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13. ABSTRACT *(Maximum 200)*

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, increased Met activity 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, with involvement of CD44 as well. 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 (uPA) expression and activity. Results of *in vivo* assays have indicated that although increased OPN expression was not associated with increased tumor-take, some OPN-transfected 21NT cells acquired metastatic potential. These studies implicate OPN in the malignancy of human breast cancer, and suggest potential mechanisms to include integrin (and CD44)-mediated cell adhesion and migration phenomena, as well as induction of uPA and cellular invasiveness.

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FOREWORD

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Alan Tuck 23/06/99

PI - Signature Date

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

INTRODUCTION

The goal of this research was to determine the functional role of osteopontin (OPN), a secreted phosphoprotein, in the malignancy of human breast cancer. Recent evidence (including a number of clinical studies) has strongly implicated 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 has been poorly understood. This information is important to obtain, as potential utility lies 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. The purpose of this project was 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. This was accomplished by fulfillment of three **technical objectives: 1) to determine whether OPN expression is associated with malignancy of established human breast epithelial cell lines (21T series vs. MDA-MB-435 cells), 2) to determine whether up-regulation of OPN expression changes the malignant properties of these cells, and 3) to examine how OPN affects other measurable properties associated with malignancy.** These studies have shown not only that level of OPN expression is associated with degree of malignancy of breast cancer cells, but that OPN serves a functional role in cell adhesion, migration, and invasiveness. Molecular mechanisms of these effects have also been elucidated.

ANNUAL SUMMARY

This work has made use of four breast epithelial cell lines, known to differ in degree of malignancy. The 21T series include: 21PT - immortal, non-tumorigenic; 21NT - tumorigenic, non-metastatic; and 21MT-1 - weakly metastatic. As a basis of comparison, MDA-MB-435 cells were also used (tumorigenic, highly metastatic). Work related to **Objective 1** 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. Furthermore, those of the 21T series that are more malignant (21MT-1>21NT>21PT) were shown by Western analysis of conditioned media to secrete higher levels of OPN protein. Interestingly, the predominant form of OPN secreted by the 21T series is of high molecular weight (~ 97 kDa), whereas MDA-MB-435 cells in addition secrete lower molecular weight (predominantly 66 kDa) forms. The high molecular weight species may represent either a very heavily post-translationally modified, or a conjugated (most likely by transglutaminase) form. In keeping with this interpretation, we found 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. Although the biologic activity of the various different MW forms of OPN is at present unknown, it is clear that cells of differing malignancy do express different relative amounts. The finding that 21PT and 21NT cells expressed relatively low basal levels of OPN also rendered these cells good candidates for upregulation by transfection with an OPN-containing expression vector (as below). *In vivo* growth properties were assessed in comparison with the OPN-transfected cells, as described below.

In order to address **Objective 2**, we established stable transfectant populations (pooled and cloned) of both 21PT and 21NT cells, using an OPN-expression vector and the control (neo-containing pcDNA3) vector. We were unable to obtain stable transfectants of 21MT-1 cells (due mainly, we think, to their inherent genetic instability). However, the lack of OPN-transfected 21MT-

1 did not significantly impact on the success of this objective, and 21MT-1 cells were used as a basis of comparison (tumorigenic, weakly metastatic cell line of same lineage as 21PT and 21NT). A number of G418-resistant pooled and cloned transfectant populations of 21PT and 21NT cells were obtained and screened for expression of OPN protein (by ELISA and Western) and mRNA (by Northern). Those OPN-transfected populations expressing the highest levels of OPN were compared against vector-control only-transfected cells in both *in vitro* (below) and *in vivo* assays. Results of the *in vivo* assays in female nude mice have indicated that although increased OPN expression was not associated with increased tumor-take (in the mammary fat pad), some OPN-transfected 21NT cells acquired metastatic potential (lung micrometastases 12 months after injection). A major reason that the OPN-transfected cells were not more metastatic in nude mice may be the lack of expression of $\alpha v\beta 3$ integrin, (which MDA-MB-435 cells do express [see below] and which has been shown by other groups to be important in the metastasis of breast cancer), although this hypothesis has yet to be tested.

Work related to **Objective 3** has shown that although exogeneous OPN does not significantly increase growth ability in low serum, it does support cell adhesion of the 21T series and MDA-MB-435 cells. In addition, cellular adhesiveness to OPN was found to be related to degree of malignancy (i.e. MDA-MB-435>21MT-1>21NT>21PT). The 21T series and MDA-MB-435 cells also demonstrated directed cell migration towards exogenous OPN in transwell assays. Synergistic effects on cell migration were found using HGF or EGF in combination with OPN. Time course experiments have shown that induction of 21PT, 21NT and MDA-MB-435 cells with exogenous OPN results in increased Met (HGF Receptor) kinase activity followed by increased Met RNA and protein expression. Induction of TGFα RNA was also found. These findings provide multiple points of potential interaction ("cross-talk") between OPN-induced integrin and growth factor-mediated pathways.

We have found that cell adhesion and migration of 21T series and MDA-MB-435 cells to exogenous OPN are mediated via cell surface integrins, with apparent involvement of CD44 as well (as shown by experiments using RGD mutant OPN and blocking experiments using anti-integrin and anti-CD44 antibodies). MDA-MB-435 cells show $\alpha v\beta 3$ integrin-dependent cell adhesion and migration, whereas 21T series cells use $\alpha v\beta 5$ and $\beta 1$ (not $\alpha v\beta 3$) integrins.

In cell invasion assays, MDA-MB-435 cells showed a more pronounced response to exogenous OPN than 21PT or 21NT cells. In time course experiments, OPN induction was found to be associated with increased urokinase-type plasminogen activator (uPA) mRNA expression and enzyme activity of conditioned media. Furthermore, for 21PT and 21NT cells, transient increases in cathepsin B,D and L mRNA were also detected following overnight treatment with exogenous OPN, although no change in MMP-2 or MMP-9 mRNA levels was found. OPN-transfected 21PT and 21NT cells both showed increased invasiveness through Matrigel. Interestingly, the OPN-transfected 21NT clone expressing the highest levels of OPN showed a degree of invasiveness approaching that of MDA-MB-435 cells. On Northern analysis, of all the protease mRNAs examined, uPA was found to show the closest association with level of OPN expression of the transfected cells. Using the OPN-transfected 21NT cells expressing the highest level of OPN, and showing the greatest degree of cellular invasiveness, we have shown that anti-uPA and anti-uPA receptor antibodies, as well as plasminogen activator inhibitor 1 (PAI-1) and aprotinin (a uPA inhibitor) block the invasiveness of these cells to a level comparable to that of the untransfected parental cell line. In this manner, we have shown the OPN-induced increased invasiveness of these cells to be uPA-dependent. Finally, both OPN-transfected 21PT and 21NT cells showed capacity for growth in soft agar, whereas the control vector-transfected cells did not.

These studies together thus supply strong evidence for functional roles of OPN in the malignancy of breast cancer, with effects on cell adhesion, migration, and invasion phenomena.

Further, they supply evidence for the feasibility of approaches based on inhibiting the mechanisms of these effects (eg. blocking integrin-mediated events with anti-integrin antibody; blocking OPN-induced cell invasion with anti-OPN or anti-uPA antibodies or uPA inhibitors), and provide direction for future work aimed at elucidating the specific molecular mechanisms by which the increased cell migration and invasiveness are achieved.

APPENDIX

1) RESEARCH ACCOMPLISHMENTS

- OPN mRNA and protein levels determined for 21PT, 21NT, 21MT-1 and MDA-MB-435. Level of OPN mRNA and protein found to relate to degree of malignancy.
- 21T series and MDA-MB-435 cells tested in adhesion assay. Degree of OPN binding found also to relate to degree of malignancy. Studies with blocking antibodies show adhesion of 21T series and MDA-MB-435 cells to OPN is both integrin and CD44-dependent. Adhesion of 21T series cells is αvß5 and ß1-dependent. Adhesion of MDA-MB-435 cells is αvß3, αvß5, and ß1-dependent.
- Cell migration assays show 21T series and MDA-MB-435 cells respond to both human recombinant and native OPN. Experiments with blocking antibodies show cell migration of 21T series cells and MDA-MB-435 to be integrin and CD44-dependent. Cell migration of 21PT and 21NT to OPN is αvß5 and ß1-dependent, whereas that of MDA-MB-435 is αvß3-dependent.
- EGF and HGF found to show synergy with OPN in induction of cell migration. OPN found to induce Met (HGF receptor), and TGFα mRNA expression. OPN also found to induce Met kinase activity and increased Met protein expression.
- Exogenous (hr) OPN found to induce cell invasion through Matrigel: MDA-MB-435 > 21NT > 21PT.
- hrOPN found to induce urokinase (uPA) mRNA expression and uPA activity in conditioned media (Zymogram analysis) of 21PT, 21NT and MDA-MB-435 cells.
- hrOPN found to induce transient increases in cathepsin B,D and L (but not MMP-2 or MMP-9) mRNA in 21PT and 21NT cells.

- hrOPN at varying concentrations (50-150 μg/ml) does not significantly alter growth ability in low serum of 21T series or MDA-MB-435 cells.
- Stable transfectants of 21PT and 21NT generated using OPN-containing and control vectors.
- 21PT and 21NT transfectants expressing high levels of OPN (mRNA and protein) found to be more invasive through Matrigel and express higher levels of uPA (not cathepsin B, D, or L, MMP-2 or MMP-9) mRNA. This increased invasiveness blocked by anti-uPA antibody and uPA inhibitors.
- OPN-transfected 21NT (not 21PT) cells found to show increased growth ability in soft agar.
- Some OPN-transfected 21NT (not 21PT) cells found to have acquired metastatic ability in nude mice (although no alteration of primary tumor take or growth rate).

2) REPORTABLE OUTCOMES

A number of publications and abstracts related to this work are listed below. Publications 1-3, although not included in the "Statement of Work", were accomplished with the help of protected time afforded by this award.

Publications:

1) Tuck, A.B., O'Malley, F.P., Singhal, H., Tonkin, K.S., Harris, J.F., and Chambers, A.F. (1997) Osteopontin and p53 expression are associated with tumor progression in a case of bilateral, invasive mammary carcinomas. Arch. Pathol. Lab. Med., 121: 578-584.

2) Singhal, H., Bautista, D.S., Tonkin, K.S., O'Malley F.P., Tuck, A.B., Chambers, A.F., and Harris, J.F. (1997) Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. Clin. Cancer Res., 3: 605-611.

3) Tuck, A.B., O'Malley, F.P., Singhal, H., Harris, J.F., Tonkin, K.S., Kerkvliet, N., Saad, Z., Doig, G.S., and Chambers, A.F. (1998) Osteopontin expression in a group of lymph node negative breast cancer patients. Int. J. Cancer, 79: 502-508.

4) Tuck, A.B., Arsenault, D.M., O'Malley, F.P., Hota, C., Ling, M., Wilson, S.M., and Chambers, A.F. (1999) Osteopontin induces increased invasiveness and plasminogen activator expression of human mammary epithelial cells. Oncogene, in press.

5) Tuck, A.B., Elliott, B.E., Hota, C. Tremblay, E., and Chambers, A.F. Osteopontin (OPN)-induced migration of human mammary epithelial cells (MEC) is integrin-dependent and associated with increased hepatocyte growth factor receptor (Met) activity. Manuscript in prep.

Abstracts:

1) Tuck, A.B., O'Malley, F.P., Hota, C., and Chambers, A.F. The role of osteopontin in the malignancy of human breast carcinoma: involvement in cell adhesion, migration, and invasiveness of a progression series of mammary epithelial cell lines. Proceedings of the U.S. Army Breast Cancer Research Program, "Era of Hope" Meeting, Oct. 31 - Nov. 4, 1997, Vol. II, page 697.

2) Tuck, A.B., O'Malley, F.P., Hota, C., and Chambers, A.F. Osteopontin induces integrin-dependent, directed cell migration of tumorigenic and non-tumorigenic breast epithelial cells, and enhances their migration response to epidermal growth factor (EGF) and hepatocyte growth factor (HGF). Breast Cancer Research and Treatment, 46: Abstract #363, 1997.

3) Tuck, A.B., Arsenault, D.M., O'Malley, F.P., Hota, C., Wilson, S.M., and Chambers, A.F. Osteopontin induces increased cellular motility and invasiveness of human mammary epithelial cell lines. Ontario Association of Pathologists, 61st Annual Meeting, London, Ontario, June 12-13, 1998.

4) Tuck, A.B., Arsenault, D.M., O'Malley, F.P., Hota, C., Wilson, S.M., and Chambers, A.F. Osteopontin upregulation is associated with increased cellular invasiveness and urokinase expression of human mammary epithelial cell lines 21PT and 21NT. VII International Congress of the Metastasis Research Society, San Diego CA, Abstract #PB6.11, page 59, Oct. 7-10, 1998.

5) Tuck, A.B., Elliott, B.E., Hota, C., Tremblay, E., and Chambers, A.F. Induction of mammary epithelial cell motility by osteopontin is integrin-dependent and is associated with increased hepatocyte growth factor receptor activity. U.S. and Canadian Academy of Pathology, 88th Annual Meeting, San Francisco CA, Abstract #165, page 31A, March 20-26, 1999.

6) Tuck, A.B., Arsenault, D.M., O'Malley, F.P., Hota, C., Wilson, S.M., Ling, M.C., and Chambers, A.F. Osteopontin induces increased invasiveness and urokinase expression of human mammary epithelial cell lines 21PT and 21NT. Canadian Breast Cancer Research Initiative, "Reasons for Hope" meeting, Toronto, Canada, June 17-19, 1999.

7) Bramwell, V., Doig, G., Tuck, A., Vandenberg, T., Tomiak, A., Perera, F., Tonkin, K., Harris, J., O'Malley, F., and Chambers, A. The role of the secreted phosphoprotein osteopontin (OPN) in metastatic breast cancer: Interim Report. Canadian Breast Cancer Research Initiative, "Reasons for Hope" meeting, Toronto, Canada, June 17-19, 1999.

8) Tuck, A.B., Hota, C., and Chambers, A.F. Osteopontin (OPN)-induced increase in human mammary epithelial cell (MEC) invasiveness is urokinase (uPA)-dependent. Abstract submitted to the 22nd Annual San Antonio Breast Cancer Symposium, December 8-11, 1999.

Funding applied for (and received) based on this award:

1) Source: Canadian Breast Cancer Research Institute (National Cancer Institute of Canada)

Title: The role of the secreted phosphoprotein osteopontin in metastatic breast cancer: clinical and experimental studies; Drs. Chambers, Tuck, Bramwell, Doig, Tonkin, O'Malley and Harris.

Amount/year: $128,625 (CAN).

Period: 1997-2000.

2) Source: Victoria Hospital Research Development Fund, RDF-031-95.

Title: "Functional role of osteopontin in malignancy of human breast cancer", Drs. O'Malley and Tuck.

Amount/year: $12,000 (CAN).

Period: 1996-1997.

3) Source: London Health Sciences Centre Internal Research Fund, IRF-061-96.

Title: The role of osteopontin in the tumorigenicity and metastatic ability of human breast epithelial cells; applicants Drs. O'Malley and Tuck.

Amount/year: $13,000 (CAN).

Period: 1996-1997.

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

Alan B. Tuck, MD, PhD; Frances P. O'Malley, MB; Hemant Singhal, MB; Katia S. Tonkin, MB; John F. Harris, PhD; Diosdado Bautista, PhD; Ann F. Chambers, PhD

• ***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.**

(*Arch Pathol Lab Med.* 1997;121:578–584)

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 expression 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

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From the London Health Sciences Centre, London Regional Cancer Centre, and University of Western Ontario, London, Ontario, Canada.

