associated with shorter survival ($P < 0.001$) when patients were grouped in terciles for plasma OPN. This was also demonstrated when using a Cox proportional hazards model. Median plasma OPN levels were significantly increased for three or more sites of involvement (median, 232 μg/liter; $n = 13$) versus 1 or 2 metastatic sites (medians, 129 and 130 μg/liter; $n = 29$ and 28, respectively). Plasma OPN levels were correlated with other biochemical markers related to the extent of disease, such as serum alkaline phosphatase, aspartate succinate aminotransaminase, and albumin ($r = 0.81, 0.62$, and -0.56, respectively; all $P < 0.001$).

This study demonstrates a statistically significant elevation in plasma OPN in the majority (~70%) of a large series of patients with metastatic breast cancer when compared (95th percentile) to healthy women or patients who had completed adjuvant treatment for early-stage breast cancer. Furthermore, this is the first study to demonstrate that higher OPN levels in patients with metastatic breast cancer may be associated with an increased number of involved sites and decreased survival.

INTRODUCTION

OPN[3] is a secreted, integrin-binding phosphoprotein that has been implicated in various normal and pathological processes (reviewed in Refs. 1–4). OPN is expressed by many normal cells including various epithelial cells as well as activated macrophages. OPN is a tumor-associated protein secreted by many tumor cells in culture (reviewed in Refs. 4 and 5). In addition, OPN has been identified in a variety of types of human carcinomas, where its expression was localized primarily to macrophages (6). OPN expression was found to be higher in breast carcinomas than in benign breast lesions (7, 8). OPN also has been detected in a variety of human body fluids including blood, urine, and milk (5, 9–13).

Using Western blot analysis with polyclonal antibodies, Senger *et al.* (5) reported elevated levels of OPN in the plasma and serum of a small number of patients (10 of 13) with a variety of disseminated carcinomas. Included in that study were results from a single patient with metastatic breast cancer; no clinical data were available for the patients in that study. Further work on the significance of plasma OPN levels in metastatic cancer were hampered in part by the lack of specific antibodies to allow the development of a fast and reliable immunoassay. We generated high-avidity monoclonal antibodies to native osteopontin (14) and developed an ELISA for the quantitative measurement of OPN levels in plasma (15) and urine (12). In normal women, we found that plasma OPN levels were independent of hormonal influences of the menstrual cycle (15). Here we used this assay to quantify OPN plasma levels in 70 patients with metastatic breast cancer and appropriate control groups to determine if OPN levels are elevated in metastatic breast cancer, and if OPN levels are associated with clinicopathological findings or survival.

MATERIALS AND METHODS

Patients. This study was conducted on female patients aged >18 years who were being followed at the London Re-

[3] The abbreviations used are: OPN, osteopontin; AST, aspartate succinate aminotransaminase.

gional Cancer Centre, London, Ontario, Canada. All patients had initially presented with histologically confirmed primary invasive breast cancer and at the time of this study were being actively treated for metastatic breast cancer. The diagnosis of metastatic breast cancer had been established by clinical examination, and appropriate biochemical and radiological examination. Biopsy confirmation of metastases was not obtained. Clinical and laboratory information was extracted from the clinical patient records.

The patient control group consisted of individuals with previously treated early breast cancer who were being seen for routine clinical follow-up after completion of primary treatment according to centre guidelines for stage of disease. A minimum of six months had elapsed since the completion of all primary treatment. There was no clinical or laboratory evidence of either local or distant recurrence.

The second control group consisted of healthy, female employees at the London Regional Cancer Centre, who volunteered to participate in the study (15). Twenty-one were pre- or peri-menopausal with mean age of 39.7 ± 6.7 year (SD), and fourteen were postmenopausal with a mean age of 52.1 ± 6.3 year (SD) at the time of entry to that study.

Plasma Samples. A 5 ml sample of blood was obtained by venipuncture into a vacutainer containing EDTA as anticoagulant. Patient samples were obtained at the time of routine venipuncture for clinical or diagnostic testing. The samples were centrifuged at 2000 rpm at 4°C for 15 minutes. The separated plasma was removed, aliquoted and frozen at −20°C for future analysis. OPN was assayed using a quantitative ELISA developed in our laboratory (15). Laboratory personnel performing the OPN assays had no knowledge of the clinical status of the patients.

ELISA for Plasma OPN. We developed a capture ELISA based on high affinity mouse monoclonal (14) and rabbit polyclonal antibodies developed against a recombinant human OPN-GST fusion protein (GST-hOPN) (16) that recognize native human OPN. This assay has been described in detail elsewhere (15) except that here we performed the initial capture of OPN from plasma at 4°C, and we purified by affinity chromatography rabbit anti-OPN antibodies. Briefly, Maxisorp immunoplates (Life Technologies, Burlington, Ontario, Canada) were coated with mouse monoclonal antibody mAb53 (100 μl/well, 10 μg/ml), then blocked with 1% BSA in ST buffer (0.15 M NaCl, 0.01 M Tris, pH 8.0) with 0.05% Tween 20 (Bio-Rad, Mississauga, Ontario, Canada). The wells were extensively washed with the ST-Tween 20 buffer prior to loading 100 μl of patient plasma at various dilutions (at least an 8-fold range) in ST-Tween 20 buffer +1% BSA. The samples were incubated for two h at 4°C for the primary antigen capture step. Sequential incubations at 37°C of 100 μl followed by washing were performed with (a) rabbit anti-OPN antibodies (0.8 μg/ml); (b) biotinylated goat anti-rabbit IgG (1:2000 dilution, Jackson Immunological Laboratories Inc., West Grove, PA). After washing, streptavidin conjugated alkaline phosphatase (1:2000, Jackson Immunological Laboratories Inc., West Grove, PA) was added for 30 minutes at 37°C. The wells were washed with buffer and 100 μl of p-nitrophenyl phosphate (1 mg/ml in 100 mM Tris pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) was added and the signal was allowed to develop at room temperature over 4−6

minutes. The reaction was stopped with 50 μl of 0.2 M Na_2EDTA (pH 8.0). A Bio-Rad plate reader was used to quantify the color signal. Recombinant GST-hOPN fusion protein (16) was used as standard. Internal controls of plasma samples were used to normalize OPN values obtained from independent experiments.

The immunoassay conditions were optimized for the concentrations of coating with mAb53, rabbit antibodies against OPN, second antibodies, and developing reagents for the kinetics and temperature conditions described above. Mixing experiments of plasmas of patients with high and low OPN levels indicated that the immunoassay was linear under these conditions. The ELISA had a plateau signal, and for some plasmas, a prozone effect was observed for low dilutions of plasma. Thus, it was important to examine a range of plasma dilutions to interpolate OPN values from the log-linear portion of the standard curve, as we have described previously (15).

Western Blot Analysis. Blood OPN was also analyzed by SDS-PAGE and Western blotting (15) in representative samples to independently verify quantification by ELISA and to examine the molecular forms of OPN detected in the various plasmas. OPN was immunoadsorbed by incubating 100 μl of plasma sample with 5–10 μl of 50% slurry of CNBr-activated Sepharose beads (Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada) that had been conjugated with saturating levels of mAb53. Immunoadsorption with the beads was in a 500-μl volume of binding buffer, followed by washing with binding buffer containing 0.2% NP40 buffer. Adsorbed proteins were released by adding gel loading buffer and boiling for 5 min. For protein fractionation, 12% denaturing SDS-PAGE was used. Fractionated proteins were electrophoretically transferred to a nylon membrane for immunoblotting with a semi-dry system (Millipore Canada, Missisauga, Ontario, Canada), using conditions as described (15).

The blot was blocked with 3% BSA (in ST buffer) for at least 2 h at room temperature. After extensive washing with ST-Tween buffer, the blot was incubated with biotinylated mAb53 antibody (0.2 μg/ml) in 5 ml of ST-Tween buffer for 2 h at room temperature, using an incubator with a bottle rotator. The blot was extensively washed and then incubated for 30 min with streptavidin-horseradish peroxidase conjugate purchased from Jackson Immunological Laboratories. OPN-specific bands were detected by the enhanced chemiluminescence ECL system (Amersham Canada, Oakville, Ontario, Canada). X-ray film was exposed for 10–20 s. Immunoreactive bands were quantified using the Personal Densitometer SI and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Analysis of Results. The curve-fitting feature of Sigma-Plot (Jandel Scientific, San Jose, CA) was used to determine the best-fit parameters of the titration curve of the recombinant protein standard to the exponential rise function, and these parameters were used to interpolate unknown values as described previously (14, 15). In view of the non-Gaussian distribution of data for the plasma OPN levels in the patients with metastatic disease, nonparametric tests (Mann Whitney U test, Kruskal-Wallis one way ANOVA by ranks, and Spearman rank correlation coefficient) were used to compare data between different groups using the analysis package of SigmaStat (Jandel Scientific, San Jose, CA). The Kaplan-Meirer adjusted survival

estimates, Cox proportional hazards model, and the Martingale residuals were computed with the SAS statistics package (SAS Institute, Inc., Cary, NC).

RESULTS

We measured plasma OPN in 70 patients with metastatic breast cancer and compared these to 44 patient controls who had previously completed treatment for early-stage breast cancer and 35 healthy women. We have shown previously that OPN plasma levels in normal women have a median of 31 μg/liter (range, 14–64 μg/liter) when the assay was performed at 37°C. In the current study, the initial antigen capture step was performed at 4°C, resulting in increased sensitivity, with the advantage of requiring smaller volumes of plasma, and a systematic increase of ~1.5-fold in calculated OPN levels using the recombinant standard. Under these assay conditions, the median plasma OPN level was 47 μg/liter (range, 22–122 μg/liter) in the healthy women's group and 60 μg/liter (range, 15–117 μg/liter) in the patient control group. The median of patients with metastatic disease was 142 μg/liter (range, 38–1312 μg/liter) and was significantly different ($P < 0.0001$, Mann Whitney U test) from that of both control groups.

The histogram of the OPN values in the study population and controls is shown in Fig. 1. The plasma OPN levels in women with metastatic breast cancer were not normally distributed and showed a very skewed distribution to large values (~30-fold range); only the distribution of the patient control group appeared to be Gaussian. The distributions for the control groups showed no significant statistical differences between the healthy volunteers and the patient control group. Sixty-nine % (48 of 70) and 71% (50 of 70) of patients with metastatic breast cancer had plasma OPN values greater than the 95th percentile of the distribution of healthy women (101 μg/liter) and patient controls (91 μg/liter), respectively.

We tested whether the survival of patients with metastatic breast cancer was related to the OPN plasma level. Fig. 2 shows that the Kaplan-Meier adjusted survival estimates of patients grouped into three according to tercile OPN levels (lower, middle, and upper thirds with OPN ≤117, 118–203, and >203 μg/liter, respectively) differed significantly ($P < 0.001$). In this cohort of 69 patients, we had a minimum follow-up period of 14 months. According to these Kaplan-Meier adjusted survival curves, the median survival was ~650 (extrapolated), 420, and 170 days for OPN values from the lower, middle, and upper thirds, respectively. Thus, the survival curves based on tercile OPN categories suggest that increasing OPN levels are associated with decreasing survival. Using a Cox proportional hazards model treating OPN as a continuous variable, there was strong evidence of an association between increasing OPN and decreasing survival ($\chi^2 = 20$, $P < 0.0001$). We found no evidence of a threshold effect of OPN when we evaluated the graphical pattern of Martingale residuals obtained from the Cox proportional hazards model (data not shown). In addition, the median plasma OPN level differed significantly ($P < 0.002$) between the patients living (128 μg/liter) and those who died (203 μg/liter) during follow-up. Thus, increased plasma OPN levels were significantly associated with shorter survival using a variety of statistical analyses.
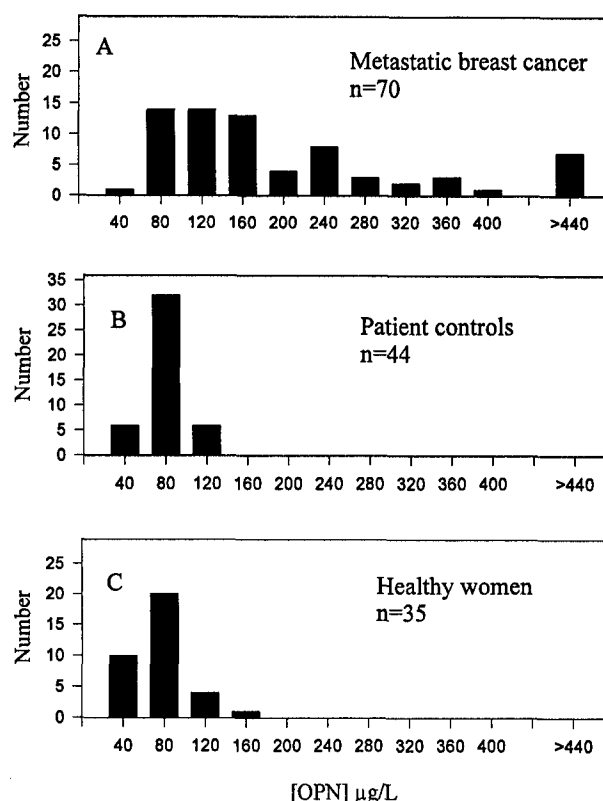

Fig. 1 Frequency distribution of plasma OPN in women diagnosed with metastatic breast cancer, treated for primary breast cancer, and normal controls. The average OPN level was calculated from plasma samples using the immunoassay described in "Materials and Methods" and displayed as a histogram with the upper bound of each interval on the *abscissa*. *A*, frequency distribution of women diagnosed with metastatic breast cancer: $n = 70$, median OPN level is 142 μg/liter, range is 38 to 1300 μg/liter. *B*, women treated for primary breast cancer: $n = 44$, median OPN is 60 μg/liter, range is 15 to 117 μg/liter. *C*, normal women: $n = 35$, median OPN is 47 μg/liter, range is 22 to 122 μg/liter. The normality test (Kolmogorov-Smirnov) indicated that only the distribution for the breast cancer-treated group was normal. The Mann-Whitney U test indicated that the median of the breast cancer metastasis group differed from the two control groups ($P < 0.001$); however, the medians of normal women and breast cancer-treated groups did not differ ($P > 0.1$).

We examined whether plasma OPN levels were related to the total number of organ sites involved or to the site of metastasis. The median OPN level was 129 μg/liter (range, 63–556; $n = 29$), 130 μg/liter (range, 50–1109; $n = 28$), and 232 μg/liter (range, 92–545; $n = 13$) with one, two, or three or more organs with metastases, respectively. The difference in median OPN levels in patients with three involved sites compared with either two or one metastatic site was statistically significant ($P < 0.05$, ANOVA on ranks). This result suggests that OPN plasma levels may be an indicator of the extent of disease because patients with multiple metastatic sites would be expected to have a higher tumor burden than patients with only a single affected site. We also found that the plasma OPN levels in 29 patients with a single organ site involvement did not differ significantly between subgroups divided according to site of involvement. Median OPN values of patients with bone ($n =$
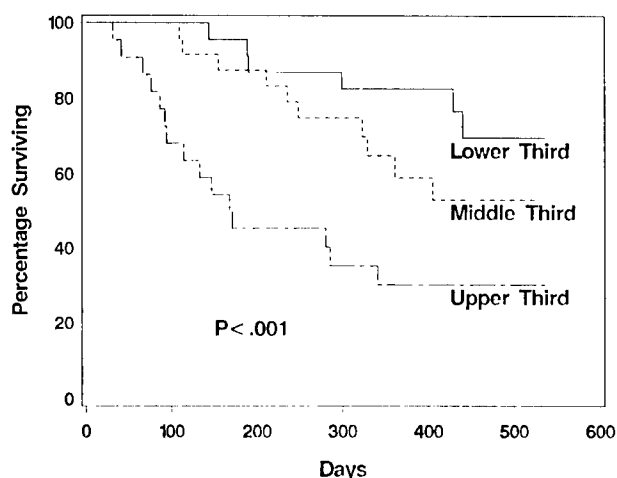
Fig. 2 Survival of patients with metastatic breast cancer grouped into tercile plasma OPN levels. The Kaplan-Meier adjusted percentage surviving is shown as a function of time (days) after OPN plasma levels were determined for patients (n = 69, one lost to follow-up). Patients were grouped into three categories for the tercile levels of OPN (upper, middle, and lower thirds were >203, 118-203, and ≤117 μg/liter, respectively). The survival curves of patients grouped in the tercile OPN levels were significantly different (P < 0.001, log rank test).

Fig. 3 Correlations between OPN levels and biochemical markers. Correlations between OPN levels and various biochemical markers are shown for alkaline phosphatase (A: r = 0.81, P < 3 × 10⁻¹⁰, n = 39), albumin (B: r = -0.56, P < 2 × 10⁻⁴, n = 39), and AST (C: r = 0.62, P < 2 × 10⁻⁵, n = 39) for those patients with metastatic breast cancer for whom we had complete blood data at the time of plasma sampling. The units shown on the *ordinate* for alkaline phosphatase, albumin, and AST are units/liter, g/liter, and units/liter, respectively, and the data were obtained from routine clinical measurements using serum samples. The units shown on the *abscissa* for OPN are ng/ml (= μg/liter), and the data were obtained using the ELISA described in "Materials and Methods" from one experiment (n = 4 replicates) for plasma samples taken at the same time.

14), liver (n = 2), lung (n = 8), and other single sites (n = 5) of metastasis were of 162 (range, 63-556), 103 (range, 76-128), 124 (range, 69-214), and 103 (range, 77-145) μg/liter, respectively. OPN levels were elevated significantly in patients with metastases to bone (P < 0.01), lung (P < 0.01), and all other sites (P < 0.003) when compared to normal volunteers. Clearly, metastasis to bone as well as other visceral sites can be associated with elevated plasma OPN levels.

We next examined for correlations of plasma OPN with other biochemical indicators of presence and extent of metastatic disease. OPN plasma levels were positively correlated with serum alkaline phosphatase (r = 0.81; Fig. 3A) and AST (r = 0.62; Fig. 3C) and negatively correlated with serum albumin (r = -0.56; Fig. 3B; all Ps < 0.001). Because these biochemical parameters are used to reflect the extent of disease, these results suggest that OPN could be related to tumor burden. We found no significant correlation with serum calcium (P > 0.3, n = 39; data not shown) or serum lactate dehydrogenase (P > 0.3, n = 14; data not shown).