The content of this article does not necessarily reflect the position or policy of the Canadian or US government, and no official endorsement should be inferred.

Reprint requests to Department of Pathology, London Health Sciences Centre, Victoria Hospital, South St Campus, 375 South St, London, Ontario, Canada N6A 4G5 (Dr Tuck).

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-

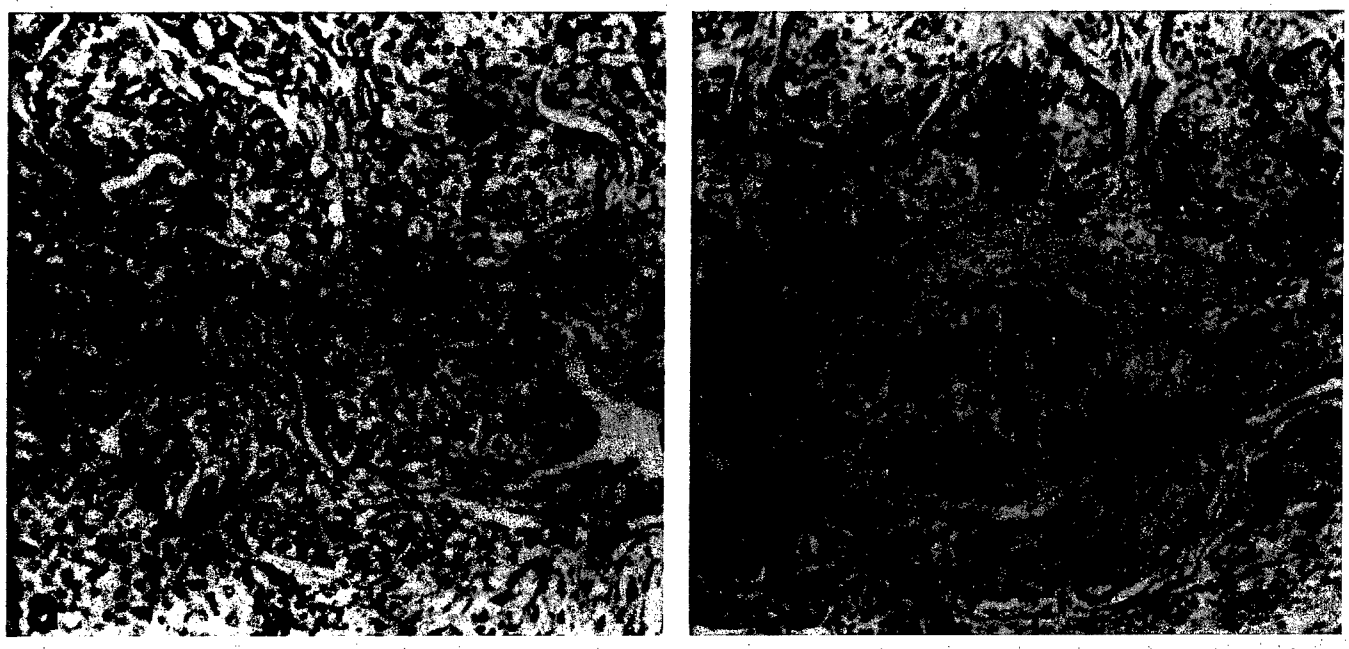


Fig 1.—*Histology of the right (a) and left (b) primary breast tumors. Both are infiltrating mammary carcinomas of no special type, SBR grade II/III (hematoxylin-eosin, original magnification ×250).*

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

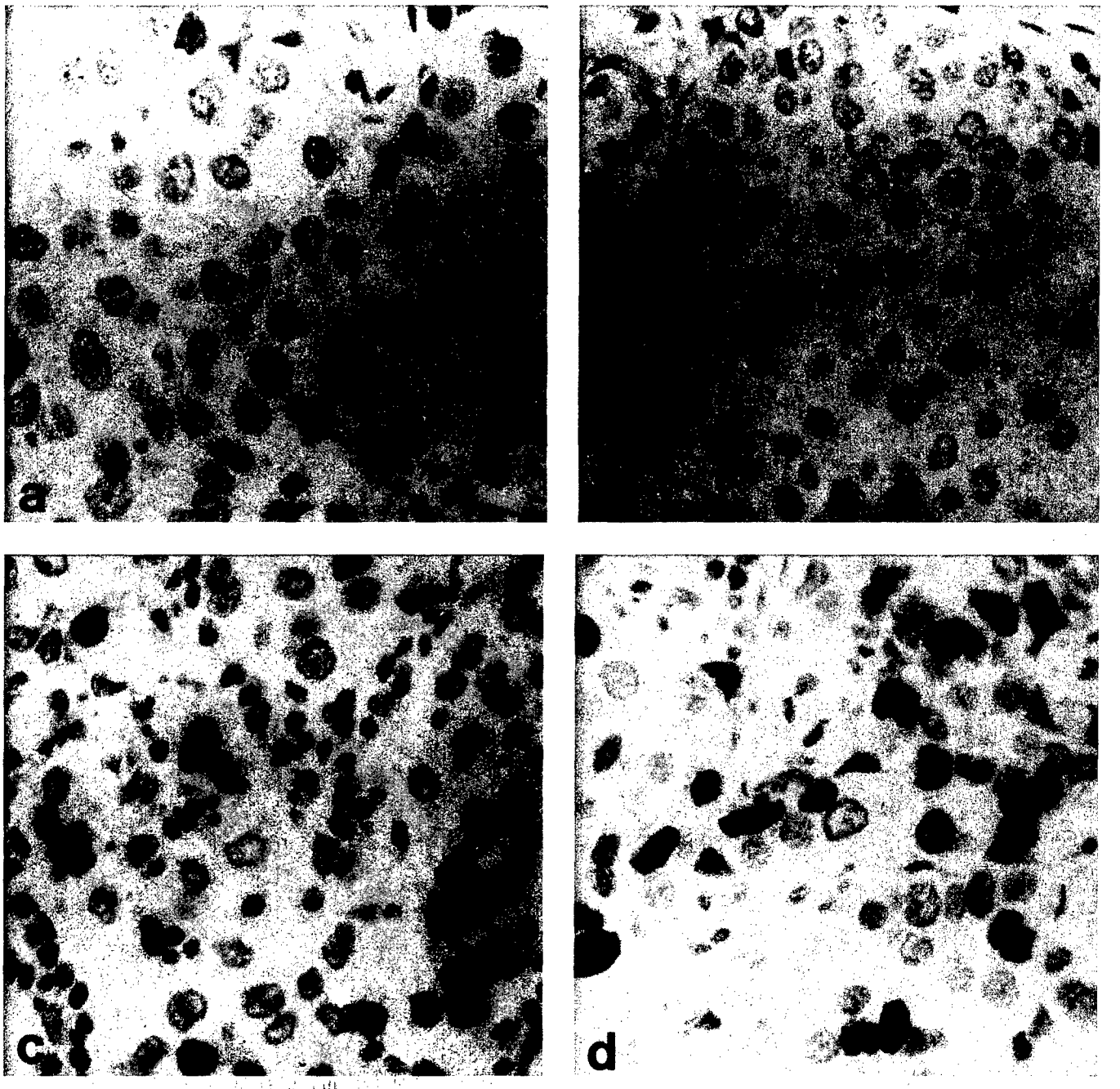
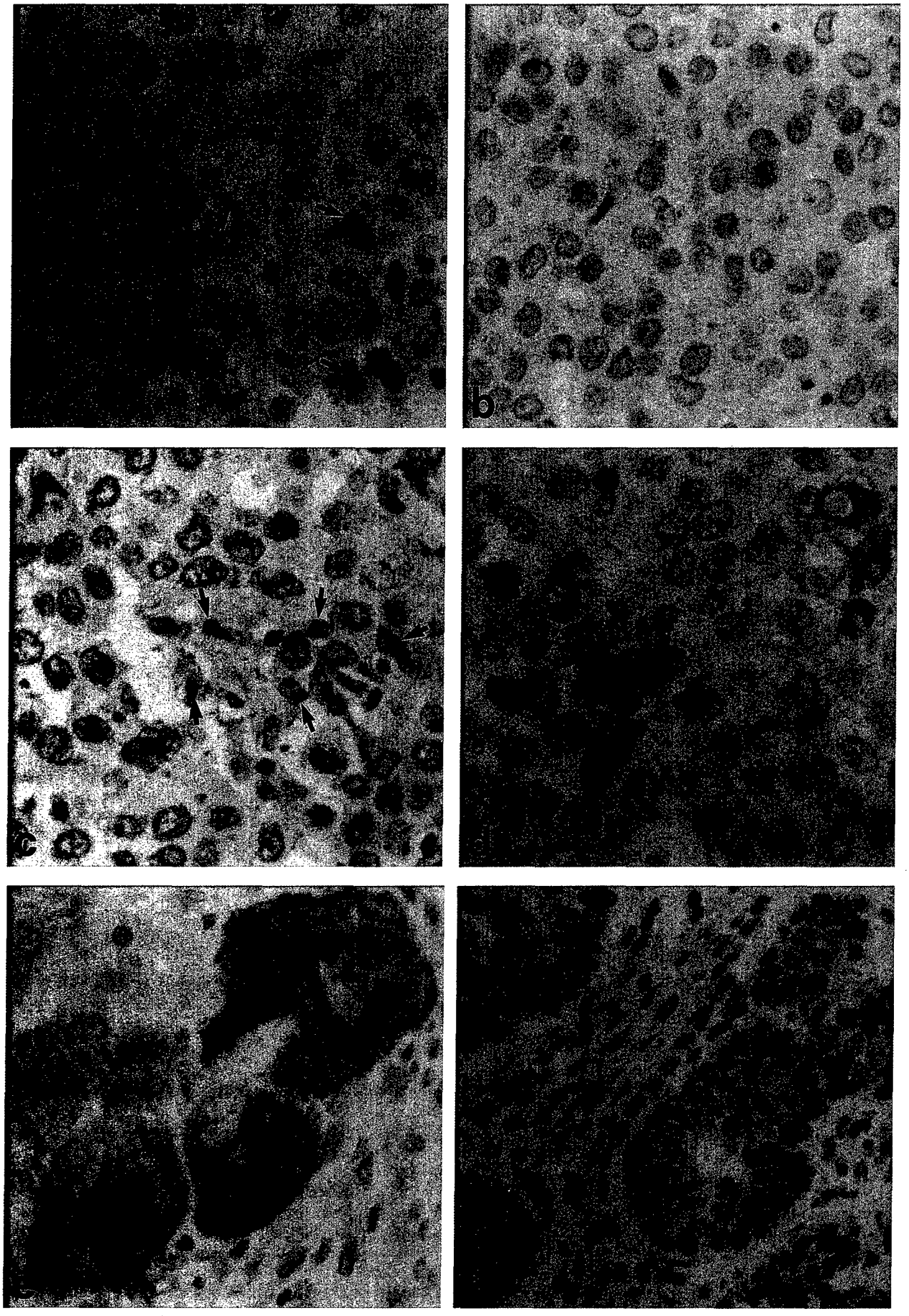


Fig 2.—*Immunohistochemistry (IHC) for p53. a, Right-sided primary breast tumor showing focal strong nuclear positivity for p53 (IHC score 6; 6 [observer 1; observer 2]). b, Left-sided primary breast tumor showing negative staining for p53 (IHC score 0; 0). c, Metastatic carcinoma cells in right axillary 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 resulting 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,


b

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 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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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).*

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References

1. Cattoretti G, Rilke F, Andreola S, et al. p53 expression in breast cancer. *Int J Cancer.* 1988;41:178–183.
2. Ostrowski JL, Sawan A, Henry L, et al. p53 expression in human breast cancer related to survival and prognostic factors: an immunohistochemical study. *J Pathol.* 1991;164:75–81.
3. Davidoff AM, Humphrey PA, Iglehart JD, et al. Genetic basis for p53 overexpression in human breast cancer. *Proc Natl Acad Sci USA.* 1991;88:5006–5010.
4. Thor AD, Moore DH, Edgerton SM, et al. Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J Natl Cancer Inst.* 1992;84:845–854.
5. Allred DC, Clark GM, Elledge R, et al. Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J Natl Cancer Inst.* 1993;85:200–206.
6. Silvestrini R, Benini E, Daidone MG, et al. p53 as an independent prognostic marker in lymph node–negative breast cancer patients. *J Natl Cancer Inst.* 1993;85:965–970.
7. Martinazzi M, Crivelli F, Zampatti C, Martinazzi S. Relationship between p53 expression and other prognostic factors in human breast carcinoma: an immunohistochemical study. *Am J Clin Pathol.* 1993;100:213–217.
8. MacGrogan G, Bonichon F, de Mascarel I, et al. Prognostic value of p53 in breast invasive ductal carcinoma: an immunohistochemical study on 942 cases. *Breast Cancer Res Treat.* 1995;36:71–81.
9. O'Malley FP, Saad Z, Kerkvliet N, et al. The predictive power of semiquantitative immunohistochemical assessment of p53 and c-*erb* B2 in lymph node negative breast cancer. *Hum Pathol.* 1996;27:955–963.
10. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/*neu* protooncogene in human breast and ovarian cancer. *N Engl J Med.* 1989;244:707–712.
11. Thor AD, Schwartz LH, Koerner FC, et al. Analysis of c-*erb* B-2 expression in breast carcinomas with clinical follow-up. *Cancer Treat Res.* 1989;49:7147–7152.
12. Paik S, Hazan R, Fisher ER, et al. Pathological findings from national surgical adjuvant breast project (Protocol D-06): prognostic significance of c-*erb*B-2 protein overexpression in primary breast cancer. *J Clin Oncol.* 1989;8:103–112.
13. Borg A, Tandon AK, Sigurdsson H, et al. HER-2/*neu* amplification predicts poor survival in node-positive breast cancer. *Cancer Res.* 1990;50:4332–4337.
14. Dati C, Muraca R, Tazartes O, et al. c-*erb*B-2 and *ras* expression levels in breast cancer are correlated and show a co-operative association with unfavourable clinical outcome. *Int J Cancer.* 1991;47:833–838.
15. Gullick WJ, Love SB, Wright C, et al. c-*erb*B-2 protein over-expression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. *Br J Cancer.* 1991;63:434–438.
16. Lovekin C, Ellis IO, Locker A, et al. c-*erb*B-2 oncoprotein expression in primary and advanced breast cancer. *Br J Cancer.* 1991;63:439–443.
17. McCann A, Dervan PA, O'Regan M, et al. Prognostic significance of

c-*erb*B-2 and estrogen receptor status in human breast cancer. *Cancer Treat Res.* 1991;51:3296–3303.

18. Winstanley J, Cooke T, Murray GD, et al. The long term prognostic significance of c-*erb*B-2 in primary breast cancer. *Br J Cancer.* 1991;63:447–450.

19. Kamel OW, Franklin WA, Ringus JC, et al. Thymidine labeling index and Ki-67 growth fraction in lesions of the breast. *Am J Pathol.* 1989;134:107–113.

20. Isola JJ, Helin HJ, Helle MJ, et al. Evaluation of cell proliferation in breast carcinoma: comparison of Ki-67 immunohistochemical study, DNA flow cytometric analysis, and mitotic count. *Cancer.* 1990;65:1180–1184.

21. Wintzer H-O, Zipfel I, Schulte-Mönting J, et al. Ki-67 immunostaining in human breast tumors and its relationship to prognosis. *Cancer.* 1991;67:421–428.

22. Weidner N, Moore DH II, Vartanian R. Correlation of Ki-67 antigen expression with mitotic figure index and tumor grade in breast carcinomas using the novel "paraffin" reactive MIB1 antibody. *Hum Pathol.* 1994;25:337–342.