We also examined for correlations with hematological parameters. Plasma OPN levels were negatively correlated with hemoglobin (r = -0.35, P < 0.026, n = 41) and total lymphocytes (r = -0.35, P < 0.026, n = 41) and positively correlated with absolute neutrophil count (r = 0.35, P < 0.027, n = 41; data not shown). There were no significant relationships between OPN levels and platelet count, monocyte count, or eosinophil count (data not shown).

We also examined the relative amounts and molecular forms of OPN by immunoadsorption and Western blotting. Fig. 4A shows a Western blot analysis using mAb53 for both immunoadsorption and development of seven representative plasma samples spanning the range of OPN values found in patients with metastatic breast cancer. The multiple molecu-
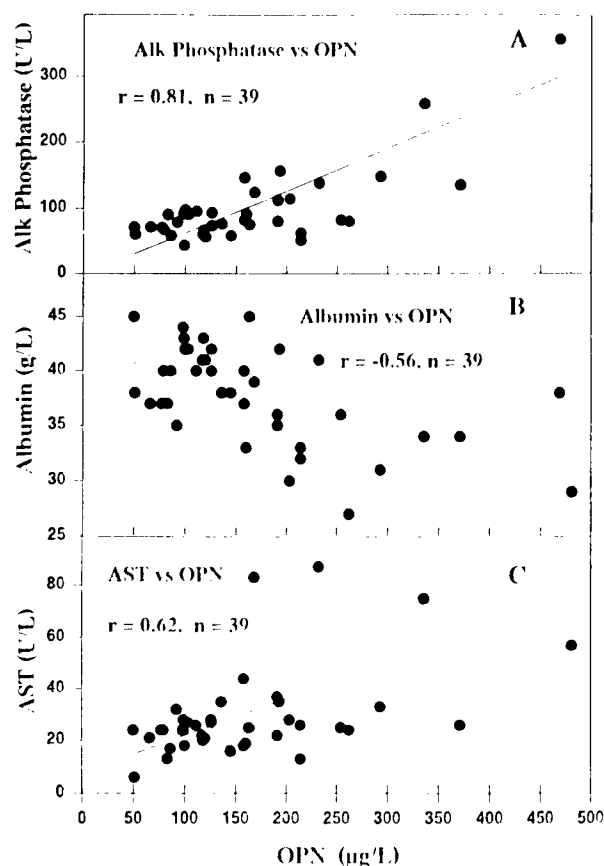
lar forms detected on these representative blots, using a single monoclonal antibody, are similar to those found in plasma OPN of healthy women (15). In examining the molecular forms of OPN in Western blots for patients with a single site of known metastasis, there was no apparent relationship of the forms and the site of metastasis or the OPN level (data not shown). Fig. 4B shows the relationship between OPN levels measured by ELISA (μg/liter) and quantitative Western blotting (densitometry units). Statistical analysis using the Pearson product moment correlation indicated a strong correlation between the two methods of quantifying OPN (r = 0.83, P < 0.001). Thus, the method of Western immunoblotting to measure relative OPN amounts was consistent with the ranking of OPN levels using our antigen capture ELISA using GST-hOPN as standard.
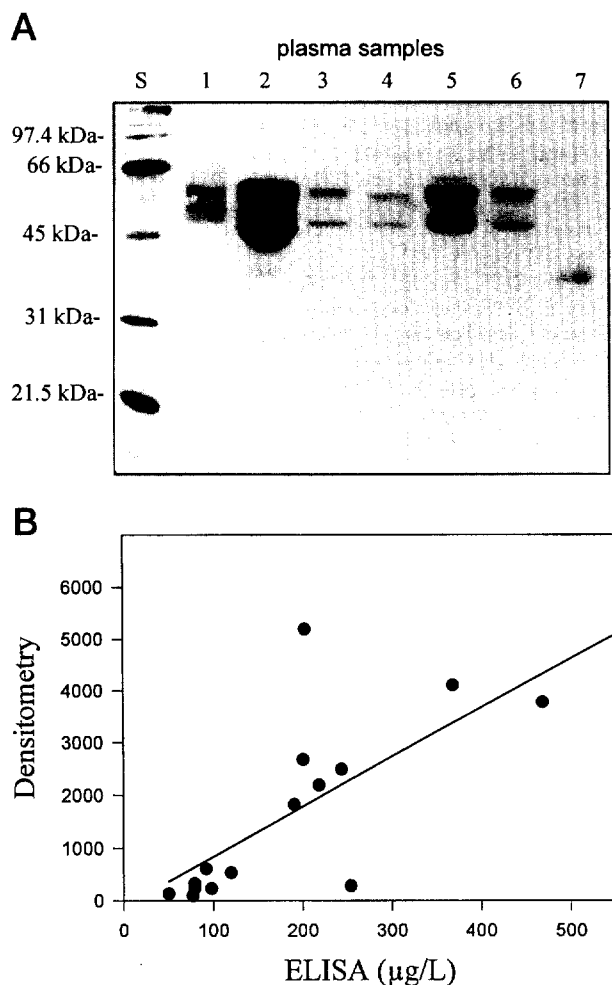
A

plasma samples

S 1 2 3 4 5 6 7

97.4 kDa-
66 kDa-

45 kDa-

31 kDa-

21.5 kDa-

B

Fig. 4 Western blot analysis of plasma OPN and comparison between ELISA and densitometry for quantification of plasma OPN levels. Plasma OPN was immunoadsorbed to mAb53-conjugated Sepharose, eluted, and fractionated in 12% SDS-PAGE as described in "Materials and Methods." *A*, representative plasma samples from seven individuals (*Lanes 1–7*) are shown. The measured OPN levels of these samples by ELISA were 254, 699, 77, 79, 244, 120, and 98 ng/ml, respectively. *B*, linear regression analysis of the relationship between the measurement of OPN by ELISA and volume densitometry. Pearson correlation coefficient of the regression line was 0.83 ($P < 8 \times 10^{-8}$) for 16 representative plasma samples including those samples shown in *A*.

DISCUSSION·

We have demonstrated that plasma OPN is significantly higher in ~70% patients with metastatic breast cancer ($n = 70$) compared with patients who are on clinical follow-up after completing all adjuvant therapy ($n = 44$) for early-stage breast cancer or healthy volunteers ($n = 35$). These results are consistent with the suggestion of Senger *et al.* (5) that plasma OPN levels may be elevated in metastatic disease. Furthermore, this is the first study to demonstrate significantly shorter survival for patients with metastatic breast cancer with increasing plasma OPN levels. The survival curves were statistically different when patients were grouped in terciles for plasma OPN ($P < 0.001$). Similarly, when OPN was treated as a continuous vari-

able in a Cox proportional hazards model, there was a strong association between increasing OPN levels and decreasing survival. Graphical analysis of Martingale residuals showed no evidence of a threshold effect. The median survival was ~650 (extrapolated), 420, and 170 days for OPN values in the lower, middle, and upper thirds, respectively. In addition, we found an association between higher median plasma OPN levels and number of involved metastatic disease sites. Because patients with multiple metastatic sites would be expected to have a higher tumor burden than patients with only a single affected site, this would suggest that plasma OPN levels are an indicator of extent of disease. This is also suggested by the correlation of high plasma OPN with biochemical and hematological indicators believed to reflect poor prognosis, such as elevated serum alkaline phosphatase and AST, and low serum albumin and hemoglobin.

Because patient prognosis is largely related to tumor burden, a plasma assay that is reflective of extent of disease could be of great potential clinical utility. Presently, tumor burden is estimated clinically by a combination of physical findings and performance status, radiological tests, and hematological and biochemical parameters (such as bone marrow involvement, coagulopathy, and abnormal liver enzymes). None of these, in isolation, is sensitive enough to be used to monitor extent of disease or effectiveness of therapy. The need for such an assay becomes critical in clinical assessment regarding response to treatment, and hence in decision-making regarding continuation or the need to instigate change in therapy.

To this end, there has been a search for valid reproducible serum/plasma markers that could be used as indicators of extent of disease and response to treatment, not only for breast carcinomas, but for other cancers as well. There has been some success with regards to specific tumors [*e.g.*, CA 125 in the case of ovarian carcinoma (17–19), HCG and α-fetoprotein in the case of nonseminomatous germ cell tumors (20), PSA for prostatic carcinoma (21), CEA for colonic carcinoma (22), and serum hormone levels in various endocrine neoplasms]. In the case of breast carcinoma, a number of potential serum markers are presently undergoing evaluation [*e.g.*, CA 15.3 (23–25), mucin-like carcinoma-associated antigen and CA-549 (26–29), mucin-related antigens CAM 26 and 29 (30), CEA (31), and hepatocyte growth factor (32)]. However, none of these markers has been proven to satisfy the criteria necessary for routine use in clinical monitoring of the majority of patients with metastatic breast cancer.

OPN shows promise in this regard, because it is elevated in the majority of patients (at least 70%) with metastatic disease, appears to vary with tumor burden (as measured by number of metastatic sites in this study), shows little intra-individual variability in level upon repeated sampling in healthy women (15), and is readily measured in plasma by our recently developed ELISA assay (15). This assay depends on the epitope specificity of mAb53 (15), and our levels of OPN may reflect the availability and exposure of this epitope rather than the concentration of OPN. In addition, by its nature as a secreted phosphoprotein whose level of expression is apparently increased in breast cancer (6–8), OPN would have an immediate advantage over those molecules that are not biologically secreted into the extracellular milieu.

In this study, we have demonstrated an association between elevated plasma OPN levels and poor prognosis in patients with metastatic breast cancer. The source of OPN in plasma, the mechanism by which plasma OPN levels are elevated in patients with metastasis, and the effects of increased plasma OPN levels remain to be elucidated. OPN in plasma could be derived from a number of cells and tissues, including activated inflammatory cells, vascular tissue, bone, or tumor cells; the physiological consequences to cancer patients of elevated blood OPN from any of these sources is not known. OPN expression by tumor cells has been functionally linked to increased malignancy of the cells in experimental studies [i.e., antisense OPN RNA expression associated with decreased malignancy (33–35)]. OPN binds to integrins including $\alpha_v\beta_3$ via the RGD conserved amino acid sequence, and thus likely participates in integrin-mediated signal transduction in cells bearing the appropriate integrins. The nature of OPN-mediated effects at the cellular and molecular levels remains to be clarified; however, proposed mechanisms include cell-substrate adhesion, chemo/haptotaxis, and OPN-mediated protection of tumor cells against nitric oxide cytotoxicity (1, 4, 36). Although the mechanism remains to be determined, the current study demonstrates clearly that plasma OPN levels in patients with metastatic breast cancer yield important prognostic information.

The pilot study reported here suggests a potential utility for plasma OPN determination in patients with metastatic carcinoma of the breast, both in the estimation of tumor burden and as a potential marker of response to therapy. Plasma OPN could be a clinically useful parameter in monitoring the effectiveness of therapy and, potentially, the decision to change treatment. In the majority of patients with metastatic breast cancer, who do not have measurable disease, sequential plasma OPN determinations could thus provide a much needed tool to guide clinical management. Our results strongly support the need for a large prospective trial to address the utility of measuring plasma OPN levels in women with breast cancer.

ACKNOWLEDGMENTS

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Osteopontin and p53 Expression Are Associated With Tumor Progression in a Case of Synchronous, Bilateral, Invasive Mammary Carcinomas

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● **Objective.**—To examine the association between expression of osteopontin (OPN), p53, other molecular markers (Ki-67, c-*erb* B2, and estrogen receptor protein) and tumor progression in a case of synchronous, bilateral, invasive mammary carcinomas of the same histology.

Design.—Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections. Plasma OPN level was determined by a quantitative antigen capture assay.

Setting.—The patient was seen, treated, and followed up for a period of 5 years at the London Regional Cancer Centre, Ontario, Canada.

Patient.—A 60-year-old woman presented with bilateral infiltrating mammary carcinomas of the same histologic type and grade. Bilateral mastectomy and axillary node dissection showed involvement of 3 of 12 right axillary and 0 of 11 left axillary lymph nodes. She later developed a right chest wall recurrence, followed by widespread metastatic disease to the skull, liver, and left femur.

Results.—The primary tumor of the right breast was OPN- and p53-positive, whereas the tumor of the left breast was negative for both markers. The development of right axillary lymph node metastases, chest wall recurrence, and distant metastases was associated in all instances with an immunohistochemical profile of high level expression of OPN and p53. Plasma assay for OPN at the time of last admission showed a markedly elevated OPN level.

Conclusions.—Increased p53 expression was found to be associated with increased tumor aggressiveness. The association of increased OPN expression with increased malignancy in breast cancer is a novel finding and raises the possibility of a role for OPN in tumor progression, as well as the potential for this marker in predicting clinical aggressiveness.

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In recent years, there has been an intensive search for molecular markers of tumor aggressiveness in breast cancer. In addition to hormone receptor status, some candidate molecules showing promise in this regard include p53,[1-9] c-*erb* B2,[9-18] and Ki-67.[19-22]

Mutations of *p53* appear to represent the most common cancer-related change at the gene level.[23] Overexpression of p53 protein (most often associated with point mutations) has been found in 16% to 52% of sporadic breast cancers and is associated with a poor prognosis.[1-3] Immunopositivity for p53 has been found to be an independent prognostic indicator in both lymph node–negative[4-6,8,9] and lymph node–positive[2,4,7,8] patients.

Osteopontin (OPN) is a secreted phosphoprotein that is expressed by a limited number of normal cells and tissues (including lactating mammary gland, developing bone, kidney, activated T cells and macrophages, and smooth muscle cells).[24,25] Osteopontin has also been identified by several groups as a tumor-associated protein,[26] and its ex-

pression has been linked to the malignancy of *ras*-transformed fibroblasts.[27-29] Recently, OPN has been shown to be expressed by human mammary carcinoma (MDA-MB-435) cells.[30,31] The RGD (arginine-glycine-aspartic acid) sequence of OPN has been shown to mediate cell-substrate attachment in these cells, as well as chemotaxis in a cell migration assay.[30,31] In the clinical setting, there have been reports of increased plasma levels of OPN in human breast cancer,[32-34] and of increased OPN in primary breast tumors.[35-37] The significance of increased OPN expression (either plasma or tumor levels) in predicting the biological behavior of a tumor is unknown at present, but is of great potential clinical impact in prognostication and planning of treatment strategies.

We report here a case of synchronous, bilateral, infiltrating mammary carcinomas, which showed very similar histologic appearances, but which had distinct immunohistochemical profiles. Lymph node and distant metastases were uniformly found to be associated with positivity for both p53 and OPN. Plasma levels of OPN were significantly elevated over an established normal range, suggesting that the level of OPN in either plasma or primary tumor may be predictive of propensity for metastasis.

REPORT OF A CASE

A case of bilateral, synchronous, invasive mammary carcinoma of no special type (invasive ductal carcinoma, not otherwise specified) is reported, in which immunophenotyping for OPN and p53, as well as a number of other markers was performed. The

natural course of the two primary tumors seemed quite different, one behaving in a more indolent and the other in a more aggressive fashion. Increased levels of OPN and p53 expression (as determined by immunohistochemistry) were associated with the more aggressive tumor and its metastases.

The woman described herein presented first at 34 years of age with a 0.5-cm, hard, nodular mass of the left breast. This mass was biopsied and found to represent a nonspecific giant cell granulomatous inflammation; it showed no evidence of malignancy, no evidence of proliferative breast disease (with or without atypia), and no identifiable infectious cause. She next presented 26 years later (at 60 years of age) with a mammographic lesion in each breast. Needle localization biopsy of the lesions showed both to be gritty and stellate on gross examination. The tumor of the right breast measured 2.5 cm in greatest dimension, and the tumor of the left measured 2.3 cm. Sections of both masses showed infiltrating mammary carcinoma of no special type, combined histologic Scharff-Bloom-Richardson (SBR) grade II/III. Both showed an associated solid and cribriform, intermediate and focal high-grade ductal carcinoma in situ, with involvement of ducts both within and away from the region of involvement by invasive carcinoma (extensive intraductal component positive). Furthermore, both showed microscopic evidence of lymphatic channel invasion (without evidence of vascular invasion), and both were estrogen receptor–positive.

The following month, the patient returned for bilateral simple mastectomy and axillary node dissection. Examination of the right breast tissue showed residual ductal carcinoma in situ with no evidence of residual invasive carcinoma. The left breast tissue showed no evidence of residual malignancy. Examination of the axillary lymph nodes showed involvement of 3 of 12 right axillary and 0 of 11 left axillary lymph nodes. Adjuvant treatment was initiated at this time, involving six cycles of CMF (cyclophosphamide, methotrexate, 5-fluorouracil).

Four years following the initial diagnosis of malignancy, the patient presented with a right chest wall recurrence. The following year she was found to have developed widespread metastatic disease, including skull and liver metastases, as well as a subcapital pathologic fracture of the left femoral head, for which she underwent a total hip arthroplasty. The histology of both the chest wall and femoral head metastasis was that of a poorly differentiated metastatic adenocarcinoma, consistent with a breast primary. The femoral head metastasis was found to be estrogen receptor–negative by immunohistochemistry. Further treatment included radiotherapy to the left femoral head and skull, as well as a single cycle of CEF (cyclophosphamide, epirubicin, 5-fluorouracil) chemotherapy. The patient died of her disease several months later the same year.