23. Vogelstein B. A deadly inheritance. *Nature.* 1990;348:681–682.

24. Butler WT. The nature and significance of osteopontin. *Connect Tissue Res.* 1989;23:123–136.

25. Denhardt DT, Butler WT, Chambers AF, et al. Osteopontin: role in cell signalling and adhesion. *Ann N Y Acad Sciences.* 1995;760:1–388.

26. Senger DR, Perruzzi CA, Papadopoulos A. Elevated expression of secreted phosphoprotein I osteopontin (2ar) as a consequence of neoplastic transformation. *Anticancer Res.* 1989;9:1291–1299.

27. Craig AM, Nemir M, Mukherjee BB, Chambers AF, Denhardt DT. Identification of the major phosphoprotein secreted by many rodent cell lines as 2ar/osteopontin: enhanced expression in H-*ras*-transformed 3T3 cells. *Biochem Biophys Res Commun.* 1988;157:166–173.

28. Craig AM, Bowden GT, Chambers AF, et al. Secreted phosphoprotein mRNA is induced during multi-stage carcinogenesis in mouse skin and correlates with the metastatic potential of murine fibroblasts. *Int J Cancer.* 1990;46:133–137.

29. Chambers AF, Behrend EI, Wilson SM, Denhardt DT. Induction of expression of osteopontin (OPN; secreted phosphoprotein) in metastatic, *ras*-transformed NIH 3T3 cells. *Anticancer Res.* 1992;12:43–48.

30. Xuan JW, Hota C, Chambers AF, et al. Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. *J Cell Biochem.* 1994;54:247–255.

31. Bautista DS, Xuan JW, Hota C, et al. Inhibition of RGD-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin. *J Biol Chem.* 1994;269:23280–23285.

32. Senger DR, Perruzzi CA, Gracey CR, et al. Secreted phosphoproteins associated with neoplastic transformation: close homology with plasma proteins cleaved during blood coagulation. *Cancer Res.* 1988;48:5770–5774.

33. Singhal H, Bautista D, Tonkin K, O'Malley F, Chambers AF, Harris JF. Plasma levels of osteopontin in primary and metastatic breast cancer. *Can J Surg.* 1995;38(4, suppl):A14. Abstract 358.

34. Singhal H, Tonkin KS, Vandenberg TA, et al. Prospective evaluation of the prognostic significance of elevated plasma osteopontin in metastatic breast cancer. *Proc Am Soc Clin Oncologists.* 1996;15:504. Abstract 1614.

35. Brown LF, Papadopoulos-Sergiou A, Berse B, et al. Osteopontin expression and distribution in human carcinomas. *Am J Pathol.* 1994;145:610–623.

36. Hirota S, Ito A, Nagoshi J, et al. Expression of bone matrix protein messenger ribonucleic acids in human breast cancers: possible involvement of osteopontin in development of calcifying foci. *Lab Invest.* 1995;72:64–69.

37. Bellahcene A, Castronovo V. Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. *Am J Pathol.* 1995;146:95–100.

38. Bautista DS, Saad Z, Chambers AF, et al. Quantification of osteopontin in human plasma with an ELISA: basal levels in pre- and postmenopausal women. *Clin Biochem.* 1996;29:231–239.

39. Muss HB, Thor AD, Berry DA, et al. c-*erb*B-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. *N Engl J Med.* 1994;330:1260–1266.

40. Chambers AF, Wilson SM, Kerkvliet N, et al. Osteopontin in lung cancer. *Lung Cancer.* 1996;15:311–323.

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-

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[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 SigmaPlot (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.

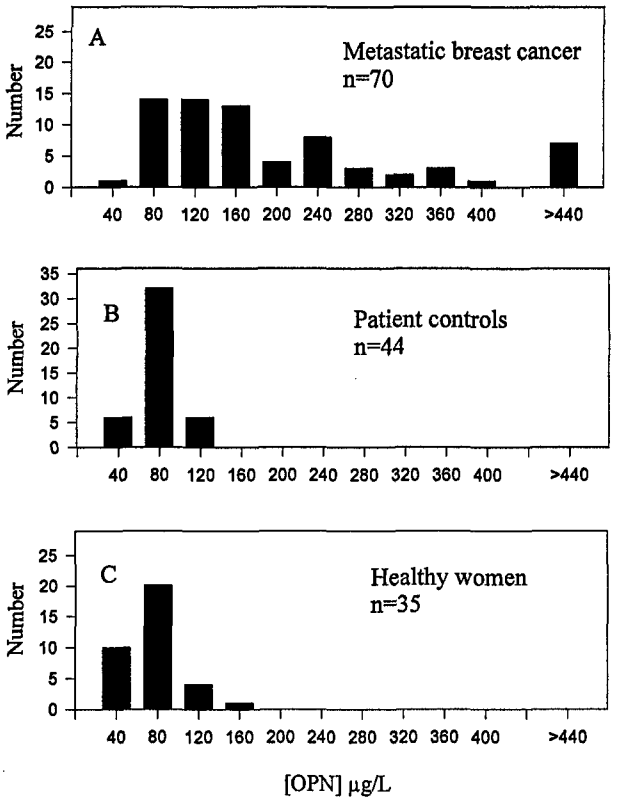

Fig. 1 Frequency distribution of plasma OPN in women diagnosed with metastatic breast cancer, treated for primary breast cancer, and normal controls. The average OPN level was calculated from plasma samples using the immunoassay described in "Materials and Methods" and displayed as a histogram with the 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 ($n =$

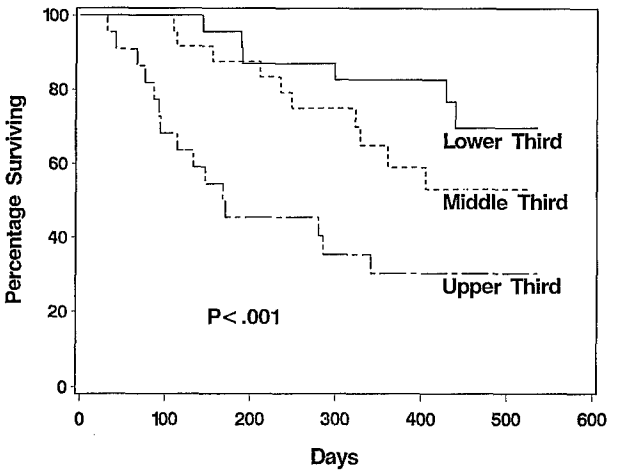

Fig. 2 Survival of patients with metastatic breast cancer grouped into tercile plasma OPN levels. The Kaplan-Meier adjusted percentage surviving is shown as a function of time (days) after OPN plasma levels were determined for patients (n = 69, one lost to follow-up). Patients were grouped into three 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).

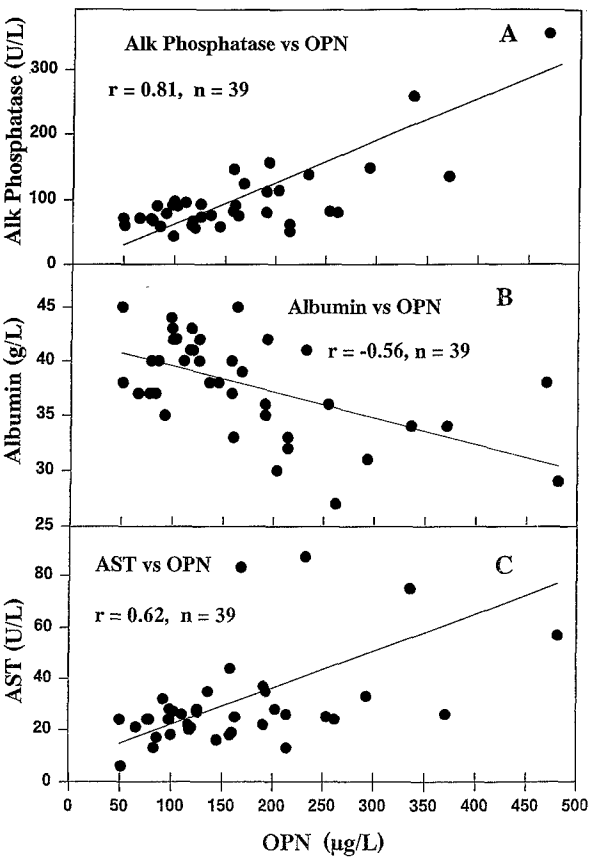

Fig. 3 Correlations between OPN levels and biochemical markers. Correlations between OPN levels and various biochemical markers are shown for alkaline phosphatase (*A:* $r = 0.81$, $P < 3 \times 10^{-10}$, $n = 39$), albumin (*B:* $r = -0.56$, $P < 2 \times 10^{-4}$, $n = 39$), and AST (*C:* $r = 0.62$, $P < 2 \times 10^{-5}$, $n = 39$) 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. 3*A*) and AST ($r = 0.62$; Fig. 3*C*) and negatively correlated with serum albumin ($r = -0.56$; Fig. 3*B*; 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. 4*A* 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 molecular 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. 4*B* 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.

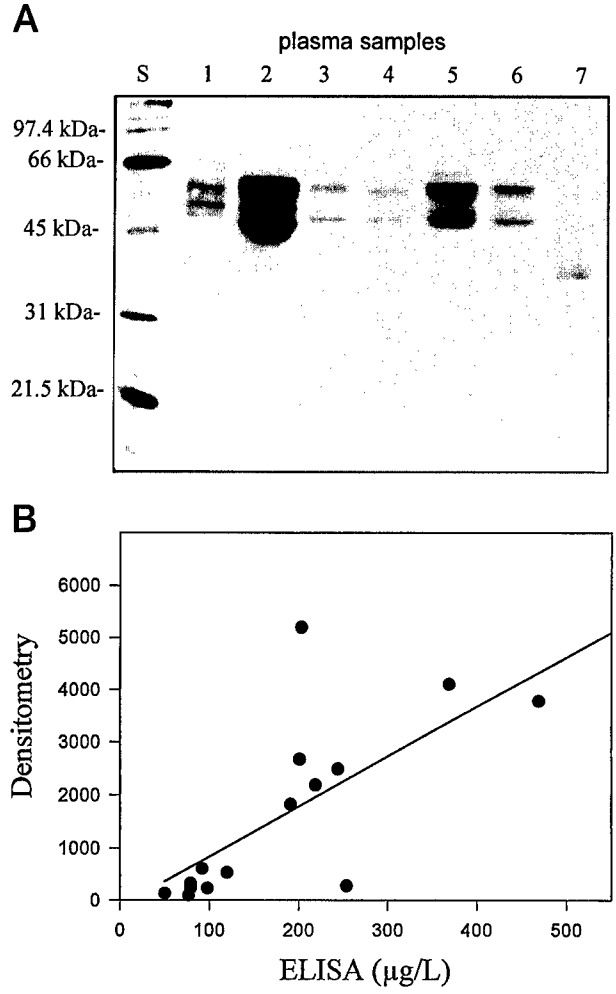

Fig. 4 Western blot analysis of plasma OPN and comparison between ELISA and densitometry for quantification of plasma OPN levels. Plasma OPN was immunoadsorbed to mAb53-conjugated Sepharose, eluted, and fractionated in 12% SDS-PAGE as described in "Materials and Methods." *A*, representative plasma samples 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 variable 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.

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REFERENCES

1. Denhardt D. T., and Guo X. Osteopontin: a protein with diverse functions. FASEB J., *7:* 1475–1482, 1993.

2. Giachelli, C. M., Schwartz, S. M., and Liaw, L. Molecular and cellular biology of osteopontin: potential role in cardiovascular disease. Trends Cardiovasc. Med., *5:* 88–95, 1995.

3. Patarca, R., Saavedra, R. A., and Cantor, H. Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. Crit. Rev. Immunol., *13:* 225–246, 1993.

4. Denhardt, D. T., Butler, W. T., Chambers, A. F., and Senger, D. R. (eds.). Osteopontin. Role in Cell Signalling and Adhesion. Ann. NY Acad. Sci., *760:* 1–388, 1995.

5. Senger, D. R., Perruzzi, C. A., and Papadopoulos, A. Elevated expression of secreted phosphoprotein I (osteopontin, 2ar) as a consequence of neoplastic transformation. Anticancer Res., *9:* 1291–1299, 1989.

6. Brown, L. F., Papadopoulos-Sergiou, A., Berse, B., Manseau, E. J., Tognazzi, E., Perruzzi, C. A., Dvorak, H. F., and Senger, D. R. Osteopontin expression and distribution in human carcinomas. Am. J. Pathol., *145:* 610–623, 1994.

7. Bellahcene, A., and Castronovo, V. Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. Am. J. Pathol., *146:* 95–100, 1995.

8. Hirota, S., Ito, A., Nagoshi, J., Takeda, M., Kurata, A., Takatsuka, Y., Kohri, K., Nomura, S., and Kitamura, Y. Expression of bone matrix protein messenger ribonucleic acids in human breast cancers. Possible involvement of osteopontin in development of calcifying foci. Lab Invest., *72:* 64–69, 1995.

9. Senger, D. R., Perruzzi, C. A., Gracey, C. R., Papadopoulos, A., and Tenen, D. G. Secreted phosphoproteins associated with neoplastic transformation: close homology with plasma proteins cleaved during blood coagulation. Cancer Res., *48:* 5770–5774, 1988.

10. Shiraga, H., Min, W., VanDusen, W. J., Clayman, M. D., Miner, D., Terrell, C. H., Sherbotie, J. R., Foreman, J. W., Przysiecki, C., Nielson, E. G., and Hoyer, J. R. Inhibition of calcium oxalate crystal growth *in vitro* by uropontin: another member of the aspartic acid-rich protein superfamily. Proc. Natl. Acad. Sci. USA, *89:* 426–430, 1992.

11. Worcester, E. M., Blumenthal, S. S., Beshensky, A. M., and Lewand, D. L. The calcium oxalate crystal growth inhibitor protein produced by mouse kidney cortical cells in culture is osteopontin. J. Bone Miner. Res., *7:* 1029–1036, 1992.

12. Bautista, D. S., Denstedt, J., Chambers, A. F., and Harris, J. F. Low molecular weight variants of osteopontin generated by serine proteinases in urine of patients with kidney stones. J. Cell. Biochem., *61:* 402–409, 1996.

13. Lopez, C. A., Olson, E. S., Adams, J. C., Mou, K., Denhardt, D. T., and Davis, R. L. Osteopontin expression detected in adult cochleae and inner ear fluids. Hear Res., *85:* 210–222, 1995.

14. Bautista, D. S., Xuan, J. W., Hota, C., Chambers, A. F., and Harris, J. F. Inhibition of Arg-Gly-Asp (RGD)-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin. J. Biol. Chem., *269:* 23280–23285, 1994.

15. Bautista, D. S., Saad, Z., Chambers, A. F., Tonkin, K. S., O'Malley, F. P., Singhal, H., Tokmakejian, S., Bramwell, V., and Harris, J. F. Quantification of osteopontin in human plasma with an ELISA: basal levels in pre- and postmenopausal women. Clin. Biochem., *29:* 231–239, 1996.

16. Xuan, J. W., Hota, C., and Chambers, A. F. Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. J. Cell. Biochem., *54:* 247–255, 1994.

17. Rustin, G. J. S., Crawford, M., Lambert, J., Ledermann, J., Coleman, R., and Meely, K. Trial of oral hexalen for relapsed ovarian carcinoma: comparison of CA125 and BORTC response definitions. Proc. Am. Soc. Clin. Oncol., *14:* 269, 1995.

18. Saksela, E. Prognostic markers in epithelial ovarian cancer. Int. J. Gynecol. Pathol., *12:* 156–161, 1993.

19. Sevelda, P., Schemper, M., and Spona, J. CA 125 as an independent prognostic factor for survival in patients with epithelial cancer. Am. J. Obstet. Gynecol., *161:* 1213–1216, 1989.