MATERIALS AND METHODS

Immunohistochemistry was performed on 4-μm sections of routinely fixed (10% formaldehyde), paraffin-embedded tissues. The sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 7 minutes. An antigen retrieval method was used on all slides prior to immunostaining. This involved heating tissue sections in citrate buffer (10 mmol/L, pH 6.0) in a microwave oven (600 W) for 7 minutes. Immunostaining was performed using a streptavidin-biotin complex method (Zymed histostain kit, Dimension Laboratories, Toronto, Ontario, Canada). Nonspecific staining was blocked by incubating slides with 5% normal goat serum. The following antibodies were used: anti-human OPN (monoclonal antibody mAb53 against the recombinant GST-human OPN fusion protein[31], anti-human p53 (monoclonal antibody DO7, Dako Corp, Carpinteria, Calif, 1:50), anti-human estrogen receptor protein (monoclonal antibody ER1D5, Dako Corp, 1:50), anti-human c-erb B2 (monoclonal antibody CB11, Novacastra Laboratories, UK, 1:40), and anti-human Ki-67 (polyclonal antibody A047, Dako Corp, 1:50). For each antibody, the slides were incubated for 1 hour at room temperature. Slides were then rinsed with phosphate-buffered saline, and a biotinylated secondary antibody was applied for 15 minutes, followed

by a phosphate-buffered saline rinse and treatment with the streptavidin-enzyme conjugate for 10 minutes. The chromogen used was aminoethyl carbazol (reddish-brown signal), and slides were counterstained with Mayer's hematoxylin. Immunostained slides were evaluated by light microscopy by two independent observers. A proportion score and intensity score were assigned for each antibody, using the semiquantitative system described by Allred et al.[5] The proportion score represented the estimated fraction of positively staining tumor cells (0 indicates none; 1, <1 in 100; 2, 1 in 100 to 1 in 10; 3, 1 in 10 to 1 in 3; 4, 1 in 3 to 2 in 3; and 5, >2 in 3). The intensity score represented the estimated average staining intensity of positive tumor cells (0 indicates none; 1, weak; 2, intermediate; and 3, strong). The overall amount of positive staining was then expressed as the sum of the proportion and intensity scores (ranges were 0 for negative staining and 2–8 for positive staining) (reported as total score observer 1; observer 2).

As a negative control in each case, the immunohistochemical procedure was performed with the omission of the primary antibody. Positive controls in each case included tissue sections previously known to express high levels of the test antigen. Pattern of staining also provided an indirect internal control for specificity of staining (ie, nuclear staining for Ki-67, p53, and ERP; cytoplasmic for OPN; and membranous for c-erb B2). An added control for OPN involved immunohistochemical staining of cell pellets of MDA-MB-435 cells (a human breast carcinoma cell line known by Western analysis and enzyme-linked immunosorbent assay to express high levels of OPN[30]).

Plasma level of OPN was determined at the time of the last admission by a quantitative antigen capture sandwich assay.[38] In this approach, the monoclonal antibody (mAb53) against OPN is adsorbed to plastic and acts as an antigen capture reagent during the first incubation with solutions of human plasma or known standard dilutions of human OPN. In the second incubation, a rabbit polyclonal antibody against human OPN is added. In the third step, biotinylated goat anti-rabbit antibody is added, followed by streptavidin-alkaline phosphatase to prepare for the signal development with substrate. Plasma level was compared with reference standards from normal women (negative controls).[38]

RESULTS

In spite of the histologic similarity of the right and left breast primary tumors (see Fig 1), their immunohistochemical profile differed (Table). Both were negative for c-erb B2 expression, in contrast to strong positivity found in a comedo-type carcinoma in situ control (not shown). Each of the primary tumors was estrogen receptor–positive, and each showed comparable positivity for the cell proliferation marker, Ki-67. In contrast, the two tumors were quite different for p53 and OPN expression. Nuclear staining for p53 was quite strong (6; 6 [total score observer 1; observer 2]) in the malignant cells of the right breast primary (both in situ and invasive), whereas it was undetectable (0; 0) in those of the left breast primary. Cytoplasmic positivity for OPN was seen in the malignant (in situ and invasive) cells of the right breast primary tumor (5; 4), but was undetectable in those from the left (0; 0). Scattered infiltrating macrophages and lymphocytes showed positivity for OPN in both primary tumors. Tumor cell positivity for p53 (Fig 2) and OPN (Fig 3) was also found in the right chest wall recurrence and at the metastatic sites, including right axillary lymph nodes and bone. The highest level of expression of both p53 and OPN was found at the most remote site (the left femoral head) (p53, 8; 7; OPN, 6; 6), at the latest point in time following the initial diagnosis (suggesting the possibility of tumor progression). A parallel decrease in estrogen receptor protein expression was seen at chronologically more ad-
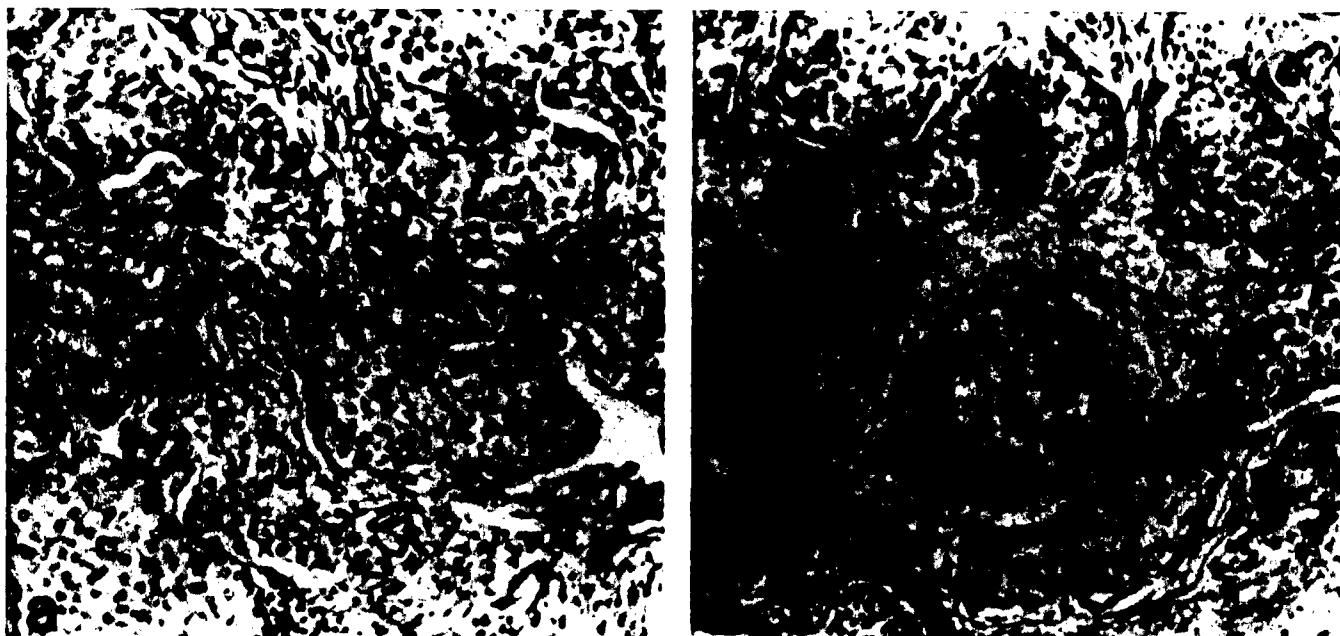
Fig 1.—Histology of the right (a) and left (b) primary breast tumors. Both are infiltrating mammary carcinomas of no special type. SBR grade II/III (hematoxylin-eosin, original magnification ×250).

Immunohistochemistry Score for Selected Markers as Determined by Two Independent Observers (Observer 1; Observer 1)*						
Tissue	Date	OPN	p53	Ki-67	c-erb B2	ERP
Left breast biopsy– benign†	June 1964	0; 0	0; 0	2; 3	0; 0	5; 5
Right breast primary tumor	October 1990	5; 4	6; 6	4; 4	0; 0	6; 6
Left breast primary tumor	October 1990	0; 0	0; 0	5; 5	0; 0	7; 6
Right axillary lymph node metastasis 1	November 1990	3; 3	6; 5	4; 5	0; 0	4; 4
Right axillary lymph node metastasis 2	November 1990	5; 4	6; 5	4; 5	0; 0	4; 4
Right chest wall recurrence	March 1994	5; 5	6; 5	8; 7	0; 0	4; 4
Bone metastasis	June 1995	6; 6	8; 7	6; 6	0; 0	2; 2

* Immunohistochemistry score determined as described in "Materials and Methods"; 0 indicates negative; positive range 2–8. OPN indicates osteopontin; ERP, estrogen receptor protein.
† Immunohistochemistry scores determined for benign epithelium.

vanced stages, with clinically insignificant levels of expression (ie, essentially estrogen receptor negative) in the malignant cells of the femoral head metastasis. Ki-67 expression was examined both by the semiquantitative scoring system (Table) and by determination of labeling indices (not shown). Both systems showed the same rank order of labeling, with equal positivity of the right and left primary tumors, a similar level of staining in the lymph node metastases, and a greater amount of labeling in both the chest wall recurrence and the bone metastasis.

At the time of the last admission (for widespread metastases with femoral head involvement and subcapital pathologic fracture), the plasma level of OPN was 324 ng/mL (normal reference range 14–64 ng/mL). This value is well above the 95th percentile as determined for normal women (50 ng/mL)[38] and is within the predicted range for patients with metastatic disease.[34] Based on our previous prospective clinical study, given this level of plasma OPN, we would have predicted a median survival of 8

months.[34] Survival in this case was indeed only a few months following this admission.

COMMENT

Instances of bilateral synchronous breast cancer offer the unique opportunity to study aspects of the cellular and molecular biology of tumors under the same (or very similar) environmental conditions at the same point in time. When both are then treated in the same fashion, at the same point in time, and at the same institution, conditions may be standardized for follow-up. In addition, where prolonged follow-up is obtained, a clinicopathologic description of tumor progression with time is made possible.

In this case, we were able to follow the immunohistochemical profile of synchronous, bilateral, invasive mammary carcinomas, as well as of the subsequent recurrence and metastases. Interestingly, although the histologic appearance of the two primary tumors was very similar, and although many of the molecular markers examined

Osteopontin Expression in Mammary Carcinomas · Tuck et al
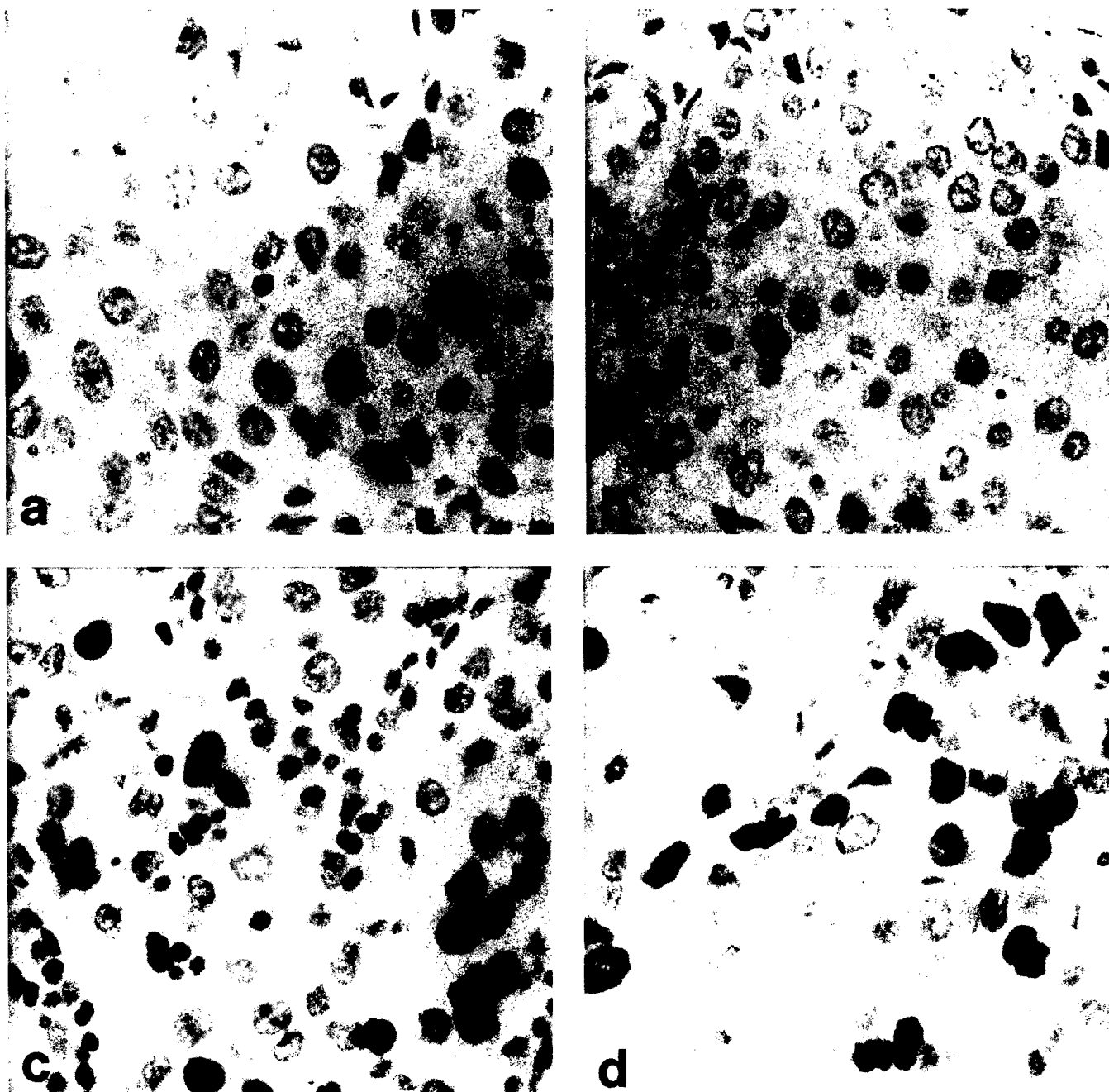
Fig 2.—*Immunohistochemistry (IHC) for p53. a, Right-sided primary breast tumor showing focal strong nuclear positivity for p53 (IHC score 6; 6 [observer 1; observer 2]). b, Left-sided primary breast tumor showing negative staining for p53 (IHC score 0; 0). c, Metastatic carcinoma cells in right axillary lymph node showing regional strong nuclear positivity for p53 (IHC score 6; 5). d, Left femoral head metastasis showing regional strong nuclear positivity for p53 (IHC score 8; 7) (original magnification ×400).*

showed similar levels of expression, p53 and OPN expression were found to be quite different. Both were detected in the cells of the primary tumor of the right breast and were not detected in cells of the primary tumor of the left. Similarly, both were consistently found at elevated levels in the subsequent lymph node metastases, in the right chest wall recurrence, and in cells of the bony metastasis. Furthermore, the highest levels of expression for both were seen at the most remote site (the bony metastasis), at the latest point in time. This would suggest that the regional recurrence and metastases not only most likely arose from the right breast primary (expressing elevated levels of p53 and OPN), but that with tumor progression, selection re-

sulting in increased expression for both may have occurred. In parallel to this, a decreased level of expression of estrogen receptor protein was found in cells of the lymph node metastases and chest wall recurrence, with the lowest levels occurring in cells of the bone metastasis. Similarly, higher levels of Ki-67 expression were seen in the right chest wall and bone metastases than in the primary tumors or regional lymph node metastases. However, as both Ki-67 and estrogen receptor protein were expressed at similar levels in both the right and left breast primaries, neither of these markers would have been useful in predicting the relative aggressiveness of the tumors at that point in time (in contrast to both p53 and OPN,
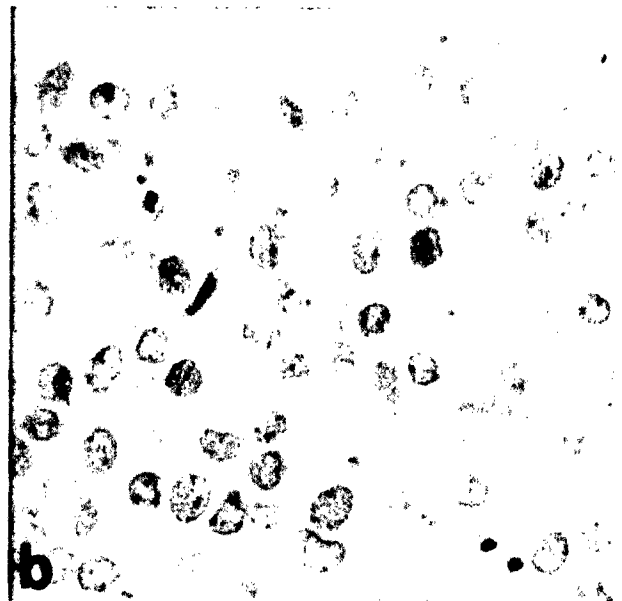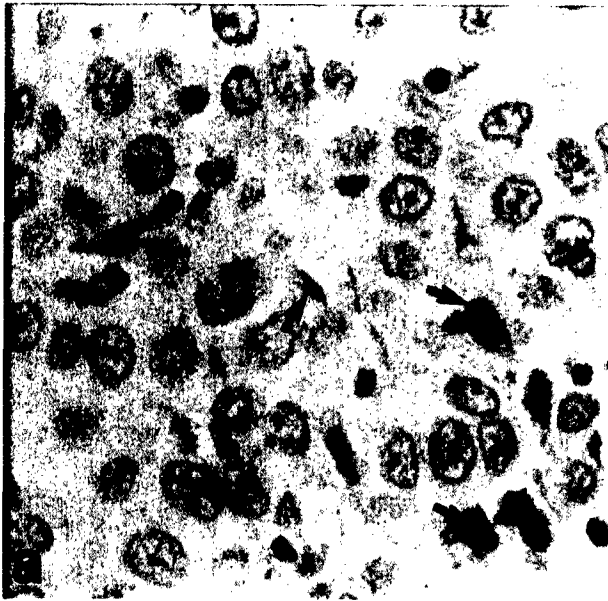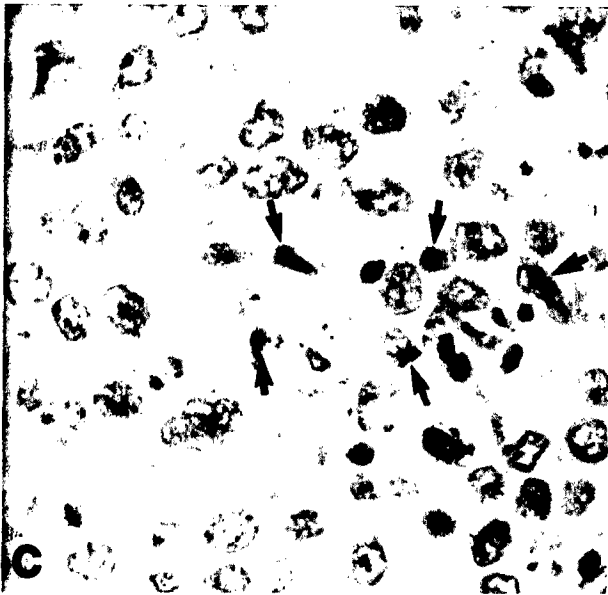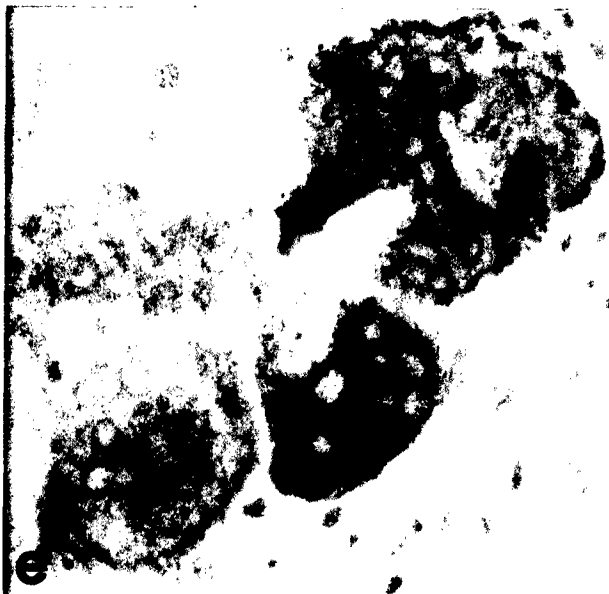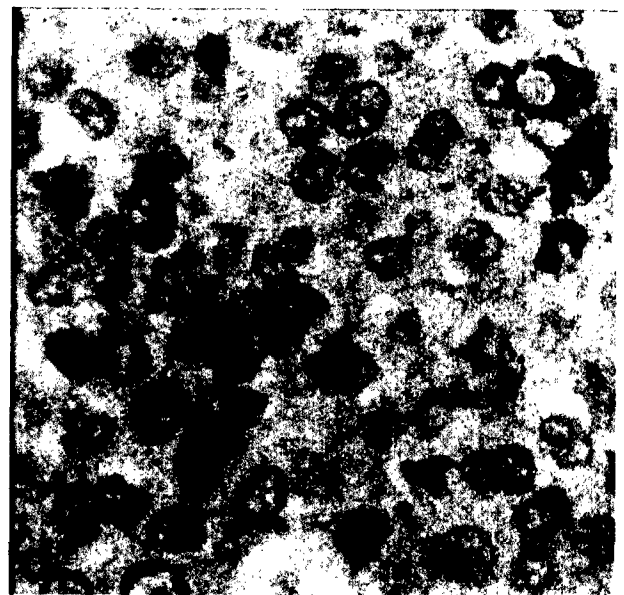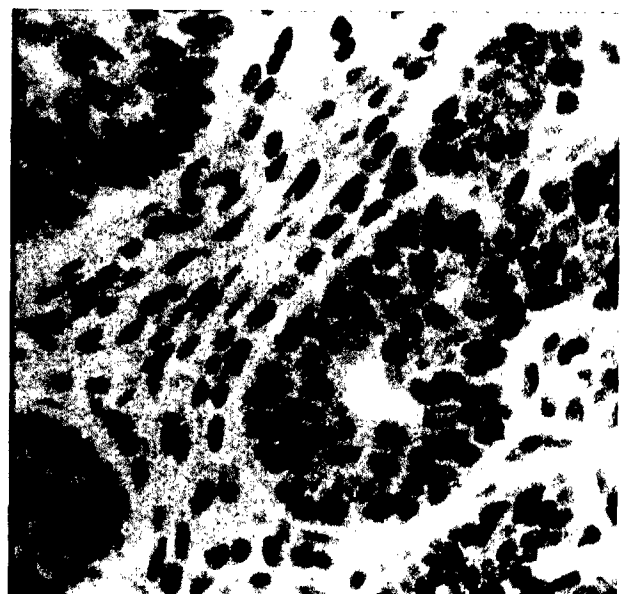
which were differentially expressed in the primary tumors).

In the case of p53, the finding of an association between the level of p53 protein expression and degree of aggressiveness of breast cancer is not new.[1-9] p53 abnormalities (mutation and, hence, prolonged protein half-life and increased protein levels) have been associated with features such as large size, high proliferative fraction, high nuclear grade, and estrogen receptor negativity in primary breast cancer,[5,8] as well as with a poor prognosis.[2,4-9] In addition, p53 has been reported to be an independent prognostic marker in both axillary lymph node–negative and –positive cases, and it has been suggested that this may make it useful in the selection of patients for adjuvant therapy.[2,4-9] In the present case, the patient would have received chemotherapy regardless, on the basis of lymph node positivity, but it is interesting to speculate whether such patients (showing high levels of p53 expression) would benefit from a more aggressive chemotherapy regimen, as has been suggested in the case of high c-*erb* B2 expression.[39]

Although a few studies have reported detectable OPN in breast carcinoma cells,[30,31,35-37] an association between the level of OPN expression and degree of malignancy has not yet been shown in breast cancer. (An association between elevated OPN levels and poor prognosis has recently been reported in lung carcinomas.[40]) We have recent evidence[33,34] that plasma OPN levels are significantly elevated in breast cancer patients harboring metastatic disease. Whether plasma OPN levels reflect the level of expression in the primary tumor, whether determination of OPN level may predict clinical course, and how OPN might effect the biological behavior of breast carcinoma cells are at present largely unknown. With regard to the latter, there is evidence that one aspect of the biological effect of OPN on human breast carcinoma cells involves cell attachment and migration-stimulating functions mediated by RGD amino acid sequences in the protein.[30,31] It has further been suggested that binding of OPN to cell surface (integrin) receptors may trigger transduction of cell growth or invasiveness-related signals.[30]

In the present case, elevated OPN protein expression was found in the more aggressive of the two histologically similar tumors. The increased OPN was seen primarily in the cytoplasm of the carcinoma cells themselves. Although scattered macrophages and lymphocytes also stained for OPN, there was no apparent qualitative or gross quantitative difference in expression by inflammatory cells between the primary tumors themselves or between primary and metastatic sites. The highest level of expression of OPN was found in malignant cells of the most distant metastatic site and at the latest point in time, suggesting the

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Fig 3.—*Immunohistochemistry (IHC) for osteopontin (OPN). a, Primary tumor of the right breast showing focal cytoplasmic positivity for OPN (arrows, IHC score 5; 4 [observer 1; observer 2]). Cytoplasmic positivity is coarsely granular and often perinuclear in location. b, Primary tumor of the left breast showing negative staining for OPN (IHC score 0; 0). c, Metastatic carcinoma cells in right axillary lymph node showing focal positivity for OPN (arrows, IHC score 5; 4). d, Left femoral head metastasis showing regional positivity for OPN (IHC score 6; 6). e, Macrophage giant cells of original (benign) left breast biopsy showing cytoplasmic positivity for OPN. f, Benign ductal epithelium of original left breast biopsy showing negative staining for OPN (original magnification ×400).*

possibility of increased expression with tumor progression. Plasma OPN determination at the time of distant metastases was markedly elevated above the normal range. Thus, as for p53 protein expression, it appears that in this case, tumor OPN expression may have predicted a more aggressive clinical course. An additional function of OPN determination, however, may lie in the ability to readily detect increased levels in the plasma. Being a secreted phosphoprotein, OPN by its nature would be expected to be more readily detected in blood than a nuclear regulatory protein, such as p53. The present case suggests the need for clinical studies to assess the potential role of OPN determination (tumor or plasma levels) as an independent prognostic indicator in human breast cancer. Work is also needed to assess changes in plasma levels of OPN with disease progression, in response to standard therapy, and perhaps following neoadjuvant therapy, to determine if a role for OPN determination may exist in therapeutic decision making as well.

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UPREGULATION OF OSTEOPONTIN IS ASSOCIATED WITH INCREASED INVASIVENESS AND PLASMINOGEN ACTIVATOR EXPRESSION OF HUMAN MAMMARY EPITHELIAL CELLS

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ABSTRACT

Osteopontin (OPN) has been associated with enhanced malignancy in breast cancer, but its functional role in this disease is poorly understood. To study the effect of OPN on cellular invasiveness, basal OPN expression was first assessed in members of a progression series of human mammary epithelial cell lines (21PT: immortalized, nontumorigenic; 21NT: weakly tumorigenic; 21MT-1: tumorigenic, weakly metastatic; MDA-MB-435 cells: tumorigenic, highly metastatic). The two lines which expressed lowest basal levels of OPN (21PT, 21NT) were then examined for upregulation of invasive behavior in response to exogenous or transfected (endogenous) OPN. Both 21PT and 21NT showed increased invasiveness through Matrigel when human recombinant (hr)OPN was added to the lower chamber of transwells. Both also showed a cell migration response to hrOPN. Populations of 21PT and 21NT cells stably transfected with an OPN-expression vector showed higher levels of cell invasiveness than control vector transfectants. Examination of transfectants for mRNA of a number of secreted proteases showed that only urokinase-type plasminogen activator (uPA) expression was closely associated with OPN expression and cellular invasiveness. Both increased cell motility and induction of uPA expression are thus potential mechanisms of increased invasiveness of breast epithelial cells in response to OPN.

INTRODUCTION

Osteopontin (OPN) is a secreted, integrin-binding glycophosphoprotein whose levels are increased in the primary tumors and plasma of patients with breast cancer (Brown *et al.*, 1994; Hirota *et al.*, 1995; Bellahcène and Castronovo, 1995; Senger *et al.*, 1988; Singhal *et al.*, 1997; Tuck *et al.*, in press). Our clinical studies have shown a relationship between plasma OPN, tumor burden, and prognosis in patients with metastatic breast cancer (Singhal *et al.*, 1997), as well as between tumor cell OPN and prognosis in patients with lymph node negative breast cancer (Tuck *et al.*, in press).

In spite of these clinical associations, little is known regarding the biological role of OPN in the progression of human breast cancer. Studies have shown that OPN may be synthesized by tumor-infiltrating macrophages (Brown *et al*, 1994; Hirota *et al.*, 1995) and by breast cancer cells themselves (Senger *et al.*, 1983; Morris *et al.*, 1993; Bautista *et al.*, 1994; Tuck *et al.*, in press), although the relative biologic significance of these different potential sources of OPN is not yet understood. Previous work (Xuan *et al.*, 1994; Xuan *et al.*, 1995; Senger *et al.*, 1996) has shown that some breast cancer cells show integrin-dependent adhesion to, and migratory response to OPN. Although these studies provide evidence that integrin binding is important in OPN-induced cell adhesion and chemotaxis of some mammary carcinoma cells, the functional significance of these events in the progression and malignancy of breast cancer in general has yet to be determined.

Cellular invasiveness through the extracellular matrix is an aspect of cell behavior important during development, tissue remodelling, and malignancy. A role for OPN has been suggested in bone development and remodeling (Denhardt *et al.*, 1995), and OPN has been

shown to increase protease activity and invasiveness of osteoclastoma cells in culture (Teti *et al.*, in press). Direct evidence for OPN involvement in cellular invasiveness in malignancy is lacking, although there are occasional reports of increased tumorigenicity and metastatic ability of rodent tumor cells with higher level expression of OPN (Oates *et al.*, 1996; Chen *et al.*, 1997), and we and others have previously reported decreased tumorigenicity upon down-regulating OPN expression of rodent tumor cells (Behrend *et al.*, 1994; Feng *et al*, 1995; Gardner *et al.*, 1994).

To invade through extracellular matrix (ECM), cells must be able to digest the ECM components in order to clear a path, and then move through the matrix into the adjacent tissue. Increased invasiveness in response to OPN thus may be expected to be due to some combination of increased protease expression and/or increased cell movement. The ability of human breast carcinoma MDA-MB-435 cells to respond to OPN by directed cell migration has been previously described (Xuan *et al.*, 1995; Senger *et al.*, 1996). However, whether this is a general phenomenon for breast epithelial cells has not been established, and the issue of whether this can then be extended to increased invasiveness through ECM has not been addressed. Similarly, aside from a suggestion that OPN may induce increased secreted protease activity of osteoclasoma cells (Teti *et al.*, in press), a direct influence of OPN on protease expression has not been shown.

The purpose of the present work was to examine the ability of cultured human breast epithelial cells at different stages of progression to synthesize OPN, as well as to determine whether OPN can affect the invasiveness and protease expression of these cells. We have made use of the 21T series of human mammary epithelial cell lines (Band *et al.*, 1990), believed to

represent different stages of tumor progression, in comparison with a highly metastatic human breast cancer cell line, MDA-MB-435 (Price et al., 1990). Evidence is shown for the ability of these cells to synthesize OPN, with highest levels of expression in cells of greater malignancy. Evidence is also shown indicating that both non-tumorigenic (21PT) and tumorigenic (21NT) cell lines migrate towards OPN and invade through basement membrane in response to OPN. Upregulation of OPN expression by tranfection with a constitutive high expression vector resulted in transfectant populations of both 21PT and 21NT cells which showed increased invasiveness through Matrigel. mRNA expression for a number of different secreted proteases was assessed in OPN-transfected cell populations. OPN transfectants showing increased invasiveness through Matrigel consistently showed upregulation of urokinase-type plasminogen activator (uPA).

RESULTS

Osteopontin expression of 21T series cells vs. MDA-MB-435 cells

OPN mRNA and protein expression were determined by Northern and Western analysis respectively, for 21T series cells (21PT, 21NT, 21MT-1) and MDA-MB-435 cells. Results of Northern analysis (Figure 1) showed that all three parental 21T series cell lines express relatively low levels of OPN mRNA, compared to highly malignant, metastatic MDA-MB-435 cells. Assay of conditioned medium by Western analysis (Figure 2a) showed that the 21T series cell lines also secrete lower levels of OPN protein than MDA-MB-435 cells, with the highest levels of expression in the 21T series seen in the weakly metastatic MT-1 cells. The predominant form of secreted OPN seen in conditioned media of the 21T series cell lines was of high molecular weight (⁻97 kDa). In addition to the ⁻97 kDa MW form, MDA-MB-435 cells also showed a significant accumulation of lower molecular weight forms (including a major 66 kDa band) in the conditioned media. Examination of cell lysates by Western analysis (Figure 2b) showed that the major intracellular form of OPN present in all four cell lines was of 66 kDa MW. Again, highest levels of OPN protein were found in the metastatic (21MT-1 and MDA-MB-435) cells.

Invasion through Matrigel of parental 21PT and 21NT cells in response to exogenous (human recombinant) OPN

As both 21PT (established, non-tumorigenic) and 21NT (tumorigenic, non-metastatic) cells express relatively low basal levels of OPN, these cells were examined further for altered invasiveness through Matrigel in response to exogenous human recombinant (hr)OPN. Invasive response of 21PT and 21NT to 100 µg/ml hrOPN is shown in Figure 3. Both 21PT and 21NT

cells showed low basal level of invasiveness through Matrigel (21NT slightly greater than 21PT). Invasiveness of both cell lines was significantly enhanced with hrOPN present in the lower chamber (21PT: $p=0.0003$, 21NT: $p<0.0001$; Student's t-test). The hrOPN-induced invasiveness of 21NT was significantly greater than that of 21PT ($p=0.0001$; Student's t-test).

Cell migration of 21PT and 21NT cells in response to hrOPN

One potential component of cellular invasive response to a chemoattractant is that of induced cell migration. Figure 4 shows the results of assay for the migratory response of parental 21PT and 21NT cells to exogenous hrOPN in the lower chamber of a transwell system, with or without the addition of 20 μg/ml of blocking anti-OPN monoclonal antibody (mAb53). Both 21PT and 21NT cells were found to respond to hrOPN by increased cell migration. This response was not significantly altered by addition of non-specific mouse IgG to the lower chamber. On the other hand, complete blocking of hrOPN-induced cell migration of both 21PT and 21NT cells was seen when anti-OPN antibody was added to the lower chamber. "Criss-cross" assay has shown that the response to OPN is indeed directed (i.e. chemo/haptotaxis vs. chemokinesis), and experiments using blocking GRGDS peptides and RGD-deletion mutant OPN have shown it to be RGD-dependent (data not shown).

Given the strong migration and invasion responsiveness of these cell lines to hrOPN when added to the culture media ("exogenous" OPN), we proceeded to establish stable transfectants of 21PT and 21NT which constitutively overexpress human OPN, in order to examine the influence of native OPN produced by the cells themselves ("endogenous" OPN).

Transfection and screening of 21PT and 21NT cells

The OPN-containing expression vector, prepared as described in Methods, was used in parallel with control experiments using the unmodified parental plasmid (pcDNA3) for LIPOFECTIN transfections (as described). Successful transfection of 21PT and 21NT cells was achieved, both with the OPN-containing construct and the unmodified pcDNA3 plasmid. Four pooled populations of G418-resistant OPN-transfected 21PT cells were obtained, designated PT/OPai, PT/OPaii, PT/OPbi, and PT/OPbii. These consisted of combined harvesting of approximately 20, 25, 20, and 20 colonies, respectively. Two pools of G418-resistant vector-only control transfectants of 21PT cells were also obtained, designated PT/Ci and PT/Cii. These pools originated from combined harvesting of approximately 70 and 50 colonies, respectively. Similarly, four pools of G418-resistant OPN-transfected 21NT cells were obtained, designated NT/OPai, NT/OPaii, NT/OPbi, and NT/OPbii, from combined harvesting of approximately 45, 25, 20, and 20 colonies, respectively. G418-resistant control transfectants of 21NT cells were obtained, designated as NT/Ci and NT/Cii. These consisted of combined harvesting of approximately 20 and 30 colonies respectively. From each of these pooled transfectant populations, 10-15 clones were also isolated, expanded, and screened for OPN expression as described below.

OPN expression by the transfected cells was screened first by assay of conditioned media using ELISA. Those OPN-transfected pools and clones of 21PT and 21NT cells expressing the highest levels of secreted OPN (by ELISA) are shown in Figure 5a, along with representative vector-only transfected controls. Four OPN-transfected cell populations were thus chosen for further study; from 21PT cells, pooled population PT/OPaii and clone 12 derived from Pool

PT/OPaii, designated PT/OPaiiC12; from 21NT cells, pooled population NT/OPbi and clone 4

derived from pool NT/OPaii, designated NT/OPaiiC4. Both pooled and cloned vector-control

transfectant populations consistently showed levels of OPN expression by ELISA that were barely

above background (BSA-only control), such that pools PT/Ci and NT/Ci were arbitrarily chosen

for further comparative analysis. Conditioned media from these transfectant populations was

then also examined by Western analysis, as shown in Figure 5b. The OPN-transfected cells

(PT/OPaiiPool; PT/OPaiiC12; NT/OPbiPool; NT/OPaiiC4) were found to secrete two major

forms of OPN, migrating at about 66 kDa and 97 kDa, while vector-transfected controls (PT/Ci;

NT/Ci) secreted low levels of OPN, predominantly of about 97 kDa.