20. Bartlett, N. L., Freiha, F. S., and Torti, F. M. Serum markers in germ cell neoplasms. Hematol.-Oncol. Clin. N. Am., *5:* 1245–1260, 1991.

21. Stamey, T. A., Yang, N., Hay, A. R., McNeal, J. E., Freiha, F. S., and Redwine, E. Prostate specific antigen as a serum marker for adenocarcinoma of the prostate. N. Engl. J. Med., *317:* 909–916, 1987.

22. Minton, J. P., Hoehn, J. L., Gerber, D. M., Horsley, J. S., Connolly, D. P., Salwan, F., Fletcher, W. S., Cruz, A. B. Jr., Gatchell, F. G., Oviedo, M., Meyer, K. K., Leffall, L. D., Jr., Berk, R. S., Stewart, P. A., and Kurucz, S. E. Results of a 400-patient carcinoembryonic antigen second-look colorectal cancer study. Cancer (Phila.), *55:* 1284–1290, 1985.

23. Nicolini, A., Colombini, C., Luciani, L., Carpi, A., and Giuliani, L. Evaluation of serum CA15–3 determination with CEA and TPA in the post-operative follow-up of breast cancer patients. Br. J. Cancer, *64:* 154–158, 1991.

24. Pirolo, F., Pacini, P., Borsotti, M., la Morgia, R., Mungai, R., Cappellini, M., Cardona, G., Cataliotti, L., Marzano, S., Neri, B., Distante, V., and Berti, P. The role of CA 15–3 and MCA monoclonal antibody assays in the detection of primary and recurrent breast cancer. Anticancer Res., *11:* 729–731, 1991.

25. Gion, M., Plebani, M., Mione, R., Penzo, C., Meo, S., and Burlina, A. Serum CA549 in primary breast cancer: comparison with CA15.3 and MCA. Br. J. Cancer, *69:* 721–725, 1994.

26. de Wit, R., Hoek, F. J., Bakker, P. J., and Veenhof, C. H. A comparison of CA-549 with CA 15–3 and MCA in patients (PTS) with metastatic breast cancer (meeting abstract). Proc. Annu. Meet Am. Soc. Clin. Oncol., *10:* A13, 1991.

27. Schlageter, M. H., Toubert, M. E., Guil, M., Espie, M., and Najean, Y. CA549: a new tumor marker in breast carcinoma. J. Tumor Marker Oncol., *6:* 43, 1991.

28. Correale, M., Abbate, I., Dragone, C. D., Tedone, T., Gargano, G., Catino, A., Paradiso, A., Addabbo, L., Musci, M. D., and De Lena, M. Serum and cytosolic levels of CA549 in breast cancer patients. Clin. Exp. Obstet. Gynecol., *20:* 264–267, 1993.

29. Chan, D. W., Beveridge, R. A., Bhargava, A., Wilcox, P. M., Kennedy, M. J., and Schwartz, M. K. Breast cancer marker Ca549: a multicenter study. Am. J. Clin. Pathol., *101:* 465–470, 1994.

30. Dominguez, G. L., Martin, C. L. M., Crespo, A., and Avila, C. MCA serum determination in breast carcinoma patients for the diagnosis of bone metastases. Int. J. Biol. Markers, *8:* 203–207, 1993.

31. Tondini, C., Hayes, D. F., Gelman, R., Henderson, I. C., and Kufe, D. W. Comparison of CA15–3 and carcinoembryonic antigen in monitoring the clinical course of patients with metastatic breast cancer. Cancer Res., *48:* 4107–4112, 1988.

32. Yamashita, J., Ogawa, M., Yamashita, S., Nomura, K., Kuramoto, M., Saishoji, T., and Shin, S. Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. Cancer Res., *54:* 1630–1633, 1995.

33. Behrend, E. I., Craig, A. M., Wilson, S. M., Denhardt, D. T., and Chambers, A. F. Reduced malignancy of ras-transformed NIH 3T3 cells expressing antisense osteopontin RNA. Cancer Res., *54:* 832–837, 1994.

34. Gardner, H. A. R., Berse, B., and Senger, D. R. Specific reduction in osteopontin synthesis by antisense RNA inhibits the tumorigenicity of transformed Rat1 fibroblasts. Oncogene, *9:* 2321–2326, 1994.

35. Su, L., Mukherjee, A. B., and Mukherjee, B. B. Expression of antisense osteopontin RNA inhibits tumor promoter-induced neoplastic transformation of JB6 epidermal cells. Oncogene, *10:* 2163–2169, 1995.

36. Denhardt, D. T., and Chambers, A. F. Overcoming obstacles to metastasis. Defenses against host defenses: osteopontin (OPN) as a shield against attack by cytotoxic host cells. J. Cell. Biochem., *56:* 48–51, 1994.

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

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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 the level of immunodetectable OPN may be associated with tumor aggressiveness. We examined OPN levels in tumors from 154 patients with LNN breast cancer who were followed for a median of 7 years (range 1.7–16.3 years). 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%. 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 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. Our results thus indicate that OPN protein can be produced by breast cancer cells *in vivo* and suggest 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 cutpoint 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 prognosis. *Int. J. Cancer (Pred. Oncol.)* 79:502–508, 1998.

Osteopontin (OPN) is a secreted, adhesive glycophosphoprotein that has been implicated in both normal (*e.g.*, bone development, immune system regulation) and pathologic (*e.g.*, 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, 1996, 1997). We have 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, 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 date, 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 [*e.g.*, 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 toward OPN in culture (Bautista *et al.*, 1994; Xuan *et al.*, 1994, 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 (LNN) 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.

MATERIAL AND METHODS

Patients

One hundred fifty-four patients with LNN breast cancer were identified from the records at the London Regional Cancer Centre (London, Canada). Available data included age, menopausal status, tumor size, biochemical estrogen receptor (ER) and progesterone receptor (PR) 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 IHC

Formalin-fixed, paraffin-embedded tumor samples were assessed for OPN expression by an immunoperoxidase technique

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(Chambers *et al.,* 1996; Casson *et al.,* 1997). Representative 4 μm sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 7 min. An antigen retrieval method was used on all slides prior to immunostaining. This involved heating tissue sections in citrate buffer (10 mM, pH 6.0) in a microwave oven (600 W) for 7 min. Immunostaining was performed using a streptavidin-biotin complex method (Zymed histostain kit, Dimension Laboratories, Toronto, Canada). Non-specific staining was blocked by incubating slides with 5% normal goat serum. The primary antibody used was the monoclonal antibody (MAb) 53, prepared against the recombinant GST-human OPN fusion protein (Bautista *et al.,* 1994). This MAb has been previously shown by Western analysis, enzyme-linked immunosorbent assay (ELISA) and IHC to efficiently and specifically detect human OPN (Bautista *et al.,* 1994, 1996; Chambers *et al.,* 1996; Casson *et al.,* 1997). The slides were incubated with primary antibody for 1 hr at room temperature. Slides were then rinsed with phosphate-buffered saline (PBS) and a biotinylated secondary antibody was applied for 15 min, followed by a PBS rinse and treatment with the streptavidin-enzyme conjugate for 10 min. The chromogen used was amino-ethyl carbazol (reddish-brown signal), and slides were counter-stained 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 OPN (1,493 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, Madison, WI) in the presence of digoxigenin-UTP (Boehringer Mannheim, Montreal, Canada). 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)*

ISH was performed essentially as described previously (Tuck *et al.,* 1996). Briefly, 4 μm paraffin sections were cut under RNase-free conditions onto Superfrost Plus (Fisher, Ottawa, Canada) slides, dewaxed in xylene and rehydrated. Permeabilization was performed by treating at room temperature sequentially with 0.2 M HCl, 0.2% Triton X-100 in PBS and 40 μg/ml proteinase K for 10 min each. Slides were then washed in 0.1× PBS, refixed for 30 min at room temperature in 4% paraformaldehyde, washed again in 0.1× PBS and acetylated with 0.25% acetic anhydride in 0.1 M 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 0.2× SSC at 55°C for 30 min, rinsed in RNase buffer [0.5 M NaCl, 10 mM 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) (1:1,000 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 (pH 9.5), 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, Burlingame, CA) was used. Color was allowed to develop for 4–6 hr 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 1) hybridizing with sense riboprobes and 2) omitting riboprobe entirely. Other controls included antisense and sense probe testing of cell blocks prepared on 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 $p \leq 0.25$ were considered eligible for inclusion in the multivariate model (Hosmer and Lemeshow, 1989). A multistep backward selection method was then used and variables were removed from the model if significance fell above $p = 0.05$. 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

TABLE I – PROFILE OF A GROUP OF 154 LNN BREAST CANCER PATIENTS

Parameter	Number (range)	%
Age (years)	Mean 52.1 (26–83)	—
Menopausal status		
Premenopausal	82	53
Postmenopausal	72	47
Tumor size (cm)	Mean 2.5 (0.1–8.0)	—
Hormone receptors		
ER positive	100/143	70
PR positive	106/143	74
Histology		
No special type	147	95
Tubular	2	1.3
Tubulolobular	1	0.6
Lobular ("classic")	2	1.3
Lobular, pleomorphic variant	1	0.6
Mucinous	1	0.6
Grade		
I	30	21
II	55	39
III	58	40
p53 positive	22	14
OPN		
Macrophage staining	108	70
Tumor cell staining	40	26

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 ER positive and 74% were PR positive. The pathology of all tumors was reviewed by us (FPO, ABT). One hundred forty-seven of the 154 invasive tumors were of "no special type" (infiltrating ductal carcinoma, not otherwise specified). The special type carcinomas included 2 tubular carcinomas, 1 tubulolobular carcinoma, 2 infiltrating lobular carcinomas of the "classic type," 1 pleomorphic variant of infiltrating lobular carcinoma and 1 mucinous carcinoma. Of those tumors for which an SBR grade could be assigned (143/154), 30 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).

IHC 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

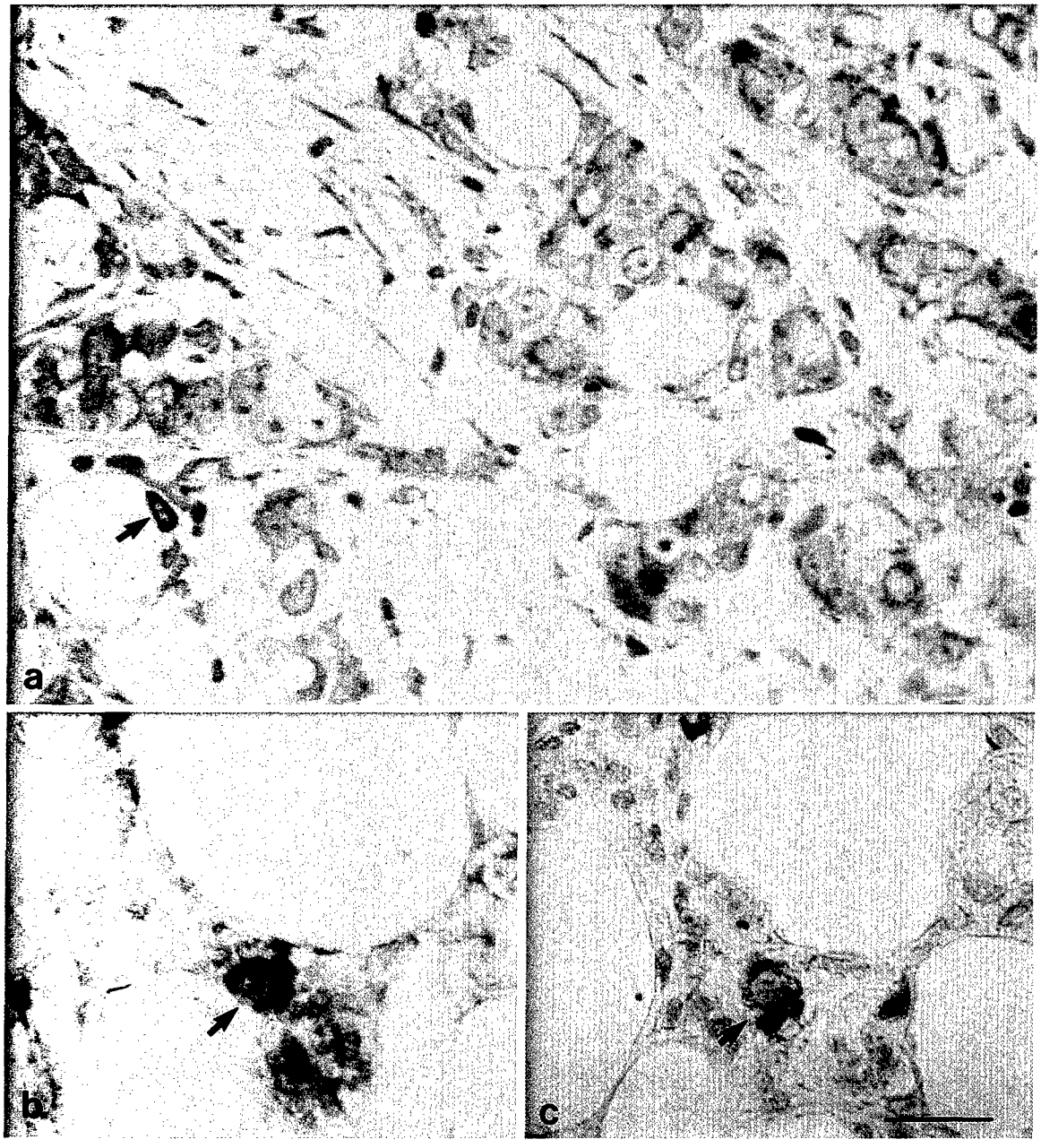

FIGURE 1 – *(a)* IHC positivity of tumor cells for OPN protein (score 6) in an infiltrating mammary carcinoma of no special type, combined histologic grade II/III. A tumor infiltrating macrophage showing staining for OPN is indicated (arrow). *(b,c)* A tumor different from that shown in *(a)*, with more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