Invasion through Matrigel of transfectants of 21PT and 21NT (vs MDA-MB-435) cells

Results of *in vitro* invasion assay for transfected cell populations of 21PT and 21NT (vs.

MDA-MB-435 cells) are shown in Figure 6. Using a 35 μg/well membrane of Matrigel and a

72 hour incubation, 21NT cells transfected with the control vector (NT/Ci) showed a basal level

of invasion which was significantly greater than that of control vector-transfected 21PT (PT/Ci)

cells. Interestingly, both pooled and cloned cell populations of OPN-transfected 21PT and 21NT

cells (PT/OPaiiPool, PT/OPaiiC12; NT/OPbiPool, NT/OPaiiC4 respectively) showed

significantly increased invasiveness over that of the respective control cell population ($p < 0.05$

for all using Student's *t*-test). The cellular invasiveness of NT/OPaiiC4 cells was found to

approach that of the highly metastatic MDA-MB-435 control cell line.

Northern analysis for protease expression of OPN-transfected vs. control 21PT and 21NT cells

mRNA levels for a number of secreted proteases were examined in OPN-transfectant (PT/OPaiiPool, PT/OPaiiC12; NT/OPbiPool, NT/OPaiiC4) and control vector transfectant (PT/Ci; NT/Ci) cell populations of 21PT and 21NT cells vs. MDA-MB-435 cells (Figure 7). As a reference, the blot has also been probed for OPN, showing high levels of OPN mRNA in the OPN-transfectants and in MDA-MB-435 cells, in keeping with the protein data.

A number of the secreted proteases showed differences in expression between the control 21PT/Ci (non-tumorigenic) and 21NT/Ci (tumorigenic) cells. Cathepsins B, D and L, and MMP-9, all showed higher level expression in 21NT/Ci than 21PT/Ci. In contrast, both cell lines expressed MMP-2 at similar levels. However, none of the cathepsins or metalloproteinases studied showed a consistent relationship of level of expression with that of OPN.

Of all the secreted proteases examined, only uPA showed a close association of expression with that of OPN. Increased uPA mRNA was seen in all of the OPN-transfectants (of both 21PT and 21NT), at a level commensurate with the level of OPN. Similarly, MDA-MB-435 cells expressed high levels of both OPN and uPA mRNA. Level of OPN and uPA mRNA expression also showed association with invasive capacity in the transwell assay (cf. Figure 5) (i.e. transfected cells expressing high levels of OPN also express high levels of uPA and invade better through Matrigel in transwell assays). uPA receptor (uPAR) expression was also examined, but was found to be expressed at comparable levels in control and OPN-transfected cells.

DISCUSSION

Although the secreted phosphoprotein OPN has been shown to be present in increased levels in the primary tumors and plasma of breast cancer patients (Brown *et al.*, 1994; Hirota *et al.*, 1995; Bellahcène *et al.*, 1995; Senger *et al.*, 1988; Singhal *et al.*, 1997; Tuck *et al.*, in press), with levels in some instances associated with prognosis (Singhal *et al.*, 1997; Tuck *et al.*, in press), little is known about whether OPN functionally affects the malignancy of human breast carcinoma cells, and if so, by what mechanism. We have here undertaken to examine the ability of members of a progression series of breast epithelial cells (21T) (Band *et al.*, 1990) (in comparison with the highly metastatic breast carcinoma cell line MDA-MB-435) to synthesize OPN, and to respond to OPN by increased invasiveness and protease expression.

Initial work to establish baseline levels of OPN mRNA and protein has shown that the 21T series cell lines, all of which are much less aggressive in nude mouse assays than MDA-MB-435 cells, as a group express lower levels of OPN (mRNA and protein) than MDA-MB-435 cells. Interestingly, assay of conditioned media by Western analysis has shown higher levels of OPN accumulation in cultures of cells known to be of greater *in vivo* malignancy (i.e. MDA-MB-435 > 21MT-1 > 21NT > 21PT). Furthermore, the predominant form of secreted OPN present in the conditioned media of the 21T series cell lines was of high molecular weight (~97 kDa), whereas MDA-MB-435 cells in addition show significant accumulation of lower molecular weight forms (including a major 66 kDa band). The high molecular weight species may represent either a very heavily post-translationally modified, or conjugated (by transglutaminase) form (Prince *et al.*, 1991; Beninati *et al.*, 1994; Sorensen *et al.*, 1994; Sorensen and Petersen, 1995; Aeschlimann *et al.*, 1996). In keeping with this interpretation is our finding that the major

intracellular form of OPN present in cell lysates (of both 21T series cells and MDA-MB-435 cells) is the low molecular weight, 66 kDa form. The relative biological activity of the different MW forms of OPN is at present largely unknown, although it has been suggested that transglutaminase crosslinking of extracellular matrix components may be important in stabilizing cellular adhesive contacts (Menter *et al.*, 1991), that sialylation and phosphorylation may modify OPN functions/activity (Shanmugam *et al.*, 1997; Saavedra *et al.*, 1995), and that the thrombin cleavage fragment containing the GRGDS sequence is more effective at promoting haptotaxis (Senger and Perruzzi, 1996).

The finding that both 21PT and 21NT cells respond to exogenously added hrOPN by increased invasion through Matrigel indicates that this responsiveness is not an exclusive property of tumorigenic (or more malignant) cells. Constitutive, high level expression of transfected OPN similarly was associated with increased invasiveness of both 21PT and 21NT transfectants. Thus, even at the earliest stage of tumor progression (established, non-tumorigenic 21PT cells), breast epithelial cells are capable of responding to OPN (either recombinant or transfected native OPN) by increased invasiveness through basement membrane. It would appear then, that the presence of OPN could thus influence malignancy even at quite early stages of malignancy and that the differences in *in vivo* malignant behavior of the 21T series cells and MDA-MB-435 cells may be related in part to their relative ability to independently express OPN, or to their relative affinity for OPN. Studies presented here further indicate that induced cell migratory behavior (chemo/haptotaxis) is a likely component of the increased invasiveness in response to OPN. This is in keeping with previous work of our own (Xuan *et al.*, 1994; Xuan *et al.*, 1995), and of others (Senger and Perruzzi, 1996), showing that MDA-MB-435 cells respond to OPN or

cleavage fragments of OPN by increased chemo/haptotactic activity. The cell migration response of 21T series cells to OPN has also been found to be directed (by "criss-cross" assay, not shown), specific (blocked by OPN-specific antibody and not non-specific IgG), and RGD-dependent (data not shown), as is that of MDA-MB-435 cells. In some cell types (eg. osteoclasts), there is evidence that OPN may modulate signal transduction pathways involving phosphatidylinositol 3-hydroxyl kinase and c-*src*, which may in turn be involved in regulating alterations in cell shape associated with cell movement (Chellaiah and Hruska, 1996; Hruska *et al.*, 1995).

In addition to the ability to move over an extracellular matrix, cells must be able to digest components of that matrix in order to invade. In the case of transfected OPN, the increase in cellular invasiveness was accompanied by increased expression of uPA. uPA in turn is known to activate a variety of proteases (either directly or indirectly by activating plasminogen) (eg. pro-MMP-1, -2, -3, -9, -14) (Baricos *et al.*, 1995; Mazzieri *et al.*, 1997; Werb, 1997) which are capable of digesting various components of the ECM (eg. fibronectin, tenascin, laminin, proteoglycans) (reviewed in Werb, 1977), as well as activating certain growth factors (eg. HGF, TGF-ß, bFGF) (reviewed in Werb, 1977; Andreasen *et al.*, 1997), some of which may also be involved in cell migration and invasion (eg. HGF). Thus, through triggering of a proteolytic cascade, with the ensuing modification of ECM components including OPN, invading cells expressing uPA are capable not only of clearing a path of migration, but of continuously modifying their environment. In addition, it has been found that uPA/uPAR complexes bind vitronectin (which in turn binds to the same αv integrins as osteopontin), and that uPAR may itself physically associate with certain integrins, such that some have suggested that uPA may

stimulate cell migration and invasion by non-proteolytic mechanisms as well (eg. by modulating adhesion interactions at focal contacts, or by triggering signal transduction pathways involved in the motility response) (Andreasen *et al.*, 1997; Yebra *et al.*, 1996).

The finding that human breast epithelial cells upregulated for OPN expression are more invasive and express elevated levels of uPA lends functional significance to clinical evidence that high level expression of both OPN and uPA (and uPAR) have been shown to be associated with poor prognosis in breast cancer (eg. Singhal *et al.*, 1997; Tuck *et al.*, in press; Andreasen *et al.*, 1997 [review]). It would appear from our work that the ability of breast epithelial cells to show increased invasiveness in response to OPN (with associated increased uPA expression) may be present even at early stages of progression (non-tumorigenic, non-metastatic 21PT cells), such that the availability of OPN in the microenvironment, or relative affinity for OPN may be critical. In this light, the ability of a tumor cell to synthesize its own OPN would be expected to afford a distinct selective advantage, allowing for increased migratory ability and invasive growth, presumably with increased propensity for metastasis. Indeed, evidence from rodent models has recently shown that increasing OPN expression in a previously benign rat mammary epithelial cell line (Rama 37) is sufficient to confer a metastatic phenotype on these cells (Oates *et al.*, 1996; Chen *et al.*, 1997). Our finding that OPN, when either supplied to or produced by breast epithelial cells, is associated with both increased invasive behavior and expression of uPA, suggests a functional mechanism by which OPN may contribute to the malignancy of breast tumors.

MATERIALS AND METHODS

Cell lines and culture

The 21T series cell lines (21PT, 21NT, 21MT-1) were obtained as a kind gift of Dr.

Vimla Band (Dana Farber Cancer Institute) (Band *et al.*, 1990). These cells were maintained

in culture in α-MEM supplemented with 10% FCS, 2mM L-glutamine (all from GIBCO-

BRL/Life Technologies, Grand Island, NY), insulin (1 μg/ml), epidermal growth factor [EGF]

(12.5 ng/ml), hydrocortisone (2.8 μM), 10 mM HEPES, 1mM sodium pyruvate, 0.1 mM

nonessential amino acids, and 50 μg/ml gentamycin (all from Sigma Chemical, St. Louis, MO)

(αHE medium). MDA-MB-435 cells were obtained as a kind gift of Dr. Janet Price (MD

Anderson Cancer Center, Houston TX), and were grown in α MEM with 10% FCS (both from

GIBCO-BRL/Life Technologies).

RNA isolation and Northern blot analysis

Cell pellets from subconfluent monolayers were mechanically homogenized (Polytron PT

1200, Brinkmannn 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 μg/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, oligolabelled [^{32}P]-dCTP cDNA

probes (labelled using a kit provided by Pharmacia, Baie d'Urfe, PQ), or with 5'[γ^{32}P]-ATP end-

labelled oligomers (labelled using a kit provided by Oncogene Science, Manhasset NY),

according to the procedures provided by the manufacturers, and as previously described (Tuck

et al., 1990; 1991).

The OPN probe used was the full-length (1493 bp) human OPN cDNA EcoRI cassette of plasmid OP-10 (Young *et al.*, 1990). Probes for human proteinase and uPAR genes included: MMP-9 (92 kDa Type IV collagenase) (1046 bp insert from plasmid p92MO1) (gift of Dr. W.G. Stetler-Stevenson), MMP-2 (72 kDa Type IV collagenase) (1117 bp insert from plasmid p3Ha) (Reponen *et al.*, 1992), cathepsin B (1.6 kb KpnI insert from plasmid pLC343) (gift of Dr. B. Sloane) (Cao *et al.*, 1994), cathepsin D (2.0 kb insert from plasmid pM13mp10), (gift of Dr. H. Rochefort) (Augereau *et al.*, 1988), cathepsin L (800 bp insert from plasmid pHCL800.1) (gift of Dr. D.T. Denhardt) (Joseph *et al.*, 1988), urokinase-type plasminogen activator (40mer antisense oligonucleotide derived from the translated sequences of exon 4) (Calbiochem/Cederlane Laboratories, Hornby, Ontario, Cat#ON333) (Riccio *et al.*, 1985), urokinase-type plasminogen activator receptor (45mer antisense oligonucleotide probe to the first 15 amino acids [not including the signal peptide]) (Roldan *et al.*, 1990). Even loading of lanes was confirmed by probing blots with a human 18s rRNA probe (p100D9; a kind gift from Dr. D.T. Denhardt).

Western blotting of cell lysates and conditioned media

Cell lysates were prepared from cell cultures (grown to 70-80% confluency on 100mm dishes) by washing each culture dish twice with cold phosphate buffered saline, followed by the addition of 500 μl cold lysis buffer (20mM Hepes pH 7.2, 5mM $MgCl_2$, 1% NP-40, 1mM DDT, 2mM PMSF, 4 μg/ml leupeptin, 4 μg/ml aprotinin). Each cell lysate was scraped from the dish, pipetted up and down to complete lysis, and spun at 16,000xg for 10 minutes to remove

insoluble material. Each supernatant was collected and total protein concentration determined by Peterson's modification of the standard Lowry assay (Peterson, 1977). 20 μg of total protein from each cell lysate was used for SDS-PAGE gel electrophoresis and immunoblotting as described below.

Conditioned media were prepared by plating cells at $5X10^5$ cells/100 mm plate in regular growth medium and incubating overnight (18 hrs) at 37°C, 5% CO_2. 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 24 hrs at 37°C, 5% CO_2. Following the incubation period, the conditioned medium from each plate was collected, and the cell debris spun out. The supernatant was concentrated by ultrafiltration in Centricon-30 mini-concentrators as per the manufacturer's protocol (Amicon Inc., Beverly, MA). Each corresponding plate was trypsinized, and a cell count performed, to allow appropriate correction in loading for cell equivalents.

Protein gel electrophoresis was done by standard SDS-PAGE methods (Sambrook et al, 1989), and immunoblotting by the enhanced chemiluminescence system (Amersham Canada, Oakville, ON). Cell lysates or conditioned media were fractionated on a denaturing SDS-PAGE gel (8% for cell lysates, 12% for conditioned media), electrophoretically transferred to nylon membrane using a semi-dry system (Millipore Canada, Mississauga, ON), and detected with biotinylated monoclonal antibody mAb53 (0.2 μg/ml) (Bautista et al., 1994), followed by streptavidin-horseradish peroxidase conjugate (Jackson Immunological Laboratories). The enhanced chemiluminescence detection system (Amersham Corp.) was used to detect immune-reactive bands. Film exposure time was 20 s. Molecular mass markers used were biotinylated

protein standards (Bio-Rad Laboratories, Hercules, CA).

Transfections

An expression vector for use in transfection was generated by cloning the full-length human OPN cDNA (from plasmid OP-10, [Young *et al.*, 1990]) into plasmid pcDNA3 (Invitrogen Corp., San Diego, CA) at the multiple cloning site between the strong, constitutive CMV immediate early gene enhancer-promoter and the (bovine) growth hormone polyadenylation and transcriptional termination signal sequences (between Not I and Apa I sites). This plasmid also contains the neomycin resistance gene, allowing for selection of stable transfectants in G418-containing medium. The control plasmid used for "vector-only" transfections consisted of the unmodified pcDNA3 plasmid. Transfections were performed using the LIPOFECTIN reagent and the procedure described by the manufacturer (Gibco-BRL/Life Technologies), using 2μg plasmid DNA for every 100μl of OPTI-MEM I in Solution A, and 10μl LIPOFECTIN reagent for every 100μl of OPTI-MEM I in Solution B. Following a 48 hr recovery period, transfected cells were subcultured into αHE medium containing 200 μg/ml (active) G418 (Gibco-BRL/Life Technologies) in order to select out stable transfectants. Plates were incubated until discrete colonies had developed, at which time both pooled populations and cloned transfectants were isolated for expansion in culture and further analysis. Conditioned medium was prepared for each transfectant population (essentially as described above, but without the need for Centricon concentration) for initial screening by ELISA assay for OPN expression. Those transfectant pooled and cloned populations expressing the highest levels of OPN were then chosen for expansion and preparation of RNA, cytosolic protein, and secreted protein (conditioned media).

ELISA for OPN protein expression by transfected cell populations

Initial screening of transfectants for OPN protein expression was performed by ELISA of conditioned medium, essentially as described previously for plasma (Bautista *et al.*, 1996; Singhal *et al.*, 1997). This is a capture ELISA based on high affinity mouse monoclonal (Bautista *et al.*, 1994) and rabbit polyclonal antibodies developed against a recombinant human OPN-GST fusion protein (GST-hOPN) (Xuan *et al.*, 1994) that recognizes native human OPN. Maxisorp immunoplates (Gibco-BRL/Life Technologies) were coated with mouse monoclonal anti-OPN antibody mAb53 (100 μl/well, 10μg/ml), then blocked with 1% BSA in ST buffer (0.15M NaCl, 0.01M Tris pH 8.0) with 0.05% Tween 20 (Bio-Rad). The wells were extensively washed with the ST-Tween 20 buffer prior to loading 100 μl of conditioned medium at various dilutions in ST-Tween 20 buffer +1% BSA. The samples were incubated for two hours at 4°C for the primary antigen capture step. Sequential incubations at 37°C of 100μl followed by washing were performed with (a) rabbit anti-OPN antibodies (0.8 μg/ml); (b) biotinylated goat anti-rabbit IgG (1:2000 dilution, Jackson Immunological Laboratories, Inc., West Grove, PA). After washing, streptavidin conjugated alkaline phosphatase (1:2000, Jackson Immunological Laboratories Inc.) was added for 30 minutes at 37°C. The wells were washed with buffer and 100μl of p-nitrophenyl phosphate (1 mg/ml in 100 mM Tris pH 9.5, 100mM NaCl and 5 mM $MgCl_2$) was added and the signal was allowed to develop at room temperature over 4-6 minutes. The reaction was stopped with 50 μl of 0.2 M Na_2EDTA (pH 8.0). A Bio-Rad plate reader was used to quantify the color signal. Recombinant GST-hOPN fusion protein (Xuan *et al.*, 1994) was used as standard, and background estimated by comparison against equivalent amounts of BSA protein. Internal controls of samples of known OPN concentration

were used to normalize OPN values obtained from independent assays.