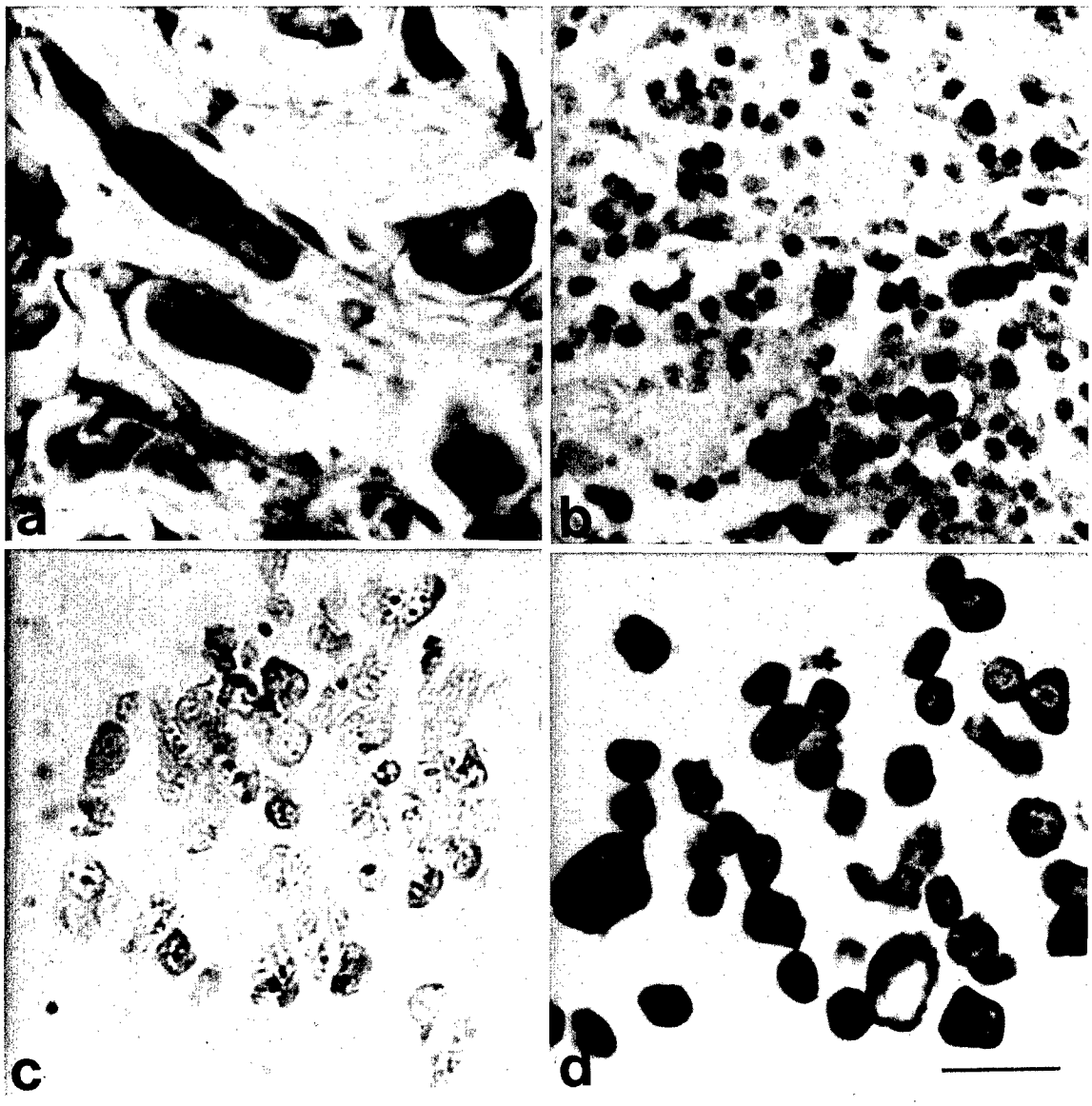

FIGURE 2 – ISH for OPN mRNA. *(a)* Strong cytoplasmic positivity for OPN is seen regionally in carcinoma cells of OPN immunopositive tumors. *(b)* Tumor-associated mononuclear inflammatory cells showing strong cytoplasmic staining for OPN mRNA. *(c)* Negative control, showing lack of cytoplasmic staining 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.

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	0.8017	0.6805
PR status	0.5127	0.4538
Grade	0.510	0.4704
p53 (score >5)	0.0074	0.003
Tumor cell OPN (score >4)	0.0025	0.0294

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 IHC scores [determined as in Allred *et al.* (1993) (see Material and 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 1*a*.

ISH was performed 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 level 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

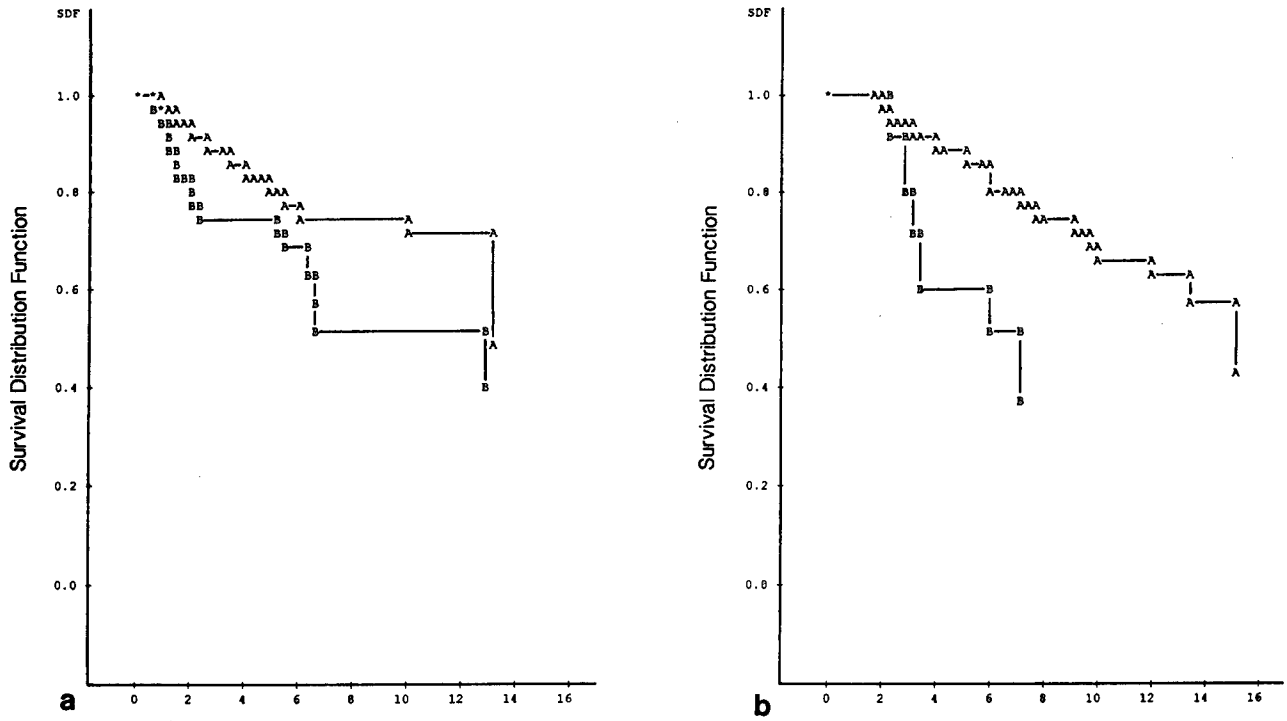

FIGURE 3 – DFS *(a)* and OS *(b)* curves for LNN breast cancer patients whose tumors were OPN positive [B] (tumor cell IHC score >4) *vs.* OPN negative [A] (tumor cell IHC score ≤4), as determined by IHC (DFS $p = 0.0025$; OS $p = 0.0294$).

macrophages and lymphocytes (Fig. 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 that expressed both OPN mRNA and protein (Fig. 1*b,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 (Fig. 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 both 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 survival (DFS) and overall survival (OS)] in this group of LNN breast cancer patients. Cutpoint analysis was performed for tumor cell OPN staining (as described in Material 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 1.7–16.3 years). Forty-five patients died of disease. Univariate survival analysis showed tumor cell OPN positivity (score >4) to be significantly associated with both decreased DFS ($p = 0.0025$) and OS ($p = 0.0294$) (Table II, Fig. 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).

Our 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 utility 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 IHC), tumor cell OPN positivity remained a significant predictor of decreased OS ($p = 0.0138$, RR = 2.971), but not DFS ($p = 0.3217$, RR = 1.634).

DISCUSSION

We have demonstrated that human breast carcinoma cells themselves (in addition to tumor infiltrating macrophages and lymphocytes) can synthesize OPN *in vivo* and that IHC 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 [*e.g.*, 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 (data not shown)]. 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 that show tumor cell positivity for OPN protein as well. However, only in our study is there ISH evidence for OPN mRNA expression also by the tumor cells. We believe this difference is most likely due to sampling and case selection. The tumor samples that we selected for screening for ISH were derived from a bank of 154 breast tumor samples. From these, 4 of the tumors chosen for ISH testing were those showing the strongest immunopositivity of the tumor cells themselves. Others (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 synthesize 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, 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 toward 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 to appreciate the nature of its role in malignancy, with a view toward the possible future identification of new therapeutic targets.

REFERENCES

ALLRED, D.C., CLARK, G.M., ELLEDGE, R., FUQUA, S.A.W., BROWN, R.W., CHAMNESS, G.C., OSBORNE, C.K. and MCGUIRE, W.L., Association of p53 protein expression with tumor cell proliferation rate and clinical outcome in node-negative breast cancer. *J. nat. Cancer Inst.,* **85,** 200–206 (1993).

BAND, V., ZAJCHOWSKI, D., SWISSHELM, K., TRASK, D., KULESA, V., COHEN, C., CONNOLLY, J. and SAGER, R., Tumor progression in four mammary epithelial cell lines derived from the same patient. *Cancer Res.,* **50,** 7351–7357 (1990).

BAUTISTA, D.S., SAAD, Z., CHAMBERS, A.F., TONKIN, K.S., O'MALLEY, F.P., SINGHAL, H., TOKMAKEJIAN, S., BRAMWELL, V. and HARRIS, J.F., Quantification of osteopontin in human plasma with an ELISA: basal levels in pre- and postmenopausal women. *Clin. Biochem.,* **29,** 231–239 (1996).

BAUTISTA, D.S., XUAN, J.W., HOTA, C., CHAMBERS, A.F. and HARRIS, J.F., Inhibition of RGD-mediated cell adhesion to osteopontin by a monoclonal antibody against osteopontin. *J. biol. Chem.,* **269,** 23280–23285 (1994).

BELLAHCÈNE, A. and CASTRONOVO, V., Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. *Amer. J. Pathol.,* **146,** 95–100 (1995).

BROWN, L.F., BERSE, B., VAN DE WATER, L., PAPADOPOULOS-SERGIOU, A., PERRUZZI, C.A., MANSEAU, E.J., DVORAK, H.F. and SENGER, D.R., Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. *Mol. Biol. Cell.,* **3,** 1169–1180 (1992).

BROWN, L.F., PAPADOPOULOS-SERGIOU, A., BERSE, B., MANSEAU, E.J., TOGNAZZI, K., PERRUZZI, C.A., DVORAK, H.F. and SENGER, D.R., Osteopontin expression and distribution in human carcinomas. *Amer. J. Pathol.,* **145,** 610–623 (1994).

CASSON, A.G., WILSON, S.M., MCCART, J.A., O'MALLEY, F.P., OZCELIK, H., TSAO, M.-S. and CHAMBERS, A.F., *ras* mutation and expression of the *ras*-regulated genes osteopontin and cathepsin L in human esophageal cancer. *Int. J. Cancer,* **72,** 739–745 (1997).

CHAMBERS, A.F., WILSON, S.M., KERKVLIET, N., O'MALLEY, F.P., HARRIS, J.F. and CASSON, A.G., Osteopontin expression in lung cancer. *Lung Cancer,* **15,** 311–323 (1996).

DENHARDT, D.T., BUTLER, W.T., CHAMBERS, A.F. and SENGER, D.R. (eds.), *Osteopontin: role in cell signalling and adhesion. Ann. N.Y. Acad. Sci.,* **760,** 388 pp (1995).

HIROTA, S., ITO, A., NAGOSHI, J., TAKEDA, M., KURATA, A., TAKATSUKA, Y., KOHRI, K., NOMURA, S. and KITAMURA, Y., Expression of bone matrix protein messenger ribonucleic acids in human breast cancers. Possible involvement of osteopontin in development of calcifying foci. *Lab. Invest.,* **72,** 64–69 (1995).

HOSMER, D.W. and LEMESHOW, S., *Applied logistic regression.* J. Wiley, New York (1989).

MACK, L., KERKVLIET, N., DOIG, G. and O'MALLEY, F.P., Relationship of a new histological categorization of ductal carcinoma *in situ* of the breast with size and the immunohistochemical expression of c-erb B2, bcl-2, and ki-67. *Hum. Pathol.,* **28,** 974–979 (1997).

MORRIS, V.L., TUCK, A.B., WILSON, S.M., PERCY, D. and CHAMBERS, A.F., Tumor progression and metastasis in murine D2 hyperplastic alveolar nodule mammary tumor cell lines. *Clin. exp. Metastasis,* **11,** 103–112 (1993).

O'MALLEY, F.P., SAAD, Z., KERKVLIET, N., DOIG, G., STITT, L., AINSWORTH, P., HUNDAL, H., CHAMBERS, A.F., TURNBULL, D.I. and BRAMWELL, V., The predictive power of semiquantitative immunohistochemical assessment of p53 and c-erb B-2 in lymph node-negative breast cancer. *Hum. Pathol.,* **27,** 955–963 (1996).

SAS INSTITUTE, INC., *SAS user's guide. Statistics, version 6* (4th ed.), Cary, NC (1990).

SENGER, D.R., ASCH, B.B., SMITH, B.D., PERRUZZI, C.A. and DVORAK, H.F., A secreted phosphoprotein marker for neoplastic transformation of both epithelial and fibroblastic cells. *Nature (Lond.),* **302,** 714–715 (1983).

SENGER, D.R. and PERRUZZI, C.A., Cell migration promoted by a potent GRGDS-containing thrombin-cleavage fragment of osteopontin. *Biochim. biophys. Acta,* **1314,** 13–24 (1996).

SENGER, D.R., PERRUZZI, C.A., GRACEY, C.F., PAPADOPOULOS, A. and TENEN, D.G., Secreted phosphoproteins associated with neoplastic transformation: close homology with plasma proteins cleaved during blood coagulation. *Cancer Res.,* **48,** 5770–5774 (1988).

SINGHAL, H., BAUTISTA, D., TONKIN, K., O'MALLEY, F., CHAMBERS, A.F. and HARRIS, J.F., Plasma levels of osteopontin in primary and metastatic breast cancer. *Canad. J. Surg.,* **38**(Suppl.), A14, Abstract 358 (1995).

SINGHAL, H., BAUTISTA, D.S., TONKIN, K.S., O'MALLEY, F.P., TUCK, A.B., CHAMBERS, A.F. and HARRIS, J.F., Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. *Clin. Cancer Res.,* **3,** 605–611 (1997).

SINGHAL, H., TONKIN, K.S., VANDENBERG, T.A., STITT, L., BAUTISTA, D.S., CHAMBERS, A.F. and HARRIS, J.F., Prospective evaluation of the prognostic

significance of elevated plasma osteopontin in metastatic breast cancer. *Proc. Amer. Soc. clin. Oncol.,* **15,** 504, Abstract 1614 (1996).

TUCK, A.B., O'MALLEY, F.P., SINGHAL, H., TONKIN, K.S., HARRIS, J.F. and CHAMBERS, A.F., Osteopontin and p53 expression are associated with tumor progression in a case of bilateral, invasive mammary carcinomas. *Arch. Pathol. Lab. Med.,* **121,** 578–584 (1997).

TUCK, A.B., PARK, M., STERNS, E.E., BOAG, A. and ELLIOTT, B.E., Coexpression of hepatocyte growth factor and receptor (met) in human breast carcinoma. *Amer. J. Pathol.,* **148,** 225–232 (1996).

WALTER, S.D., FEINSTEIN, R.R. and WELLS, C.K., Coding ordinal independent variables in multiple regression analysis. *Amer. J. Epidemiol.,* **125,** 319–323 (1987).

XUAN, J.W., HOTA, C. and CHAMBERS, A.F., Recombinant GST-human osteopontin fusion protein is functional in RGD-dependent cell adhesion. *J. Cell. Biochem.,* **54,** 247–255 (1994).

XUAN, J.W., HOTA, C., SHIGEYAMA, Y., D'ERRICO, J.A., SOMERMAN, M.J. and CHAMBERS, A.F., Site-directed mutagenesis of the arginine-glycine-aspartic acid sequence in osteopontin destroys cell adhesion and migration functions. *J. Cell. Biochem.,* **57,** 680–690 (1995).

YOUNG, M.F., KERR, J.M., TERMINE, J.D., WEWER, U.M., WANG, M.G., MCBRIDE, O.W. and FISHER, L.W., cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). *Genomics,* **7,** 491–502 (1990).

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Osteopontin induces increased invasiveness and plasminogen activator expression of human mammary epithelial cells

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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, non-tumorigenic; 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 up-regulation 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 invasiness 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. Treatment of the parental 21PT and 21NT cells with exogenous hrOPN resulted in increased uPA mRNA expression and increased urokinase activity of the conditioned media. Both increased cell ~~motility~~ and induction of uPA expression are thus potential mechanisms of increased invasiness of breast epithelial cells in response to OPN.