Cell invasion

In vitro invasiveness through Matrigel was assayed as described previously (Tuck *et al.*,

1991), 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) per

filter. The Matrigel concentration was determined by preliminary experiments using MDA-MB-

435 cells and 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 X 10^4 cells

per well in serum-free αHE medium containing 0.1% BSA. The lower chamber was filled with

serum-free culture medium with 0.1% BSA and either 10 μg/ml fibronectin (for assays involving

transfectants) or 50 or 100 μg/ml hrOPN (for assays of parental (non-transfected) cell lines).

Plates were incubated for 72 hours in a 5% CO_2 incubator at 37°C. Following incubation, the

upper wells were removed and inverted, 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 X100 magnification.

Cell migration

Cell migration assays were performed essentially as described previously (Xuan *et al.*, 1995), using 24-well transwell chambers with polycarbonate filters of 8 μm pore size (Costar, Cambridge, MA). Gelatin (Sigma) was applied at 6 μg/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 800 μl of αHE plus 0.1% BSA, with or without test proteins. Cells (5 X 10^4) were added to each upper well in αHE medium with 0.1% BSA and incubated at 37°C; the time of incubation (5hrs) for this series of cell lines was based on preliminary experiments in which optimal time for achieving countable numbers of all four parental cell lines was determined. At the end of the specified incubation time, the cells that had migrated to the undersurface of the filters were fixed in place with gluteraldehyde 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 100X magnification and representative areas were counted to determine the number of cells that had migrated through the filters. The migratory response was tested in the presence or absence of blocking anti-OPN antibody in the lower chamber (20 μg/ml anti-OPN antibody, mAb53 [9]), in order to further assess the OPN specificity of the response. Control experiments were also performed in which OPN blocking antibody in the lower chamber was replaced by non-immune mouse IgG at comparable concentration.

All cell migration and invasion assays were performed in triplicate. Statistical differences between groups were assessed using the Mann-Whitney test, *t*-test, or ANOVA, using SigmaStat (Jandel Scientific, San Rafael, CA) statistical software.

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FIGURE LEGENDS

Figure 1:   Northern analysis of OPN mRNA expression by 21T series human mammary epithelial cell lines (21PT, 21NT, 21MT-1) vs. MDA-MB-435 (highly metastatic human breast carcinoma) cells.   Total RNA (10 μg/lane) was separated, blotted, and probed as described in Methods.   The OPN mRNA message size was 1.8 kb (upper panel).   Equivalent RNA loading and integrity were verified by assessment of 18s rRNA bands (2.1 kb, lower panel).

Figure 2:   Western blot analysis of OPN protein in a) conditioned media and b) cell lysates of 21T series cells vs. MDA-MB-435 cells.   Protein was fractionated in SDS-PAGE and detected with mAb53 as described in Methods.   In a), serum-free culture supernatants were concentrated by microfiltration prior to fractionation in 12% SDS-PAGE.   The equivalent of 8-10 μg of total secreted protein was loaded after correction for cell equivalents based on cell counts at the time of collection of conditioned media (see Methods).   In b), 20 μg of total cell lysate was loaded per lane, followed by fractionation in 8% SDS-PAGE.   Molecular mass markers used were biotinylated protein standards (BioRad).

Figure 3:   *In vitro* chemoinvasiveness of 21PT (PT) and 21NT (NT) cells in response to 100 μg/ml hrOPN (OP) in the lower chamber of 8 μm pore transwells, vs. 0.1% BSA only (0).   The filter of each transwell was coated with 35 μg Matrigel as described in Methods.   Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 72 hours, after which each filter was fixed and stained, the upper surface wiped clean, and cells on the lower surface

counted microscopically. The *bar graphs* represent the mean of counts (cells invaded/well) for three separate wells and the error bars represent standard error of the mean. Both 21PT and 21NT cells show significantly increased invasion in response to hrOPN (p=0.0003, p<0.0001 respectively; Student's *t*-test). 21NT shows significantly greater hrOPN-induced invasiveness than 21PT (p=0.0001; Student's *t*-test).

Figure 4: Cell migration of 21PT (PT) and 21NT (NT) cells in transwell assay. Migration assays were performed as described in Methods, with lower chamber contents as follows: 0.1% BSA alone (0); 50 μg/ml hrOPN alone (OP); 50 μg/ml hrOPN plus 20 μg/ml non-specific IgG (OP+nIg); 50 μg/ml hrOPN plus 20 μg/ml anti-OPN antibody (aOPIg). Cells were seeded at 5 X 10⁴ cells/well in the upper chamber and incubated for 5 hours, after which each filter was fixed and stained, the upper surface wiped clean, and cells on the lower surface counted microscopically. The *bar graphs* represent the mean of four or five counts from each of three separate wells and the error bars represent the standard error of the mean. Addition of anti-OPN antibody (but not non-specific IgG) significantly reduced the OPN-induced migration of both 21PT (PT) and 21NT (NT) cells (p<0.05 for both, one-way ANOVA).

Figure 5: Relative level of OPN protein expression of 21PT and 21NT OPN-transfected cells vs. control vector-transfected cells, as determined by ELISA (a) and Western analysis (b) of conditioned media. ELISA assay and Western blotting were performed as outlined in Methods and in Figure 2. Cell lines are as follows: 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 levels of OPN protein); PT/OPaiiC12: clone 12 of OPN-transfected 21PT pool aii (clone expressing highest levels 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 levels of OPN protein); NT/OPaiiC4: clone 4 of OPN-transfected 21NT pool aii (clone expressing highest levels of OPN protein). Conditioned media from MDA-MB-435 cells is included in b) as a positive control and basis for comparison.

Figure 6: *In vitro* chemoinvasiveness of transfectant cell lines in the transwell assay. 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 expressing the highest levels of OPN (c.f. Figure 4). MDA-MB-435 cells are included as a positive control and basis for comparison. Invasion is in response to 10 μg/ml fibronectin in the lower chamber, through 8 μm pore filters precoated with 35 μg Matrigel, as described in Methods. Cells were seeded at 5 X 10^4 cells/well in the upper chamber and incubated for 72 hours, after which the upper surface of the filter was wiped clean, and the under surface fixed, stained, and counted. The *bar graphs* represent the mean of counts from each of three separate wells and the error bars represent the standard error of the mean. OPN-transfected 21PT (PT/OPaii, PT/OPaiiC12) and 21NT (NT/OPbi, NT/OPaiiC4) showed significantly increased invasiveness over the vector-only transfected controls (PT/Ci, NT/Ci) (p < 0.05 for all by Student's *t*-test).

Figure 7:   Northern analysis of transfectant cell populations described in Figures 4 and 5 (vs.

MDA-MB-435 cells) for expression of mRNA of a number of secreted proteases (and urokinase-

type plasminogen activator receptor) vs. OPN. Probes were prepared as outlined in Methods,

and included: human osteopontin (OPN); human urokinase-type plasminogen activator (uPA);

urokinase-type plasminogen activator receptor (uPAR); matrix metalloproteinase 9 (MMP-9; 92

kDa type IV collagenase); matrix metalloproteinase 2 (MMP-2, 72 kDa type IV collagenase);

cathepsin B; cathepsin D; cathepsin L; and 18s ribosomal RNA (18s rRNA). Only uPA

expression showed close association with OPN mRNA and cellular invasiveness (c.f. Figure 5).
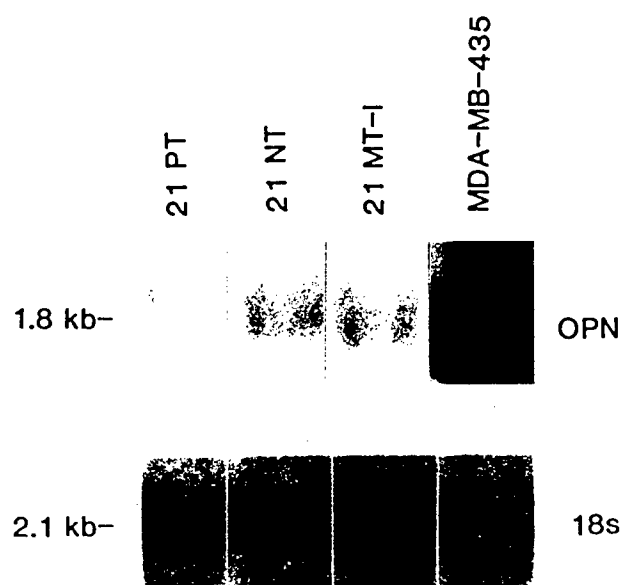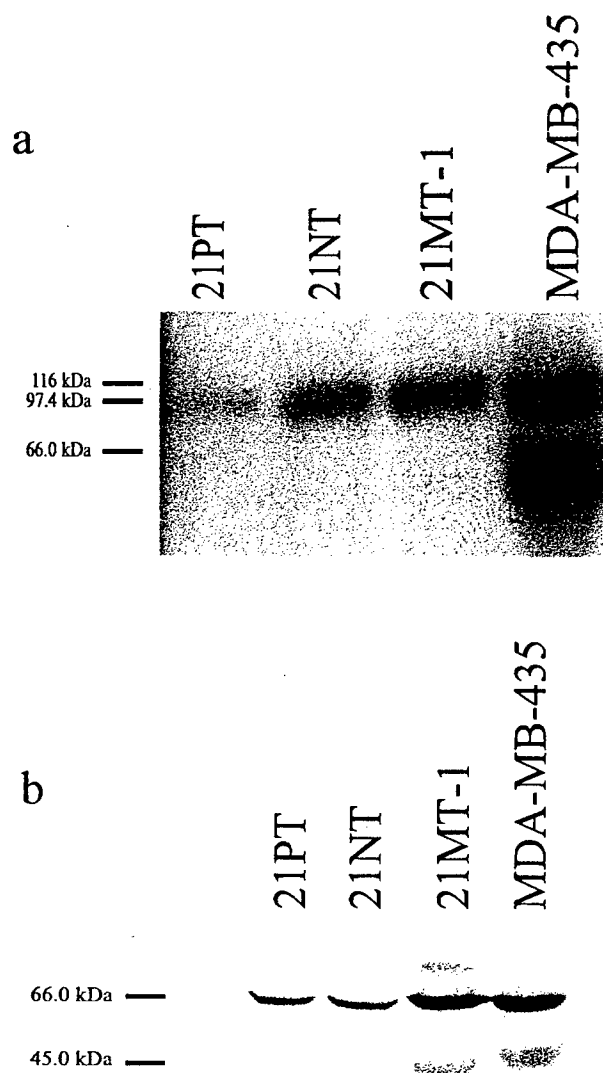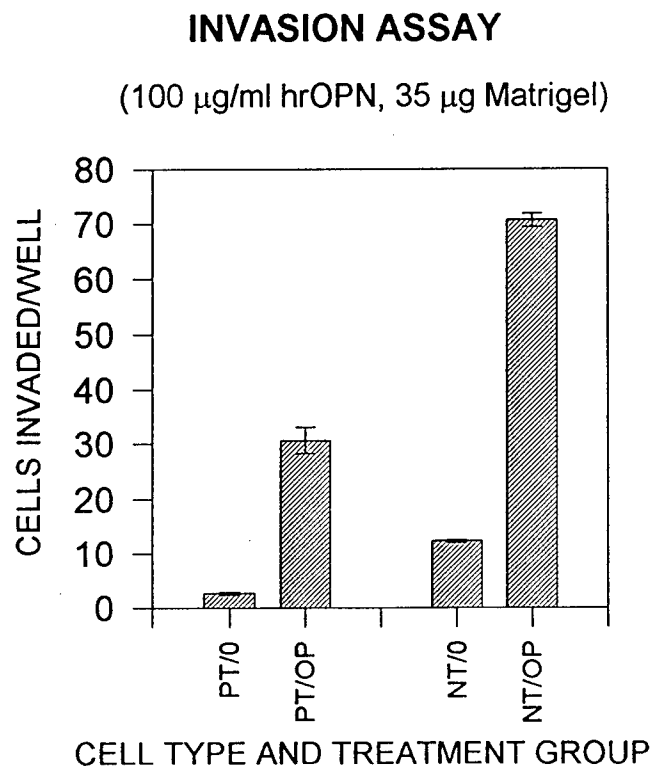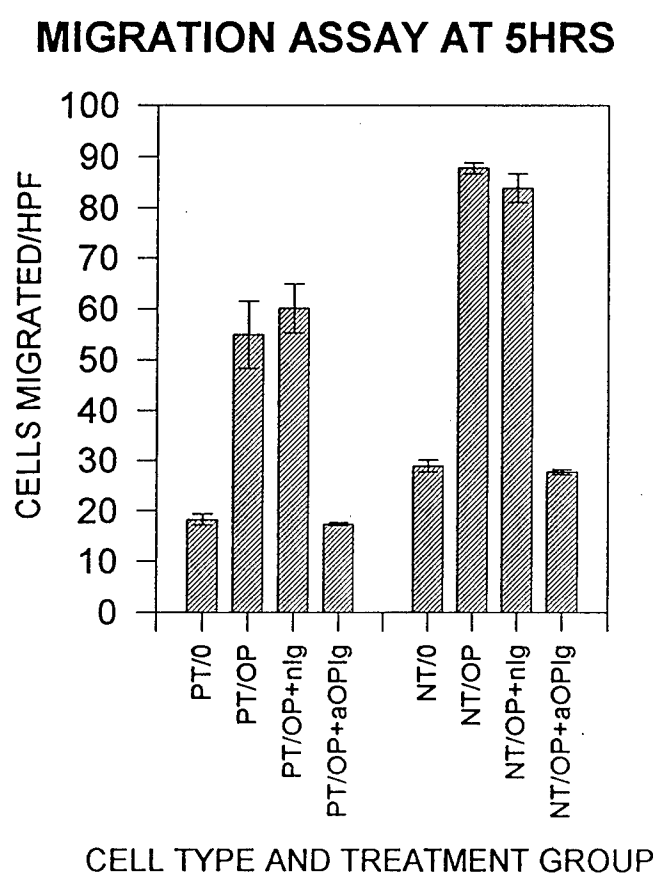
Figure 1

Figure 2

Figure 3

INVASION ASSAY

(100 µg/ml hrOPN, 35 µg Matrigel)

CELL TYPE AND TREATMENT GROUP

Figure 4

MIGRATION ASSAY AT 5HRS

Figure 5

Figure 6

INVASION ASSAY

(10 µg/ml fibronectin, 35 µg Matrigel)

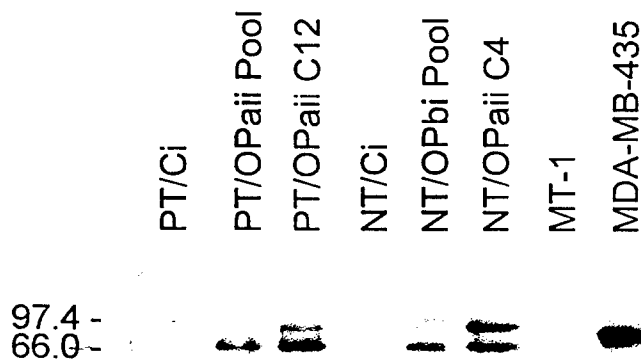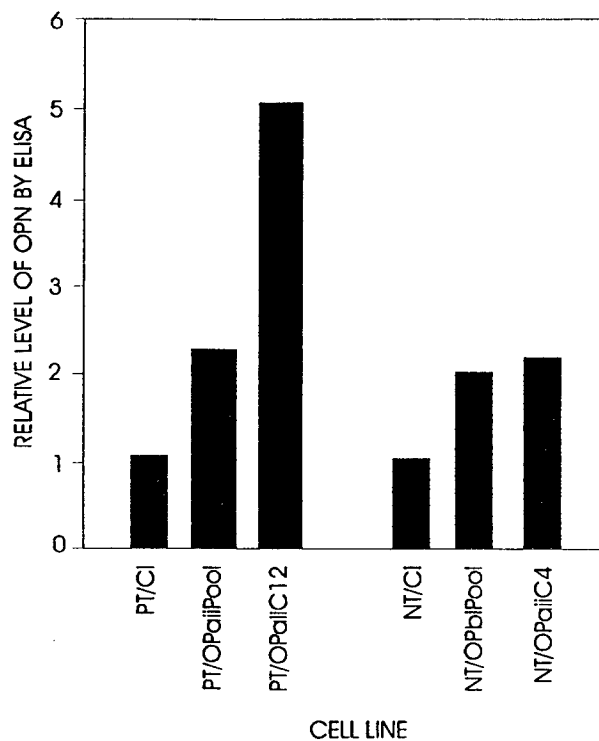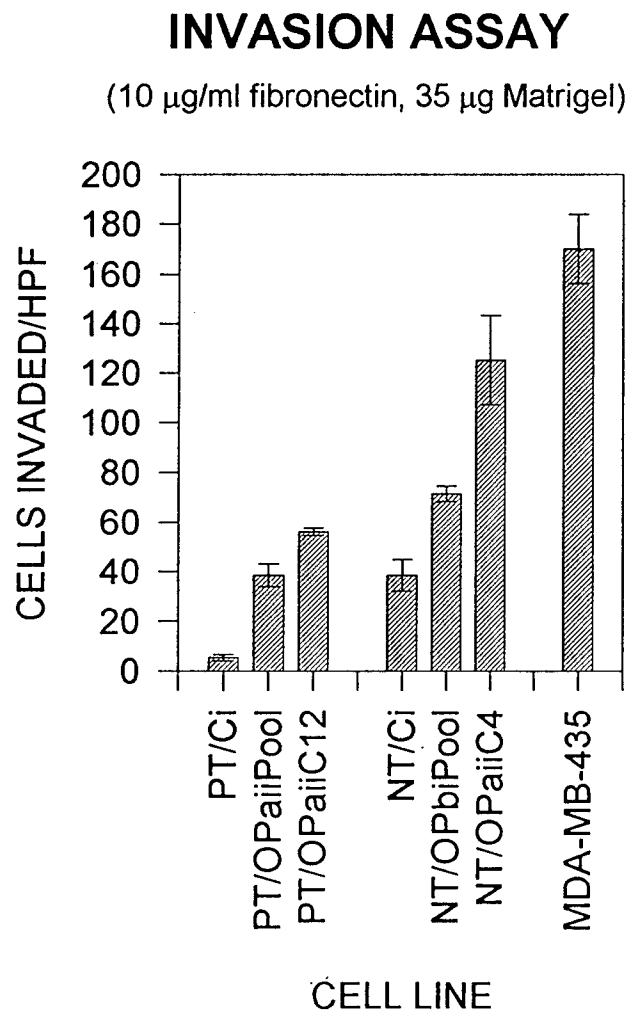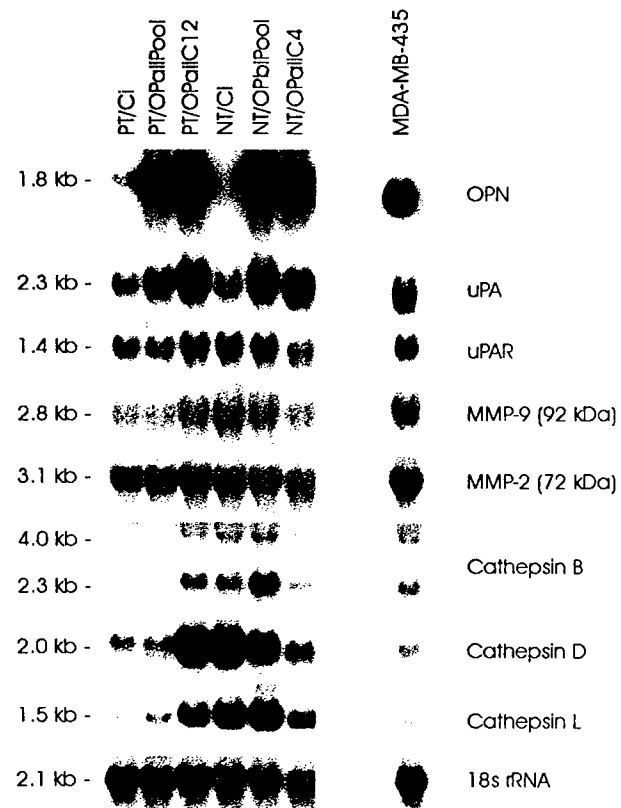# Figure 7

1

OSTEOPONTIN (OPN) EXPRESSION IN A GROUP OF LYMPH NODE NEGATIVE BREAST CANCER PATIENTS

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ABSTRACT

The aim of this study was to examine the cellular distribution of osteopontin (opn) protein (by immunohistochemical [IHC] analysis) and mRNA (by *in situ* hybridization [ISH]) in the primary tumors of lymph node negative (LNN) breast cancer patients, and to determine whether level of immunodetectable opn may be associated with tumor aggressiveness. We examined opn levels in tumors from 154 patients with lymph node negative breast cancer, who were followed for a median of 7 years (range 1.7 to 16.3). IHC staining for opn was seen in tumor infiltrating macrophages and lymphocytes in 70% of these tumors, and in the carcinoma cells themselves in 26%. *In situ* hybridization (ISH) was performed to determine cellular distribution of opn mRNA expression in sections from selected tumors. Opn mRNA was detected in groups of tumor cells, individual tumor cells, and in tumor infiltrating macrophages and lymphocytes. Matched sections showed that some tumor cells with IHC staining for opn protein were also positive for opn mRNA by ISH, in contrast with previous studies which have shown opn mRNA expression only in tumor infiltrating inflammatory cells. This study thus indicates that opn protein can be produced by breast cancer cells *in vivo*, and suggests that it may also be taken up from the environment (i.e. secreted by inflammatory cells or other tumor cells). Tumor cell IHC staining intensity was then assessed using a semiquantitative scoring system. Univariate analysis showed tumor cell opn positivity above an optimized cut point to be significantly associated with decreased disease free survival (DFS) and overall survival (OS). The results of this pilot study thus suggest that the ability of breast cancer cells to either synthesize opn, or to bind and sequester opn from the microenvironment, may be associated with tumor aggressiveness and poor patient prognosis.

INTRODUCTION

Osteopontin (opn) is a secreted, adhesive glycophosphoprotein which has been implicated in both normal (eg. bone development, immune system regulation) and pathologic (eg. transformation, kidney stone formation) processes. Opn has been found to be expressed by a number of different cell types including osteoblasts, arterial smooth muscle cells, leukocytes (particularly activated macrophages and T-cells), various types of epithelial cells and transformed cells of different lineages (Denhardt *et al.*, 1995).

In normal breast tissue, opn is expressed by secretory phase ductal epithelium, occasionally by non-lactating breast epithelial cells, and is seen to be localized on the apical (luminal) aspect of the cells (Brown *et al.*, 1992). Elevated plasma levels of opn have been reported in patients with metastatic carcinoma, including metastatic breast cancer (Senger *et al.*, 1988; Singhal *et al.*, 1995; Singhal *et al.*, 1996; Singhal *et al.*, 1997). We have recently reported an association between high plasma level of opn, increased tumor burden, and decreased survival in patients with metastatic breast cancer (Singhal *et al.*, 1996; Singhal *et al.*, 1997). Opn has also been detected in the primary tumors of patients with breast cancer (Brown *et al.*, 1994; Hirota *et al.*, 1995; Bellahcène and Castronovo, 1995), where evidence has suggested expression by tumor infiltrating macrophages. To this point, there has been no association established between levels of expression of tumor opn and prognosis in patients with breast cancer.

Although opn protein has been detected by immunohistochemistry (IHC) in the tumor cells themselves (Brown *et al.*, 1994; Bellahcène and Castronovo, 1995), a lack of *in situ* evidence for RNA expression by the cancer cells has led Brown et al. (1994) to suggest that opn

secreted by macrophages may bind to and be taken up by the tumor cells. However, there is experimental evidence that cultured mammary carcinoma cells (eg. D2HAN series [Senger *et al.*, 1983; Morris *et al.*, 1993]; MDA-MB-435 cells [Bautista *et al.*, 1994]) not only may produce opn, but that at least some (MDA-MB-435 cells) show RGD-dependent adhesion to and migration towards opn in culture (Bautista *et al.*, 1994; Xuan *et al.*, 1994; Xuan *et al.*, 1995; Senger and Perruzzi, 1996).

We have here undertaken a study to examine the expression of opn in the primary tumors of a group of 154 lymph node negative breast cancer patients. The first aim was to establish whether tumor cell immunopositivity for opn protein may be at least in part attributable to opn mRNA expression by the tumor cells themselves. Regardless of the cellular source of opn protein, the second aim was to examine whether **tumor cell** IHC staining for opn protein may be associated with tumor aggressiveness (poor survival) in this group of LNN breast cancer patients.

MATERIALS AND METHODS

Patients: One hundred and fifty four patients with lymph node negative (LNN) breast cancer were identified from the records at the London Regional Cancer Centre (London, Ontario, Canada). Data available included age, menopausal status, tumor size, biochemical ER and PR receptor status, p53 status, and definitive surgical treatment. Dates of recurrence or death were recorded as well as date of last follow-up for those who remained disease-free. All of the 154 patients showed either invasive or microinvasive mammary carcinoma.

Opn Immunohistochemistry: Formalin-fixed, paraffin-embedded tumor samples were assessed for opn expression by an immunoperoxidase technique (Chambers *et al.*, 1996; Casson *et al.*, 1997). Representative four micron sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 7 minutes. An antigen retrieval method was used on all slides prior to immunostaining. This involved heating tissue sections in citrate buffer (10mM, pH 6.0) in a microwave oven (600 watt) for 7 minutes. Immunostaining was performed using a streptavidin-biotin complex method (Zymed histostain kit-Dimension Laboratories, Toronto, ON). Non-specific staining was blocked by incubating slides with 5% normal goat serum. The primary antibody used was monoclonal antibody mAb53, prepared against the recombinant GST-human opn fusion protein (Bautista *et al.*, 1994). This antibody has been previously shown by Western analysis, ELISA and immunohistochemistry to efficiently and specifically detect human opn (Bautista *et al.*, 1994; Bautista *et al.*, 1996; Chambers *et al.*, 1996; Casson *et al.*, 1997). The slides were incubated with primary antibody for one hour at room temperature. Slides were then rinsed with PBS, and

a biotinylated secondary antibody was applied for 15 minutes, followed by a PBS rinse and treatment with the streptavidin-enzyme conjugate for 10 minutes. The chromogen used was aminoethyl carbazol (reddish-brown signal), and slides were counterstained with Mayer's hematoxylin. Immunostained slides were evaluated by light microscopy. A proportion score and intensity score were assigned, using the system described by Allred *et al* (1993), as we have used previously (O'Malley *et al.*, 1996; Mack *et al.*, 1997; Tuck *et al.*, 1997). The proportion score represented the estimated fraction of positively staining tumor cells (0 = none; 1 = <1/100, 2 = 1/100-1/10; 3 = 1/10-1/3; 4 = 1/3-2/3; 5 = >2/3). The intensity score represented the estimated average staining intensity of positive tumor cells (0 = none, 1 = weak, 2 = intermediate, 3 = strong). The overall amount of positive staining was then expressed as the sum of the proportion and intensity scores (ranges = 0 for negative staining and 2-8 for positive staining).

Plasmids: The *opn* plasmid used for generation of riboprobes (OP-10) consisted of the complete protein-encoding region of human osteopontin (1493 bp) cloned into a Bluescript SK vector at the *Eco*RI site between the T3 and T7 promoters, with the 5' end of the gene downstream to the T3 promoter (Young *et al.*, 1990).

Riboprobes: Riboprobes were generated by *in vitro* transcription from linearized templates with the appropriate phage RNA polymerase (Promega Corp., Madison, WI) in the presence of digoxigenin-UTP (Boehringer Mannheim, Montreal, PQ). Antisense riboprobes for opn were generated by transcription from the T7 promoter of plasmid OP-10, and negative control sense

riboprobes by transcription from the T3 promoter.

In situ hybridization: ISH was performed essentially as described previously (Tuck *et al.*, 1996). Briefly, four micron paraffin sections were cut under RNase-free conditions onto Superfrost Plus (Fisher, Ottawa, ON) slides, dewaxed in xylene, and rehydrated. Permeabilization was performed by treating at room temperature sequentially with 0.2M HCl, 0.2% Triton X-100 in PBS, and 40 μg/ml proteinase K for 10 min each. Slides were then washed in 0.1X PBS, refixed for 30 min at room temperature in 4% paraformaldehyde, washed again in 0.1X PBS and acetylated with 0.25% acetic anhydride in 0.1M triethanolamine HCl. Slides were then dehydrated, delipidated in 100% chloroform for 15 min, followed by treatment with absolute ethanol for 5 min and 95% ethanol for 15 min, and then air-dried. Probes were diluted in prehybridization mix to a concentration of 800 ng/ml, and 200 μl of hybridization mix was applied to each section. Slides were incubated in a humid chamber at 42°C overnight, washed in 0.2X SSC at 55°C for 30 min, rinsed in RNase buffer (0.5M NaCl, 10mM PIPES [pH 7.2], 0.1% Tween 20) at room temperature for 10 min, and incubated in 20 μg/ml RNase A (Sigma, St. Louis, MO) for 30 min at 37°C to remove unbound single-stranded RNA. Slides were washed in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) at room temperature for 10 min and blocked with 3% normal sheep serum in buffer 1 at room temperature for 30 min. To detect specific hybrids, slides were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) (1 to 1,000 dilution in buffer 1 with 3% normal sheep serum) overnight at 4°C, then washed twice (10 min each) with buffer 1, and twice (5 min each) in buffer 2 (100 mM Tris-HCl [pH9.5], 100 mM NaCl, 50 mM MgCl$_2$). Hybrids bound to anti-

digoxigenin antibody were then visualized by a color reaction containing nitroblue tetrazolium salt (NBT), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and Levamisole (0.24 mg/ml) in buffer 2. (An alkaline phosphatase substrate kit IV [BCIP/NBT] [Vector Laboratories, Burlingame, CA] was used). Color was allowed to develop for 4-6 hours in the dark. Slides were then dehydrated, washed in xylene, mounted with permount, and viewed and photographed by a light microscope. Positive areas showed brown-purple cytoplasmic staining.

Controls for specificity of probe binding included: (a) hybridizing with sense riboprobes and (b) omitting riboprobe entirely. Other controls included antisense and sense probe testing of cell blocks prepared of a cell line known to synthesize high levels of opn (MDA-MB-435 cells [Bautista *et al.*, 1994]).

Statistical Analyses: For opn and p53 IHC analysis, formal cutpoint analysis was conducted coding the variables as outlined in Walter *et al.* (1987), to detect the optimal cutpoint and thus define positivity vs. negativity for opn and p53. Univariate associations between all reported risk factors and outcomes were first assessed using chi-square tests, Fisher's exact test, or logistic regression where appropriate. All variables with a univariate p-value ≤ 0.25 were considered eligible for inclusion in the multivariate model (Hosmer and Lemeshow, 1989). A multiple-step backward selection method was then used and variables were removed from the model if significance fell above a p-value of 0.05. Those variables remaining in the model were considered to be independent predictors of outcome. All analysis was performed using SAS for Windows, version 6.08 (SAS Institute Inc., 1990).

RESULTS

A summary of the patient data base is shown in Table I. Patient ages in the study group ranged from 26 to 83 years, with a mean of 52.1 years. Fifty-three percent of the patients were premenopausal, and 47% were postmenopausal. Tumor sizes ranged from 0.1 to 8.0 cm (mean=2.5 cm). A particularly high proportion of tumors were in the 2-3 cm size range (71%). Of those tumors in which the biochemical hormone receptor status was known (93% of tumors), 70% were estrogen receptor (ER) positive, and 74% were progesterone receptor (PR) positive. The pathology of all tumors was reviewed by us (FOM, ABT). One hundred and forty seven of the 154 invasive tumors were of "no special type" (infiltrating ductal carcinoma, not otherwise specified). The special type carcinomas included two tubular carcinomas, a tubulolobular carcinoma, two infiltrating lobular carcinomas of "classic type", a "pleomorphic variant" of infiltrating lobular carcinoma, and a mucinous carcinoma. Of those tumors for which an SBR grade could be assigned (143/154), thirty patients (21%) had grade I carcinomas, 55 patients (38.5%) had grade II carcinomas, and there were 58 (40.5%) grade III tumors. Using a previously described semiquantitative method of scoring p53 immunopositivity (Allred *et al.*, 1993), and cutpoint analysis to determine the optimum cutoff score, 22 of the 154 cases (14.3%) showed p53 positivity (score >5).

Immunohistochemical staining of sections from the 154 tumors showed diffuse cytoplasmic positivity for opn in scattered tumor infiltrating macrophages and/or lymphocytes in the majority (70%) of cases. Accumulation of extracellular and macrophage-associated opn was also seen in regions of necrosis and calcification, as previously described (Hirota *et al.*, 1995). Benign epithelium showed only focal, faint staining for opn in occasional sections.

When present, opn staining in benign epithelium was usually seen concentrated at the luminal (apical) surface of the cells. Forty tumors (26%) showed opn staining of the carcinoma cells themselves, with immunohistochemical scores (determined as in Allred et al (1993) [see Methods]) ranging from 3 to 7. The opn positivity was seen mainly in an intracytoplasmic (often perinuclear) or, less commonly, in a cell surface distribution in the neoplastic cells. A representative tumor showing IHC tumor cell staining for opn is shown in Figure 1a.

In situ hybridization was performed in order to determine the cellular distribution of opn mRNA expression in sections from selected immunopositive tumors (all infiltrating mammary carcinomas of "no special type" [ductal], 4 showing high and 4 low level of tumor cell immunopositivity for opn). Non-neoplastic ductal epithelium in these sections showed only focal, faint staining. In 7/8 cases and as described previously (Brown *et al.*, 1992; Hirota *et al.*, 1995), opn mRNA was detected in scattered groups of tumor infiltrating macrophages and lymphocytes (Figure 2). In addition, opn mRNA-expressing invasive carcinoma cells were seen in all 8 cases, both as isolated cells and variably-sized clusters. The degree of opn mRNA expression by the tumor cells was heterogenous and regional. Strong focal positivity for opn mRNA was seen in the invading cells of tumors that had shown strong immunopositivity of the tumor cells. Matched (mirror-image) sections allowed identification of individual tumor cells which expressed both opn mRNA and protein (Figure 1b&c). Thus, we have shown by combined use of ISH and IHC that breast cancer cells themselves (in addition to tumor infiltrating inflammatory cells) can synthesize opn *in vivo*. (As controls for these studies: the MDA-MB-435 cells, which are known to express high levels of opn (Bautista *et al.*, 1994), showed strong positive staining when hybridized with antisense opn riboprobe, and complete lack

of staining with sense riboprobe (Figure 2). In addition, none of the tumors showed positive staining when hybridized with sense riboprobe, or when the riboprobe hybridization step was omitted entirely).

Our interest in this study was focused on the ability of **tumor cells** themselves to either synthesize or sequester opn, and on the potential biological consequences regarding tumor aggressiveness. We thus examined the relationship of **tumor cell** (vs. tumor infiltrating inflammatory cell) opn IHC score with tumor aggressiveness as measured by outcome (disease free and overall survival) in this group of LNN breast cancer patients. Cutpoint analysis was performed for tumor cell opn staining (as described in Materials and Methods), and the optimum cutoff score was >4. Using this cutoff score, 11 (7%) of tumors showed opn tumor cell positivity. Patients were followed for a median of 7 years, range 1.7 to 16.3. Forty five patients died of disease. Univariate survival analysis showed tumor cell opn positivity (score >4) to be significantly associated with both decreased disease free survival (DFS) (p=0.0025) and overall survival (OS) (p=0.0294) (Table II, Figure 3). Increased tumor cell opn, whether synthesized by the tumor cells themselves or bound and sequestered from the environment, was thus significantly associated with tumor aggressiveness (as measured by decreased survival).