Keywords: osteopontin (OPN); invasion; plasminogen activator; urokinase; mammary epithelial cells; breast cancer

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.*, 1998). 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.*, 1998).

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.*, 1998), although the relative biologic significance of these different potential sources of OPN is not yet understood. Previous work (Xuan *et al.*, 1994, 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 behaviour important during development, tissue remodeling, 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.*, 1998). 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

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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 osteoclastoma cells (Teti *et al.*, 1998), 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). Of the 21T series, 21PT cells are immortal but non-tumorigenic, whereas 21NT are immortal and tumorigenic, but non-metastatic (Band *et al.*, 1990). MDA-MB-435 cells are both highly tumorigenic and metastatic in nude mice (Price *et al.*, 1990). Evidence is shown for the ability of all 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 transfection 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 up-regulation of urokinase-type plasminogen activator (uPA). Direct evidence for up-regulation of uPA (mRNA and enzyme activity) by OPN was obtained by treatment of the parental 21PT and 21NT cells with exogenous OPN.

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 1a) 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 1b) 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). This band was confirmed as OPN by specific detection with anti-OPN polyclonal (rabbit anti-human) antibody as well (data not shown). 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 1c) showed that a 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 21T and 21NT to 100 μg/ml hrOPN is shown in Figure 2a. Both 21PT and 21NT cells showed low basal level of invasiveness through Matrigel (21NT slightly greater than 21PT).

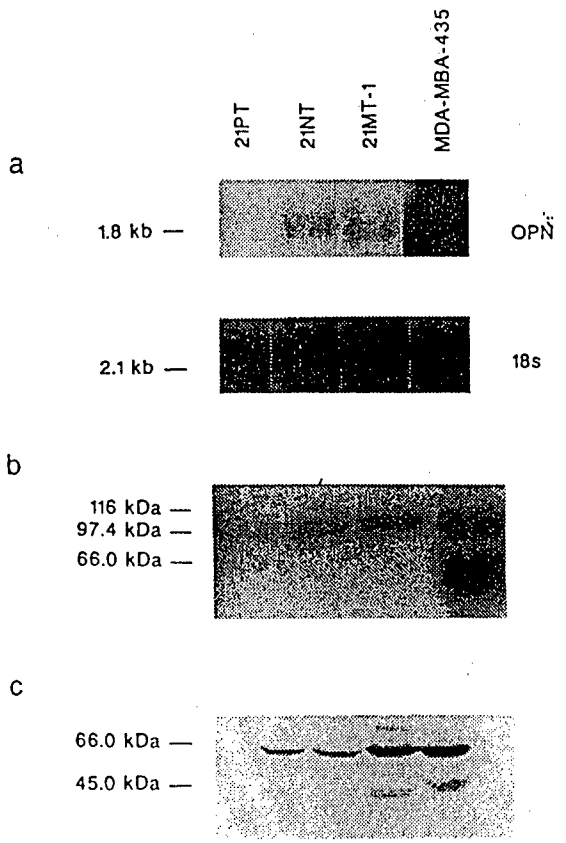

Figure 1 (a) Northern analysis of OPN mRNA expression by 21T series human mammary epithelial cell lines 21PT (non-tumorigenic) and 21NT (tumorigenic, non-metastatic) vs MDA-MB-435 (highly metastatic human breast carcinoma) cells. Total RNA (10 μg/lane) was separated, blotted, and probed as described in Materials and methods. Equivalent RNA loading and integrity were verified by assessment of 18S rRNA. (b) and (c) Western blot analysis of OPN protein expression by 21T series vs MDA-MB-435 cells. (b) Conditioned media 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. (c) 20 μg of total cell lysate was loaded per lane, followed by fractionation in 8% SDS–PAGE. Immunoblotting was performed as described in Materials and methods

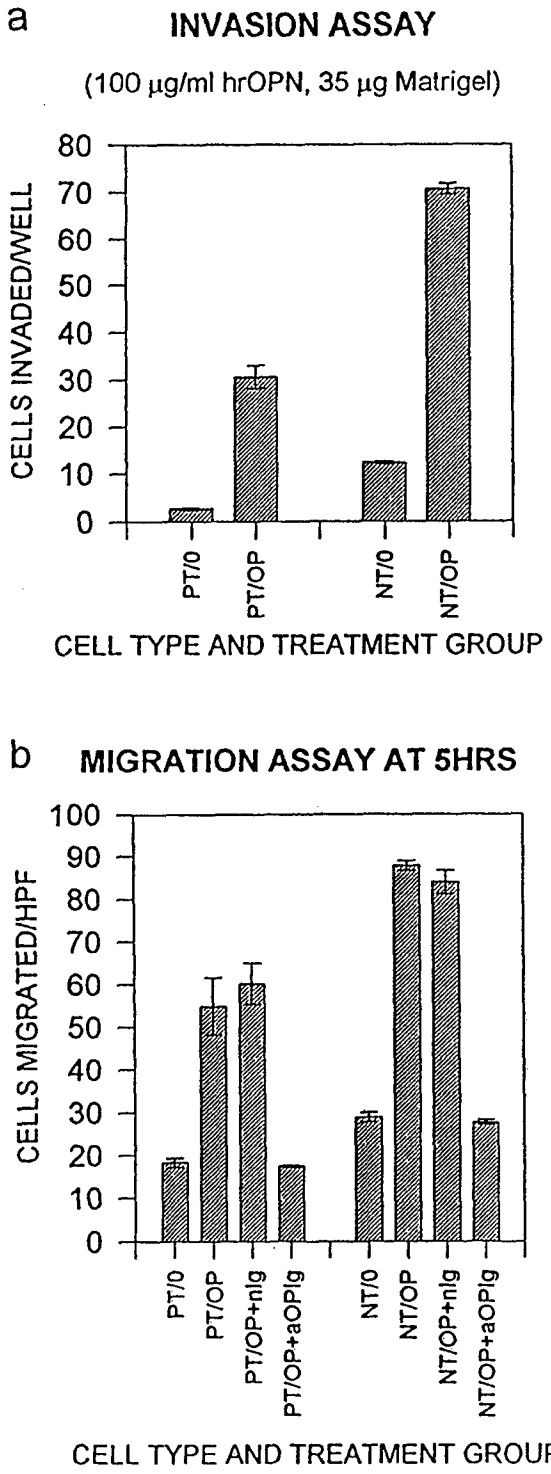

Figure 2 (a) *In vitro* chemoinvasiveness of 21PT (PT) and 21NT (NT) cells in response to 100 μg/ml hrOPN (OP) in the lower chamber of 8 μm pore transwells, vs 0.1% BSA only (0). The filter of each transwell was coated with 35 μg Matrigel. Cells were seeded at 5×10^4 cells/well in the upper chamber and incubated for 72 h, after which each filter was fixed and stained, the upper surface wiped clean, and cells on the lower surface counted microscopically. Bar graphs represent the mean of counts (cells invaded/well) for three separate wells; error bars are SEM. 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). (b) Cell migration of 21PT (PT) and 21NT (NT) cells in transwell assay. Migration assays were performed as described in Materials and 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 5×10^4 cells/well in the upper chamber and incubated for 5 h, 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 21 (NT) cells ($P < 0.05$ for both, one-way ANOVA)

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 2b 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 Materials and 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 3a, along with representative vector-only tranfected 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 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.

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 4. Using a 35 μg/well membrane of Matrigel and a 72 h 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 fact that the pooled OPN-transfectant populations and control-vector transfectants examined represent an average of at least 20–25 transfectant colonies/clones in each case, makes it highly unlikely that the differences seen are due to random clonal variations. With regards to the clones examined, it is of interest that the cellular invasiveness of NT/OPaiiC4 cells was indeed 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

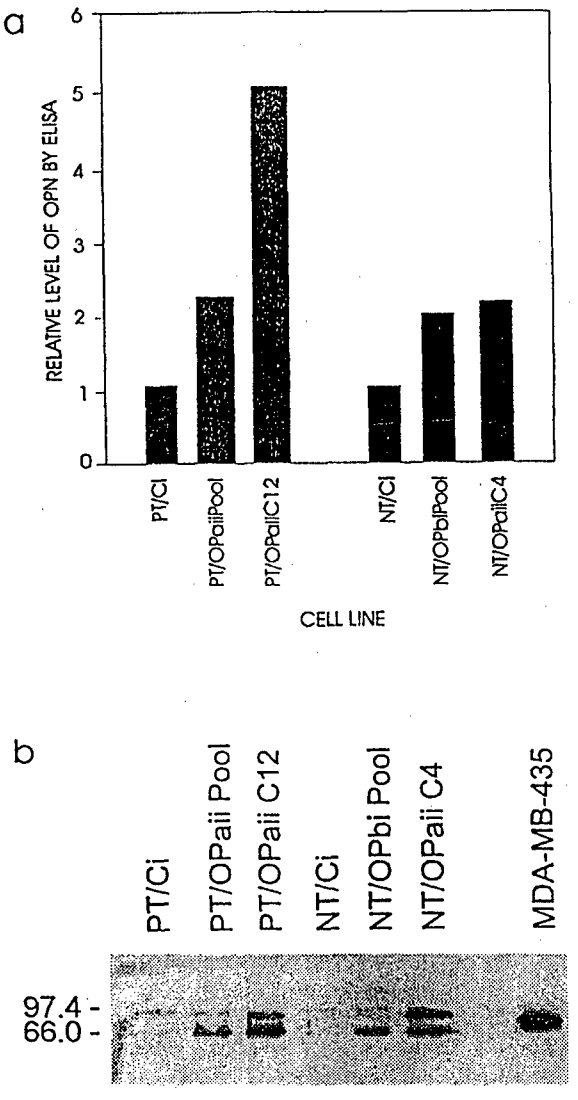

Figure 3 Relative level of OPN protein expression of 21PT and 21NT OPN-transfected cells vs control vector-transfected cells, as determined by ELISA (a) and Western analysis (b) of conditioned media. ELISA assay and Western blotting were performed as outlined in methods and in Figure 2. Cell lines are as follows: PT/Ci: pooled population (70 colonies) of 21PT cells transfected with the control vector (pcDNA3) only; PT/OPaiiPool: pooled population (25 colonies) 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 (30 colonies) of 21NT cells transfected with the control vector (pcDNA3) only; NT/OPbiPool: pooled population (20 colonies) 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

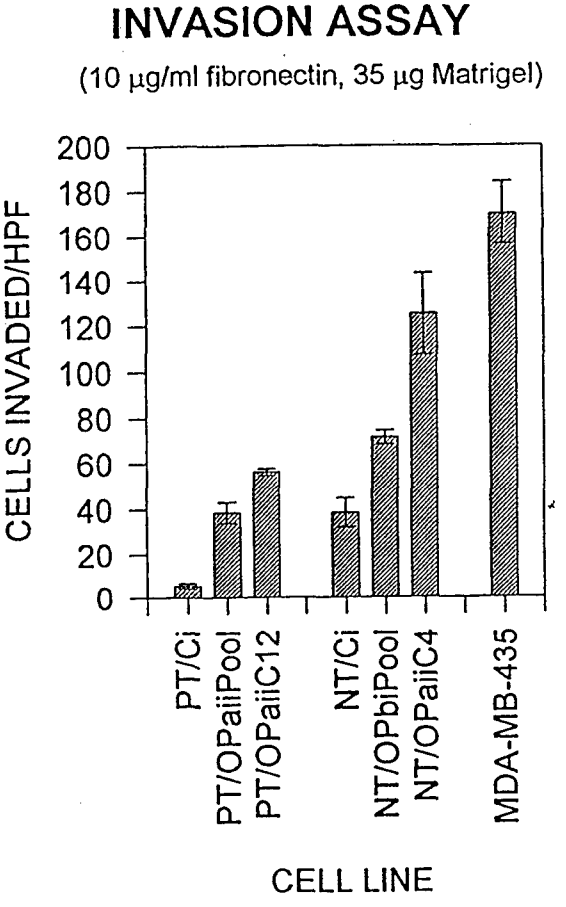

Figure 4 *In vitro* chemoinvasiveness of transfectant cell lines in the transwell assay. Vector-only transfected controls of 21PT (PT/Ci) and 21NT (NT/Ci) cells are compared with the OPN-transfected pool and clone of 21PT (PT/OPaiiPool; PT/OPaiiC12) and 21NT (NT/OPbiPool; NT/OPaiiC4) cells expressing 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 Materials and methods. Cells were seeded at 5×10^4 cells/well in the upper chamber and incubated for 72 h, 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)

21PT and 21NT cells vs MDA-MB-435 cells (Figure 5). 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.

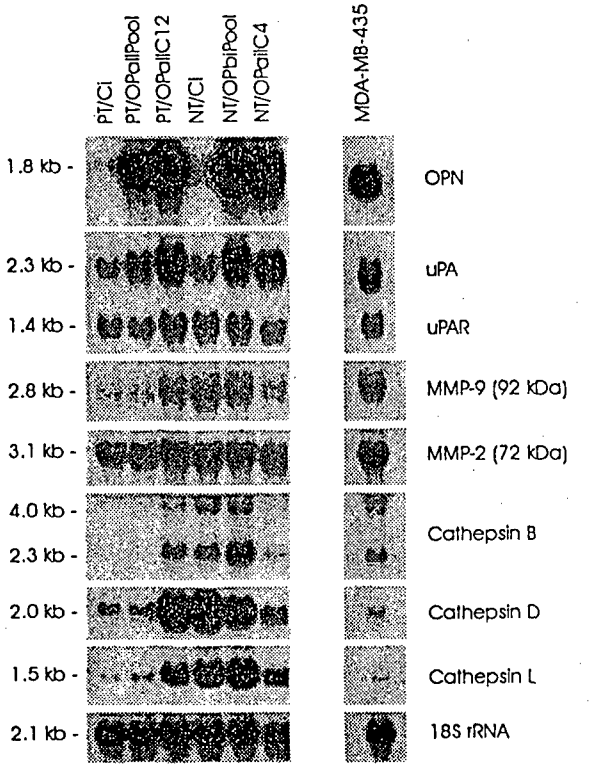

Figure 5 Northern analysis of transfectant cell populations described in Figures 3 and 4 (vs MDA-MB-435 cells) for expression of mRNA of a number of secreted proteases (and urokinase-type plasminogen activator receptor) vs OPN. Probes were prepared as outlined in Methods, and included: human osteopontin (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 4)

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 4) (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.

Induction of uPA mRNA and enzyme activity in 21PT and 21NT cells treated with exogenous hrOPN

Incubation of the parental 21PT and 21NT cell lines with exogenous hrOPN (100 μg/ml), resulted in an increase in both uPA mRNA (as determined by Northern analysis, Figure 6a) and in uPA enzyme activity in the conditioned media (as determined by

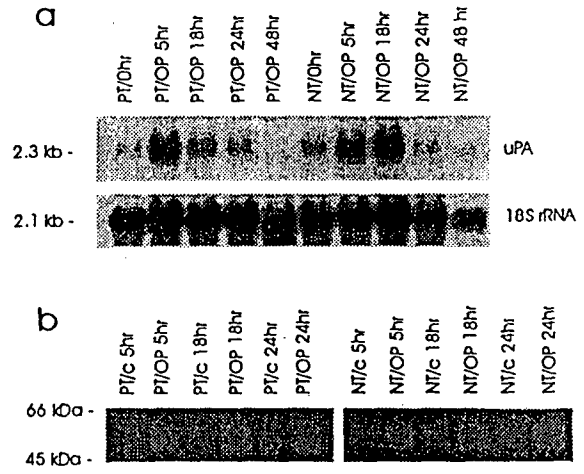

Figure 6 Induction of urokinase-type plasminogen activator (uPA) expression by 100 μg/ml hrOPN. (a) Induction of uPA mRNA expression in 21PT (PT) and 21NT (NT) cells treated with hrOPN (OP) for the times specified. Cells were harvested following induction, and RNA extraction and Northern blot analysis were performed as described in Materials and methods (10 μg/lane). Equivalent RNA loading and integrity were verified by assessment of 18S rRNA. (b) Induction of uPA enzyme activity in conditioned media from 21PT (PT) and 21NT (NT) cells incubated with (OP) or without (control (c)) hrOPN for the times specified. Zymogram analysis of conditioned media was performed as described in Materials and methods. Plasminogen-dependent proteolysis is seen as a white band on a dark background. An increase in enzyme activity was seen in conditioned media from both cell lines as early as 5 h after adding hrOPN. The band was seen in the expected MW range for uPA (approximately 55 kDa)

Zymogram analysis, Figure 6b). An increase in both uPA mRNA and enzyme activity for both cell lines was detected as early as 5 h post-induction. Incubation of the cells with hrOPN for longer periods showed that peak expression of uPA mRNA was seen for 21PT cells at 5 hr whereas that for 21NT cells was at 18 h. For both cell lines, most of the increase in uPA enzyme activity of the conditioned media was seen over the first 5 h, with only slight further accumulation with more prolonged incubations with hrOPN. The most dramatic increase in uPA enzyme activity was seen for 21PT cells, which showed lower basal (control) levels than 21NT 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*., 1998), with levels in some instances associated with prognosis (Singhal *et al*., 1997; Tuck *et al*., 1998), 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, 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. More direct evidence for up-regulation of uPA mRNA and enzyme activity by OPN has also been demonstrated here, in the experiments involving incubation of the 21PT and 21NT parental cell lines with exogenous hrOPN. 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, 1997), as well as activating certain growth factors (eg. HGF, TGF-β, bFGF) (reviewed in Werb, 1997; 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 up-regulated 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.*, 1998; Andrésen *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, 2 mM L-glutamine (all from GIBCO-BRL/Life Technologies, Grand Island, NY, USA), insulin (1 μg/ml), epidermal growth factor [EGF] (12.5 ng/ml), hydrocortisone (2.8 μM), 10 mM HEPES, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 50 μg/ml gentamycin (all from Sigma Chemical, St. Louis, MO, USA) (αHE medium). MDA-MB-435 cells were obtained as a kind gift of Dr Janet Price (MD Anderson Cancer Center, Houston, TX, USA), 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, Brinkman Instruments (Canada) Ltd., Mississauga, ON, Canada) and RNA extracted using TRIzol Reagent (Canadian Life Technologies Inc., Burlington, ON, Canada), 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, Canada). Blots were probed either with denatured, oligolabeled ^{32}P-dCTP cDNA probes (labeled using a kit provided by Pharmacia, Baie d'Urfe, PQ, Canada), or with 5'γ^{32}P-ATP end-labeled oligomers (labeled using a kit provided by Oncogene Science, Manhasset NY, USA), 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 WG Stetler-Stevenson), MMP-2 (72 kDa Type IV collagenase) (1117 bp insert from plasmid p3Ha) (Reponen *et al.*, 1992), cathepsin B (1.6 kb *Kpn*I insert from plasmid pLC343) (gift of Dr B Sloane) (Cao *et al.*, 1994), cathepsin D (20 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 DT 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, Canada, Cat#ON333) (Riccio *et al.*, 1985), urokinase-type plasminogen activator ~~response~~ (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 DT Denhardt).

Western blotting of cell lysates and conditioned media

Cell lysates were prepared from cell cultures (grown to 70–80% confluency on 100 mm dishes) by washing each culture

dish twice with cold phosphate buffered saline, followed by the addition of 500 μl cold lysis buffer (20 mM HEPES pH 7.2, 5 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 2 mM 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 16 000 *g* for 10 min to remove insoluble material. Each supernatant was collected and total protein concentration determined by Peterson's modification of the standard Lowry assay (Peterson, 1977). Twenty 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 cell/100 mm plate in regular growth medium and incubating overnight (18 h) 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 h 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, USA). 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, Canada). 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, Canada), and detected with biotinylated monoclonal antibody mAb53 (0.2 μg/ml) (raised against human recombinant OPN) (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, USA).

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, USA) 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 2 μg plasmid DNA for every 100 μl of OPTI-MEM I in Solution A, and 10 μl LIPOFECTIN reagent for every 100 μl of OPTI-MEM I in Solution B. Following a 48 h 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 level 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 recognize native human OPN. Maxisorp immunoplates (GIBCO–BRL/Life Technologies) were coated with mouse monoclonal anti-OPN 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). 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 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, USA). After washing, streptavidin conjugated alkaline phosphatase (1:2000, Jackson Immunological Laboratories Inc.) was added for 30 min 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 min. 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 (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, USA), coated with 35 μg Matrigel (Collaborative Research Inc., Bedford, MA, USA) 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 5×10^4 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 h 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 ×100 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, USA). 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 (5×10^4) were added to each upper well in αHE medium with 0.1% BSA and incubated at 37°C; the time of incubation (5 h) 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 100× 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 (Bautista *et al.*, 1994)) 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, USA) statistical software.

Induction of 21PT and 21NT cell uPA mRNA and enzyme activity by exogenous hrOPN

5×10^5 cells/60 mm culture dish were seeded and kept overnight at 37°C in medium with 10% FBS. The following morning, each plate was washed 3× with OPTI-MEM without serum. Plates were then incubated with 2 ml/plate of OPTI-MEM with or without 100 μg/ml hrOPN for the specified time period. Conditioned media was then collected, and the cells trypsinized and counted. RNA extraction and Northern analysis were performed as described above.

Aliquots of conditioned medium were adjusted for cell number and diluted 1/50 for Zymogram analysis. The samples were separated by 11% SDS-PAGE, the gel impregnated with 0.1% casein and 10 μg/ml plasminogen as previously described (Simon *et al.*, 1996). The gel was then incubated in 2.5% Triton-X 100 for 2 h, followed by incubation in 50 mM Tris (pH 8.3) and 0.1 M glycine for 18 h at 37°C. The gel was fixed in 15% acetic acid and stained with 0.25% Coomassie blue. Following destaining, plasminogen-dependent proteolysis was detected as a white band on a dark background.

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References

Aeschlimann D, Mosher D and Paulsson M. (1996). *Semin. Thromb. Hemost.*, 22, 437–443.

Andreasen PA, Kjøller L, Christensen L and Duffy MJ. (1997). *Int. J. Cancer*, 72, 1–22.

Augereau P, Garcia M, Mattei MG, Cavailles V, Depadova F, Derocq D, Capony F, Ferrera P and Rochefort H. (1988). *Mol. Endocrinol.*, 2, 186–192.

Band V, Zajchowski D, Swisshelm K, Trask D, Kulesa V, Cohen C, Connolly J and Sager R. (1990). *Cancer Res.*, 50, 7351–7357.

Baricos WH, Cortez SL, el-Dahr SS and Schnaper HW. (1995). *Kidney Int.*, 47, 1039–1047.

Bautista DS, Xuan JW, Hota C, Chambers AF and Harris JF. (1994). *J. Biol. Chem.*, 269, 23280–23285.

Bautista D, Saad Z, Chambers AF, Tonkin KS, O'Malley FP, Singhal H, Tokmakejian S, Bramwell V and Harris JF. (1996). *Clin. Biochem.*, 29, 231–239.

Behrend EI, Craig AM, Wilson SM, Denhardt DT and Chambers AF. (1994). *Cancer Res.*, 54, 832–837.

Bellahcène A and Castronovo V. (1995). *Am. J. Pathol.*, 146, 95–100.

Beninati S, Senger DR, Cordella-Miele E, Mukherjee AB, Chackalaparampil I, Shanmugam V, Singh K and Mukherjee BB. (1994). *J. Biochem.*, 115, 675–682.

Brown LF, Papadopoulos-Sergiou A, Berse B, Manseau EJ, Tognazzi K, Perruzzi CA, Dvorak HF and Senger DR. (1994). *Am. J. Pathol.*, 145, 610–623.

Cao L, Taggart RT, Berquin I, Moin K, Fong D and Sloane BF. (1994). *Gene*, 139, 163–169.

Chellaiah M and Hruska K. (1996). *Mol. Biol. Cell.*, 7, 743–753.

Chen H, Ke Y, Oates AJ, Barraclough R and Rudland PS. (1997). *Oncogene*, 14, 1581–1588.

Denhardt DT, Butler WT, Chambers AF and Senger DR. (1995). *Ann. N.Y. Acad. Sci.*, 760, 388pp.

Feng B, Rollo EE and Denhardt DT. (1995). *Clin. Exp. Metastasis*, 13, 453–462.

Gardner HA, Berse B and Senger DR. (1994). *Oncogene*, 9, 2321–2326.

Hirota S, Ito A, Nagoshi J, Takeda M, Kurata A, Takatsuka Y, Kohri K, Nomura S and Kitamura Y. (1995). *Lab. Invest.*, 72, 64–69.

Hruska KA, Rolnick F, Huskey M, Alvarez U and Cheresh D. (1995). *Ann. N.Y. Acad. Sci.*, 760, 151–165.

Joseph LJ, Chang LG, Stamenkovitch D and Sukatme VP. (1988). *J. Clin. Invest.*, 81, 1621–1629.

Mazzieri R, Masiero L, Zanetta L, Monea S, Onisto M, Garbisa S and Mignatti P. (1997). *EMBO J.*, 16, 2319–2332.

Menter DG, Patton JT, Updyke TV, Kerbel RS, Maamer M, McIntire LV and Nicholson GL. (1991). *Cell Biophys.*, 18, 123–143.

Morris VL, Tuck AB, Wilson SM, Percy D and Chambers AF. (1993). *Clin. Exp. Metastasis*, 11, 103–112.

Oates AJ, Barraclough R and Rudland P. (1996). *Oncogene*, 13, 97–104.

Peterson GL. (1977). *Anal. Biochem.*, 83, 346–356.

Price JE, Polyzos A, Zhang RD and Daniels LM. (1990). *Cancer Res.*, 50, 717–721.

Prince CW, Dickie D and Krumdieck CL. (1991). *Biochem. Biophys. Res. Commun.*, 177, 1205–1210.

Reponen P, Sahlberg C, Huhtala P, Hurskainen T, Thesleff I and Tryggvason K. (1992). *J. Biol. Chem.*, 267, 7856–7862.

Riccio A, Grimaldi G, Verde P, Sebastio G, Boast S and Blasi F. (1985). *Nucleic. Acids. Res.*, 13, 2759–2771.

Roldan AL, Cubellis MV, Mascucci MT, Behrendt N, Lund LR, Dano K, Appella E and Blasi F. (1990). *EMBO J.*, 9, 467–474.

Saavedra RA, Kimbro SK, Stern DN, Schnuer J, Ashkar S, Glimcher MJ and Ljubetic CI. (1995). *Ann. N.Y. Acad. Sci.*, 760, 35–43.

Sambrook J, Fritsch EF and Maniatis T. (eds.). (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Senger DR, Asch BB, Smith BD, Perruzzi CA and Dvorak HF. (1983). *Nature*, 302, 714–715.

Senger DR, Perruzzi CA, Graccy CR, Papadopoulos A and Tenen DG. (1988). *Cancer Res.*, 48, 5770–5774.

Senger DR and Perruzzi CA. (1996). *Biochim. Biophys. Acta.*, 1314, 13–24.

Shanmugam V, Chackalaparampil I, Kundu GC, Mukherjee AB and Mukherjee BB. (1997). *Biochemistry*, 36, 5729–5738.

Simon C, Juarez J, Nicolson GL and Boyd D. (1996). *Cancer Res.*, 56, 5369–5374.

Singhal H, Bautista DS, Tonkin KS, O'Malley FP, Tuck AB, Chambers AF and Harris JF. (1997). *Clin. Cancer Res.*, 3, 605–611.

Sorensen ES, Rasmussen LK, Moller L, Jensen PH, Hojrup P and Petersen TE. (1994). *Biochem. J.*, 304, 13–16.

Sorensen ES and Petersen TE. (1995). *Ann. N.Y. Acad. Sci.*, 760, 363–366.

Teti A, Farina AR, Villanova I, Tiberio A, Tacconelli A, Sciotino G, Chambers AF, Gulino A and Mackay AR. (1998). *Int. J. Cancer*, 77, 82–93.

Tuck AB, Wilson SM and Chambers AF. (1990). *Clin. Exp. Metastasis*, 8, 417–431.

Tuck AB, Wilson SM, Khokha R and Chambers AF. (1991). *J. Natl. Cancer Inst.*, 83, 485–491.

Tuck AB, O'Malley FP, Singhal H, Harris JF, Tonkin KS, Kerkvliet N, Saad Z, Doig G and Chambers AF. (1998). *Int. J. Cancer*, 79, 502–508.

Werb Z. (1997). *Cell*, 91, 439–442.

Xuan JW, Hota C and Chambers AF. (1994). *J. Cell Biochem.*, 54, 247–255.

Xuan JW, Hota C, Shigeyama Y, D'Errico JA, Somerman MJ and Chambers AF. (1995). *J. Cell. Biochem.*, 57, 680–690.

Yebra M, Parry GCN, Strömblad S, Mackman N, Rosenberg S, Mueller BM and Cheresh DA. (1996). *J. Biol. Chem.*, 271, 29393–29399.

Young MF, Kerr JM, Termine JD, Wewer UM, Wang MG, McBride OW and Fisher LW. (1990). *Genomics*, 7, 491–502.

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THE ROLE OF OSTEOPONTIN IN THE MALIGNANCY OF HUMAN BREAST CARCINOMA: INVOLVEMENT IN CELL ADHESION, MIGRATION, AND INVASIVENESS OF A PROGRESSION SERIES OF MAMMARY EPITHELIAL CELL LINES

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Osteopontin (OPN) is a secreted, integrin-binding glycophosphoprotein whose levels are increased in the primary tumors and plasma of patients with breast cancer. Our clinical studies have shown a relationship between plasma OPN, prognosis and tumor burden in patients with metastatic breast cancer (Singhal et al., Clin. Cancer Res., 3, 605-611, 1997), as well as between tumor cell OPN and prognosis in patients with lymph node negative breast cancer (Tuck et al., manuscript submitted). The purpose of the present work is to examine the ability of cultured human breast epithelial cells at different stages of progression to synthesize OPN, as well as to determine in what manner OPN may influence the biological behavior and malignant potential of these cells.

For this work, we have made use of the 21T series of human mammary epithelial cell lines (Band et al., Cancer Res., 50, 7351-7357, 1990), believed to represent different stages of tumor progression (21PT: immortalized, nontumorigenic; 21NT: weakly tumorigenic, nonmetastatic; 21MT-1: tumorigenic, weakly metastatic). Basal levels of expression of OPN were assessed, by both Northern and Western analysis. 21PT and 21NT were then compared in cell adhesion and migration assays for response to OPN. Stable transfectants of both of these cell lines were established, using an expression vector containing OPN cDNA under the control of the strong CMV promoter. Transfectants showing constitutive upregulation of OPN were assayed for invasiveness through Matrigel (artificial basement membrane), in comparison with controls.