This work represents a pilot study assessing the potential prognostic value of IHC for tumor cell opn in a group of 154 LNN breast cancer patients. Further definitive study must be done to establish the utililty of tumor cell opn as a prognostic indicator in LNN breast cancer in general. However, we did perform multivariate analysis of this data set, to determine if tumor cell opn was independently predictive of outcome in this population. In the multivariate model, which included patient age, menopausal status, tumor size, grade, hormone receptor status, and

p53 positivity (determined by immunohistochemistry), tumor cell opn positivity remained a significant predictor of decreased overall survival (p=0.0138, RR=2.971), but not of disease-free survival (p=0.3217, RR=1.634).

DISCUSSION

This study is the first to demonstrate that human breast carcinoma cells themselves (in addition to tumor infiltrating macrophages and lymphocytes) can synthesize opn *in vivo*, and that immunohistochemical **tumor cell** positivity for opn is associated with poor clinical outcome in a group of LNN breast cancer patients. We found that tumor infiltrating macrophages and lymphocytes that are positive for both opn mRNA (by ISH) and protein (by IHC) are present both in tumors that do and do not show evidence of tumor cell immunopositivity for opn. Thus, the mere presence of opn positive inflammatory cells does not determine the presence of tumor cell opn. This distinction is important, as we have shown in this study that opn present in or on the tumor cells themselves is associated with poor survival, suggesting that the ability of the **tumor cells** to bind and sequester or synthesize opn may in turn be associated with tumor aggressiveness.

Results from cell culture have shown *in vitro* expression of OPN by a number of different transformed mammary epithelial cell lines (eg. D2HAN cells [Senger *et al.*, 1983; Morris *et al.*, 1993], MDA-MB-435 cells [Bautista *et al.*, 1994], 21T series [Band *et al.*, 1990] cell lines [Tuck et al., unpublished observations]). It is thus reasonable to expect that at least in some instances, breast cancer cells themselves may synthesize opn *in vivo* as well. In the present study, we have shown that indeed, regional expression of opn mRNA by the tumor cells may be found in immunopositive tumors, in addition to expression by tumor infiltrating macrophages and lymphocytes. Thus, tumor cell opn may be accounted for by some combination of synthesis by the cancer cells themselves, and/or uptake from tumor-infiltrating inflammatory cells.

In our work, and that of others (Brown *et al.*, 1994; Hirota *et al.*, 1995) there is

agreement that the majority of breast tumors show the presence of tumor infiltrating macrophages and/or lymphocytes that express opn mRNA and protein. In addition, both our study and that of Brown et al (1994) include a subgroup of tumors which show tumor cell positivity for opn protein as well. However, only in our study is there ISH evidence for opn mRNA expression by the tumor cells as well. We believe this difference is most likely due to sampling and case selection. The tumor samples which we selected for screening for ISH were derived from a bank of 154 breast tumor samples. From these, four of the tumors chosen for ISH testing were those showing the strongest immunopositivity of the tumor cells themselves. Other studies (Brown *et al.*, 1994; Hirota *et al.*, 1995) have examined a smaller number of breast cancers (14 and 13 respectively), and did not report specifically selecting those high in tumor-cell opn immunopositivity for ISH analysis. Our demonstration of an association between tumor cell opn and outcome suggests that at least in this group of LNN patients, those cancers containing tumor cells positive for opn behave differently than those that do not. Opn-positive tumor cells may be different through some combination of the ability to sequester the molecule from the environment (perhaps by expression of specific cell surface receptor(s) necessary for opn accumulation), and the ability to themselves sythesize the molecule. Although the functional consequences of opn on breast cancer cells have not yet been completely elucidated, there is evidence from cell culture that at least some breast cancer cells can adhere to and show increased migration in response to opn (Bautista *et al.*, 1994; Xuan *et al.*, 1994; Xuan *et al.*, 1995; Senger and Perruzzi, 1996), suggesting one potential mechanism for increased aggressive behavior of opn-positive tumors.

If opn indeed plays a role in some aspect of malignancy in breast cancer (such as in tumor

cell motility and invasiveness), then the ability of some tumor cells to themselves synthesize opn may allow the cells to favorably alter their own microenvironment. This may in turn circumvent a dependence on other cell types (such as tumor infiltrating inflammatory cells) to serve this role, and may thus represent a step in progression towards greater tumor autonomy. In this light, the finding of an association between tumor cell immunopositivity for opn and outcome in this group of LNN breast cancer patients is of both biological and clinical interest. The potential role of tumor cell opn as a prognostic indicator in LNN disease is intriguing, but must be borne out by more definitive study in a large, representative consecutive series of LNN patients. More detailed analysis of the biological effects of opn on breast cancer cells is also needed in order to appreciate the nature of its role in malignancy, with a view towards the possible future identification of new therapeutic targets.

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Table I: Profile of a group of 154 lymph node negative breast cancer patients.

Parameter	Number (Range)	%
Age	mean = 52.1 yrs (26-83)	-
Menopausal Status: Premenopausal Postmenopausal	 82 72	 53 47
Tumor Size	mean = 2.5 cm (0.1-8.0)	-
Hormone Receptors: ER positive PR positive	 100/143 106/143	 70 74
Histology: No Special Type Tubular Tubulolobular Lobular ("classic") Lobular, pleomorphic variant Mucinous	 147 2 1 2 1 1	 95 1.3 0.6 1.3 0.6 0.6
Grade: I II III	 30 55 58	 21 39 40
p53 positive	22	14
opn: Macrophage staining Tumor cell staining	 108 40	 70 26

Table II: Univariate analysis using Cox Proportional Hazards methods.

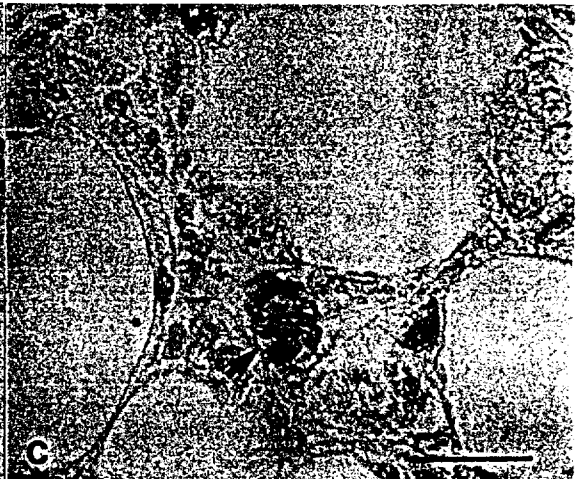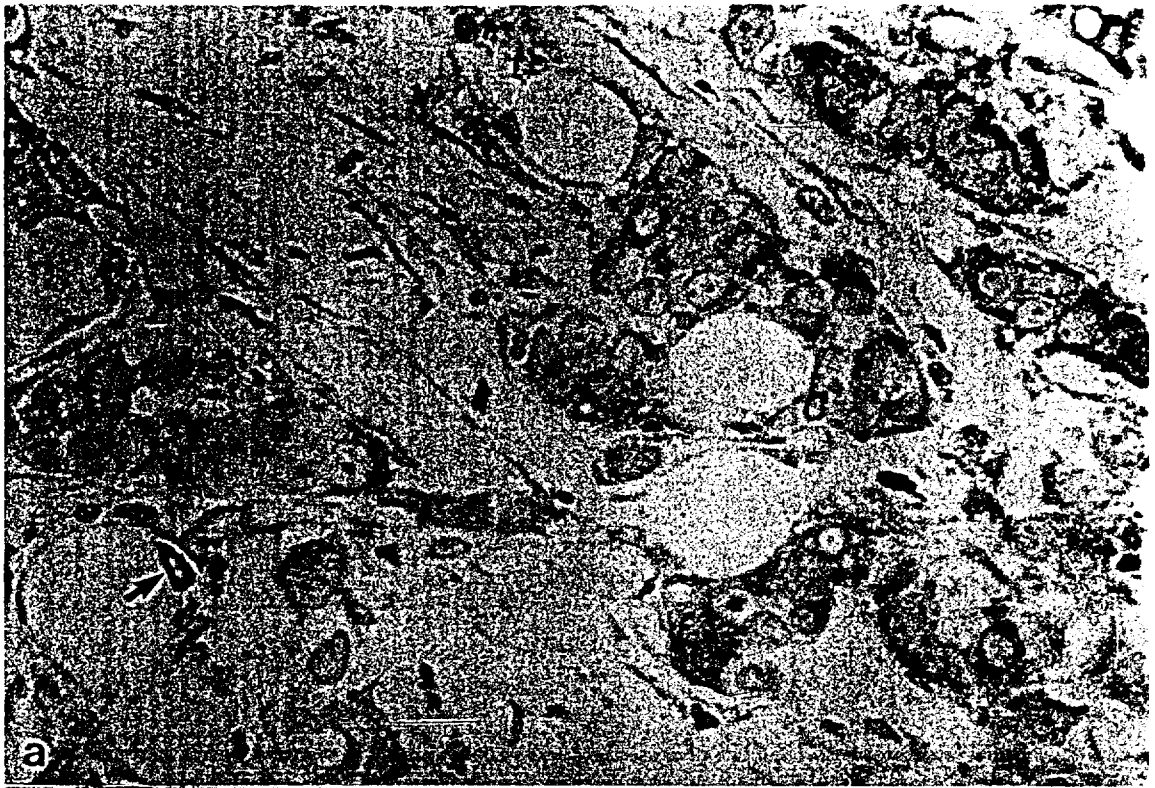| Parameter	DFS p value	OS p value
Age	0.518	0.132
Menopausal Status	0.0408	0.6182
Tumor Size	0.750	0.5673

Hormone Receptors:
  ER status
  PR status
	
	0.8017
	0.5127
		
		0.6805
		0.4538

Grade	0.510	0.4704
p53 (score > 5)	0.0074	0.003
Tumor cell opn (score > 4)	0.0025	0.0294

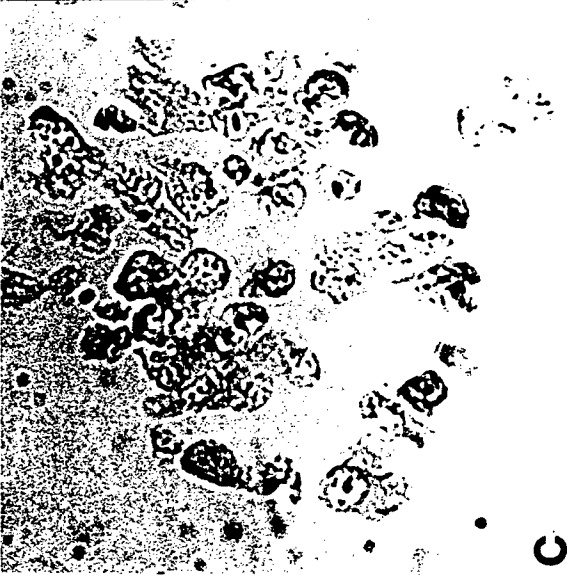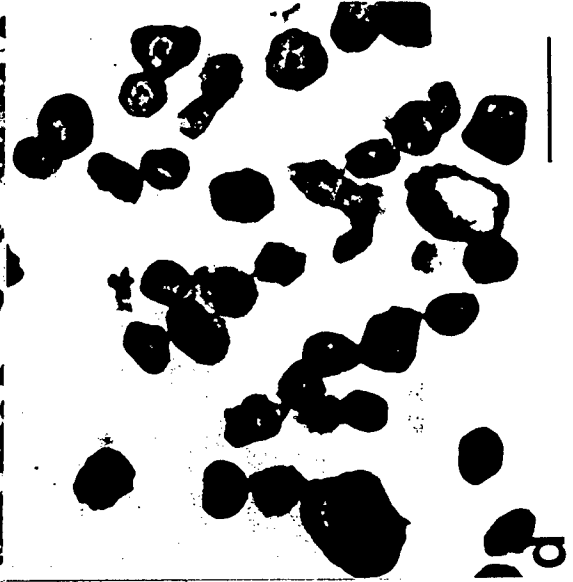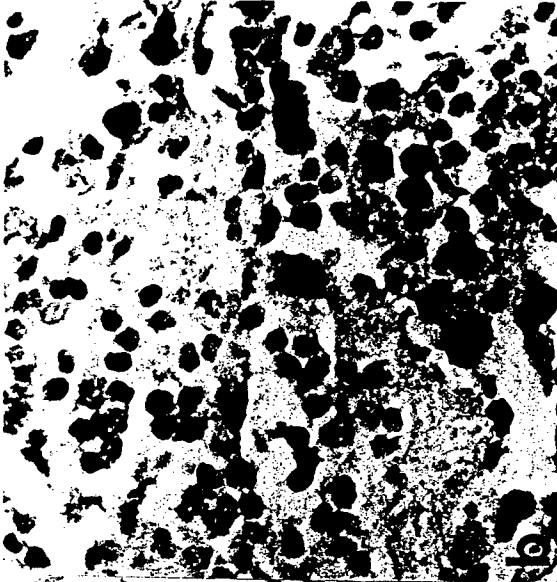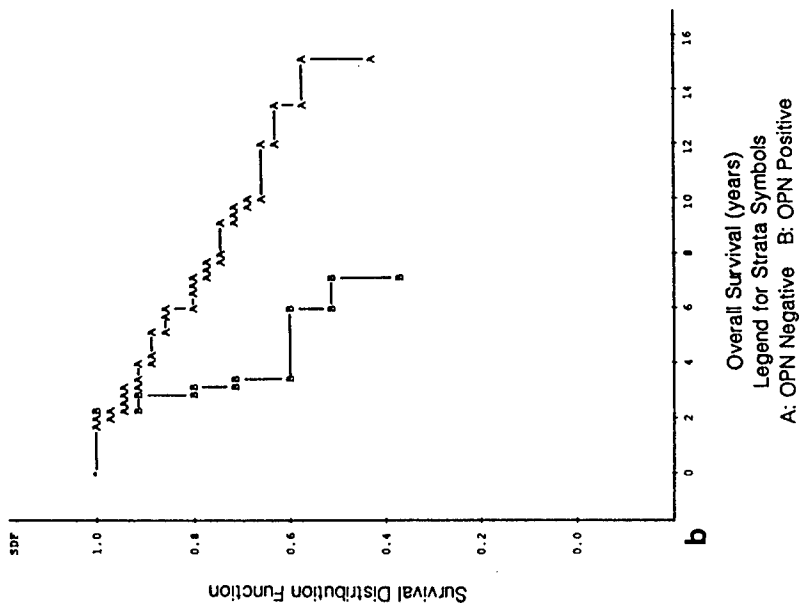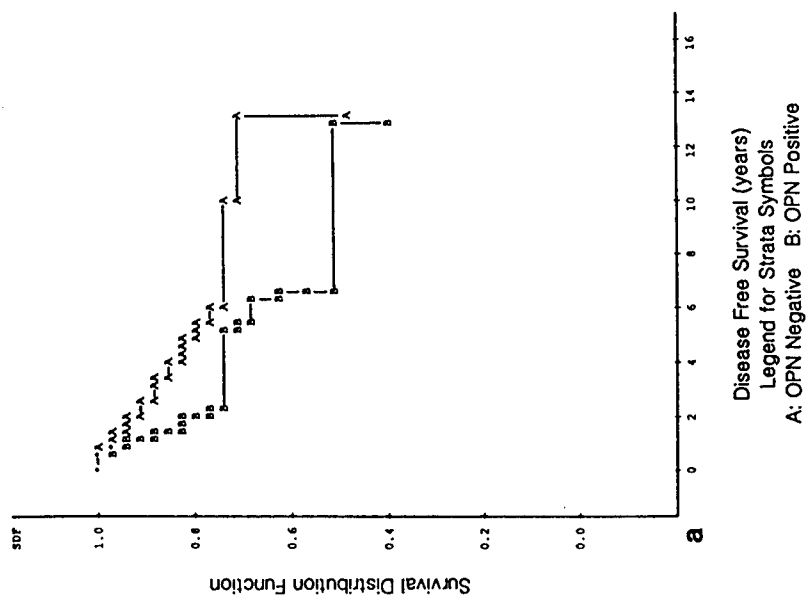DFS = Disease Free Survival; OS = Overall Survival

FIGURE LEGENDS

Figure 1: a) Immunohistochemical positivity of tumor cells for opn protein (score 6) in an infiltrating mammary carcinoma of no special type, combined histologic grade II/III. A tumor infiltrating macrophage showing staining for opn is indicated by the arrow. b) and c) A tumor different from that shown in a), with more focal tumor cell positivity for opn. Matched (mirror image) sections of the same field are shown: panel b) *in situ* hybridization (ISH) for opn mRNA; panel c) immunohistochemistry (IHC) for opn protein. The same breast carcinoma cell (arrow in each panel) is seen to stain positive for both opn mRNA and protein. Scale bar: 25 μm (a-c).

Figure 2: *In situ* hybridization for opn mRNA. a) Strong cytoplasmic positivity for opn is seen regionally in carcinoma cells of opn immunopositive tumors. b) Tumor associated mononuclear inflammatory cells showing strong cytoplasmic staining for opn mRNA. c) Negative control, showing lack of cytoplasmic staining in cultured human MDA-MB-435 cells (known by Northern analysis to express high levels of opn mRNA) when probed with "sense" riboprobe (phase contrast). d) Positive control, showing strong cytoplasmic staining for opn mRNA when cultured human MDA-MB-435 cells are probed with "antisense" riboprobe. Scale bar: 25 μm (a-d).

Figure 3: Disease free survival (DFS) (a) and overall survival (OS) (b) curves for lymph node negative breast cancer patients whose tumors were opn-positive [B] (**tumor cell** IHC score > 4) vs. opn-negative [A] (**tumor cell** IHC score ≤ 4), as determined by immunohistochemistry (DFS p=0.0025; OS p=0.0294).

b

Survival Distribution Function

SDF — 1.0, 0.8, 0.6, 0.4, 0.2, 0.0

Overall Survival (years) — 0, 2, 4, 6, 8, 10, 12, 14, 16

Overall Survival (years)
Legend for Strata Symbols
A: OPN Negative   B: OPN Positive

a

Survival Distribution Function

SDF — 1.0, 0.8, 0.6, 0.4, 0.2, 0.0

Disease Free Survival (years) — 0, 2, 4, 6, 8, 10, 12, 14, 16

Disease Free Survival (years)
Legend for Strata Symbols
A: OPN Negative   B: OPN Positive

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