Keywords: Osteopontin (OPN), Malignancy, Adhesion, Migration, Invasion

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(and over)

Results of Northern analysis have shown that all three 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 has shown that the 21T series cell lines also secrete lower levels of OPN protein than MDA-MD-435 cells, with the highest levels of expression in the 21T series seen in the weakly metastatic 21MT-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. When tested in cell adhesion assay, 21NT showed a greater avidity for OPN at low concentrations than did 21PT, and in cell migration assay 21NT showed a higher basal rate of migration than 21PT. Furthermore, both 21PT and 21NT cells showed directed migration towards human recombinant (hr)OPN, which was completely blocked by anti-OPN antibody. The integrin-dependence of the cell migration effect was demonstrated by complete inhibition with GRGDS peptide, and absence of a response using RGD deletion mutant hrOPN as the chemoattractant. OPN-induced migration was further found to complement that of epidermal growth factor, in a synergistic fashion. Pooled OPN-transfectants of 21PT and 21NT cells, constitutively upregulated for OPN, invaded significantly better through Matrigel than did controls, indicating that these cells not only show directed migration in response to OPN, but also an increased ability to invade through basement membrane in the presence of elevated levels of OPN. Possible molecular mechanisms of these effects are presently under study.

This work thus provides direct evidence for the ability of a series of transformed breast epithelial cells to synthesize OPN, and to respond to OPN by directed cell migration and invasion through basement membrane. The ability to respond to OPN in this fashion is seen both in transformed, nontumorigenic and tumorigenic cells. A trend towards higher levels of OPN synthesis in cells of greater malignancy is also evident. Thus, cells at earlier stages of progression may respond to OPN synthesized by neighboring cells (i.e. tumor infiltrating inflammatory cells) by induction of cell motility and invasiveness, whereas more malignant cells, having acquired the ability to themselves synthesize elevated levels of OPN, may have become more autonomous in this regard. *In vivo* assays are presently underway in nude mice to determine the influence of transfected OPN on tumorigenicity and metastatic ability of these cells. Understanding the functional role of OPN in the malignancy of human breast cancer will be of potential importance not only in the interpretation of prognostic information gained through determination of OPN levels, but also in the future development of therapeutic strategies aimed at blocking OPN effects.

Breast Cancer Research and Treatment, 46 : Abstract #363, 1997

361 VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION PREDICTS METASTASIS IN HUMAN BREAST CANCER.

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Vascular Endothelial Growth Factor (VEGF) is a potent angiogenic growth factor. Angiogenesis correlates with breast cancer metastasis. To investigate the role of VEGF expression in breast cancer metastasis, we counted microvessels and examined VEGF expression in two matched groups of patients (22 with metastases and 44 without). Paraffin-embedded breast cancer tissue was immunostained and the number of blood vessels and expresssion of VEGF staining compared in patients with and without nodal metastases.

Both mean microvessel numbers and VEGF expression correlated with metastatic disease. The mean numbers of microvessels were higher in the metastatic group compared to the nonmetastatic group at 400X (15.77±12.99 vs. 5.619±5.574, respectvely, p=0.0001). VEGF expression was higher in the metastatic group as compared to the non-metastatic group (P<0.0001). VEGF expression also correlated with increased angiogenesis (p=0.0007 at 400X). The combination of angiogenesis and VEGF expression was found to be more predictive of nodal metastasis than angiogenesis or VEGF expression alone.

We conclude that VEGF expression with angiogenesis, is a superior predictor of metastasis in human breast cancer compared to VEGF expression or microvessel numbers alone. In addition, our results implicate VEGF in the mechanism of angiogenesis and metastasis.

362 IMPLICATION OF P53 MUTATION IN MATRIGEL-ENHANCED GROWTH AND METASTASIS OF HUMAN BREAST CARCINOMA.

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Primary human breast carcinomas generally fail to grow as xenografts when transplanted into experimental animals; however, we obtained a 60% success rate when tumor tissues were co-injected with Matrigel in mice. Matrigel not only increased tumor take but also enhanced tumor growth and metastatic potential. Detailed analysis of original patients' tumors suggested that most with positive take in animals harbored mutant p53 protein. To further confirm association between P53 status and growth-enhancing effects of Matrigel, we evaluated in vivo growth (with/without Matrigel) of 10 different human breast carcinoma cell lines with different p53 status. Cells (1 million/animal) were injected into the dorsal flank of 4- to 6-week-old female athymic mice either as suspension in Hank's balanced salt solution (HBSS) or mixed with 1:1 volume of HBSS:Matrigel. Matrigel enhanced the tumorigenicity of all cell lines studied; however, the growth-promoting effect of Matrigel was limited to those lines with p53 mutation. Study of different integrin profiles suggested that Matrigel favored the growth of cells with relatively high expression of α6 and β4 integrins. We further studied the effects of laminin, a major component in extracellular matrix (ECM) and a ligand for α6β4 integrin, on in vivo growth of breast carcinoma cell line MAXF-401 with mutant p53. The xenografts developed from cells coinjected with laminin showed enhanced growth and were histopathologically more highly invasive than cells injected without laminin. Immunohistological studies of xenografts (originated with/without laminin) suggested that laminin treatment down-regulates the expression of α3 integrin without altering other integrin subtypes. In conclusion, mutation of p53 gene may play a significant role in modulating ECM protein–tumor cell interaction. Laminin alters the expression of tumor cell surface integrins, which in turn may increase its binding to α6 integrin. Laminin interaction with α6 integrin may transduce signals to produce increased levels of proteases/factors involved in tumor cell growth and invasion.

363 OSTEOPONTIN INDUCES INTEGRIN-DEPENDENT, DIRECTED CELL MIGRATION OF TUMORIGENIC AND NON-TUMORIGENIC BREAST EPITHELIAL CELLS, AND ENHANCES THEIR MIGRATION RESPONSE TO EPIDERMAL GROWTH FACTOR (EGF) AND HEPATOCYTE GROWTH FACTOR (HGF). Tuck AB*, O'Malley FP, Hota C, and Chambers AF, London Health Sciences Centre, London Regional Cancer Centre, and University of Western Ontario, London, Ontario, Canada N6A 4G5

Osteopontin (OPN) is a secreted, integrin-binding glycophosphoprotein whose levels are increased in primary tumors and plasma of some patients with breast cancer. We examined the role of OPN in inducing directed cell migration of human breast epithelial cell lines 21PT (non-tumorigenic) and 21NT (tumorigenic), both alone and in collaboration with EGF and HGF. Both cell lines require exogenous EGF for continuous growth in cell culture. We found that in the absence of either EGF or HGF, human recombinant (hr)OPN induced a low-level migration response of both 21PT and 21NT. Similarly, both EGF and HGF alone were found to induce cell migration of both cell lines. Addition of hrOPN to either EGF- or HGF-containing culture medium resulted in a synergistic effect on cell migration of both cell lines. Criss-cross assay showed OPN-induced cell migration to be a directional response. The response was blocked by both anti-OPN antibody and RGD peptides, while RGD deletion mutant hrOPN did not induce cell migration. We then examined cultures of 21PT and 21NT cells which had been incubated either in the presence or absence of hrOPN (X18 hours), and found that OPN induced expression of HGF receptor (HGFR/met) mRNA, but not EGF receptor (EGFR) mRNA, in both cell lines. These studies thus provide evidence that OPN induces integrin-dependent cell migration of both tumorigenic (21NT) and non-tumorigenic (21PT) breast epithelial cell lines, and also increases sensitivity of both to the migration-enhancing effects of growth factors EGF and HGF. The increased sensitivity to HGF may be due, at least in part, to upregulation of HGFR mRNA expression in response to OPN.

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-96-1-6075 ("Career Development" Award to Dr. Tuck), and by the Canadian Breast Cancer Research Initiative, the London Health Sciences Centre, and the London Regional Cancer Centre. The content of this abstract does not necessarily reflect the position or policy of the U.S. government, and no official endorsement should be inferred.

364 PRO-MMP-2 TRANSFECTION OF MDA-MD-231 CELLS: *IN VITRO* AND *IN VIVO* ANALYSIS. *Oh S.J.[1], Sung V., Aaron A.D., Bae S.N.[2], Arand G.A., Hanfelt J., Lippman M.E., and Thompson E.W. Lombardi Cancer Center, Georgetown University Medical Center, NW Washington D.C., U.S.A. and Departments of [1]Surgery and [2]Gynecology, Catholic University Medical College, Seoul, Korea.

Human breast carcinoma (HBC) cell lines which show increased invasiveness in conjunction with a vimentin-positive phenotype can be induced to activate MMP-2. To investigate the biological implications of this pathway, we stably transfected the vimentin-positive MDA-MB-231-BAG HBC cells with a pro-MMP-2 cDNA expression vector. Multiple clones were shown to produce pro-MMP-2 and activate it in response to ConA, TPA or collagen type I gel. MMP-2-transfected clones showed no advantage over the parental cells or vector control clones in the Boyden chamber chemoinvasion assay or Matrigel outgrowth assay, perhaps reflecting their inability to activate the MMP-2. Individual clones were tested for bone and organ colonization after intracardiac injection using PCR, and mice were monitored radiologically for osteolysis. Extensive bone metastasis and osteolysis was seen in 3 of 4 mice with one of the MMP-2-transfected clones, and extensive lung metastasis in 3 of 4 from another. No metastasis were developed by the remaining MMP-2-transfected clone, and low levels of metastasis to brain, spleen, bone and lung were seen with parental cells and vector control clones. Exact Pearson chi square analysis indicated that MMP-2 may facilitate either lung or bone metastasis in conjunction with additional clonal traits. Additional analyses will be performed to confirm and extend these results.

Ontario Association of Pathologists, 61st Annual Meeting, London, Ontario, June 12-13, 1998.

OSTEOPONTIN INDUCES INCREASED CELLULAR MOTILITY AND INVASIVENESS OF HUMAN MAMMARY EPITHELIAL CELL LINES

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To determine a functional role for osteopontin in breast cancer, we assessed the effect of osteopontin (OPN) on motility and invasiveness of three human mammary epithelial cell lines: 21PT (non-tumorigenic), 21NT (tumorigenic, non-metastatic), MDA-MB-435 (tumorigenic, metastatic). 21PT and 21NT expressed low, and MDA-MB-435 high basal levels of OPN. In transwell assay, both 21PT and 21NT showed increased directed cell motility and invasiveness through Matrigel in response to human recombinant OPN. Cell motility was specifically inhibited by anti-OPN and anti-integrin (anti-αvß5 and ß1) antibodies. In contrast, MDA-MB-435 cells showed a motility response that was inhibited by anti-αvß3, but not anti-αvß5 or ß1 antibodies. Stable transfectants of 21PT and 21NT cells were generated by transfection with either control vector (pcDNA3), or the same plasmid with full-length human OPN downstream of the constitutive CMV promoter. Pooled and cloned transfectants were isolated and assessed for OPN protein levels. The highest OPN-expressing pool and clone of both 21PT and 21NT were examined in transwell assays for invasion through Matrigel. All OPN-overexpressing transfectants showed increased invasiveness, with 21NT-derived cells more invasive than those from 21PT. The OPN-transfected 21NT clone expressing the highest level of OPN showed invasive ability which approached that of MDA-MB-435 cells. These data provide evidence that OPN may play a role in motility and invasiveness of human mammary carcinoma, and that different integrins may be involved in the motility response of cells at different stages of progression.

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VII International Congress of the Metastasis Research Society, San Diego CA, Abstract # PB6.11 Oct. 7-10, 1998!

PB6.08

Soluble fibrin increases platelet adherence to tumor cells *in vivo*: A possible role in metastasis?

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Anticoagulant or antiplatelet therapies have reduced metastasis in some clinical trials and animal tumor models. Many cancer patients have activated blood coagulation with elevated levels of soluble fibrin (sFn). We previously demonstrated that sFn increases platelet adherence to A375 melanoma cells in both static and flow conditions. Since platelet binding facilitates blood-borne metastasis, we examined the effect of sFn on tumor-induced thrombocytopenia in an animal model. SFn was produced by adding thrombin (0.25 U/ml) to purified fibrinogen (Fg; 0.5 mg/ml) in the presence of Gly-Pro-Arg-Pro-amide (GPRP; 2 mmol/l). Immediately before injection, A375 cells were incubated with (a) culture medium (control); (b) sFn; (c) Fg (0.5 mg/ml) with GPRP or (d) thrombin with GPRP. Treated or untreated cells were injected i.v. into 24 Beige nude Xid mice, 12 of which were therapeutically anticoagulated (warfarin). Platelets were counted before and 15 minutes after tumor injection. In untreated animals, A375 cells (untreated or treated with sFn, Fg or thrombin) similarly decreased the count (40–46%). In warfarin-treated animals untreated or Fg-treated cells only minimally decreased platelet count (5% and 7% respectively). In contrast, platelets fell by 22% and 13% following injection of sFn- and thrombin-treated cells respectively ($P < 0.05$). A375 cells possess potent procoagulant activity, thus masking any effect of sFn in the non-anticoagulated animals. This effect was removed by anticoagulation and we conclude that thrombocytopenia induced by sFn-treated tumor cells was caused by sFn-mediated platelet/tumor cell adherence *in vivo*. These results suggest that elevated sFn levels in cancer may be a risk factor for metastasis.

PB6.09

Distribution and survival of melanoma cells in mouse lung

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Metastases in mouse lung form primarily at the organ surface, and any interior tumors are found adjacent to large airways or vessels. This selective distribution could result from (1) initial positions of cell arrest (2) preferential survival of cells at specific locations; (3) preferential growth of cells at favoured sites. We hypothesized that early survival of cancer cells is a function of organ architecture and that survival increases with proximity to surfaces of major structures. We quantified initial cell distribution and subsequent survival at the surface and in the interior using two melanoma cell lines (highly metastatic B16F10, poorly metastatic B16F1). Fluorescently labelled cells mixed with 9-µm polystyrene reference microspheres were targeted to lung via the vena cava of anethetized syngeneic mice. The microspheres became trapped in capillaries indefinitely, and by comparing the pre-injection ratio of cells to microspheres to that found in tissue, cell survival was determined. Digitized images of 70 µm lung sections taken at 1 h or 24 h after injection provided co-ordinate positions of cells, microspheres and tissue structures. Initial distribution was uniform, with survival of 78% (B16F1) and 98% (B16F10) at 1 h. By 24 h, survivals were 71 and 83%, respectively. For both cell lines, survival at 1 h was independent of position of initial arrest. However, by 24 h, survival decreased markedly at distances > 50 µm from the lung surface or internal structures > 100 µm in diameter. These data indicate that contrary to general expectations, most B16 cells survive in the lung for at least 24 h. Moreover, site-specific metastasis in lung is not due to initial distribution, but rather to factors promoting survival or growth of cells near surfaces. (Support: NCI Canada, NSERC, CFUW, SFC)

PB6.10

Type I collagen promotes primary human ovarian epithelial carcinoma cell adhesion, migration and proMatrix metallaproteinase-2 activation

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Epithelial ovarian carcinoma is a highly malignant disease which presents at an advanced stage with widespread intraperitoneal metastases. Short-term primary cultures of malignant human ovarian epithelium (OVCA) were used to analyze the effect of specific extracellular matrix (ECM) proteins on the invasive phenotype. Analysis of cell:matrix adhesive profiles indicated that OVCA adhere preferentially to type I collagen. Immunoprecipitation analyses demonstrated the presence of the collagen-binding α2β1 integrin in biotin labelled ovarian carcinoma cell membranes and cellular adhesion was inhibited by blocking antibodies directed against the α2 and β1 integrin subunits. The α2β1-binding peptide Asp-Gly-Glu-Ala (DGEA) was also active at blocking adhesion to collagen relative to the control peptide Ala-Gly-Glu-Ala (AGEA). Analysis of cell motility on protein coated colloidal gold coverslips demonstrated that ovarian carcinoma cells migrate preferentially on type-I collagen coated surfaces. Type I collagen promoted migration in a concentration dependent, saturable manner, with maximal migration observed at a collagen coating concentration of 50 µg/ml. Migration on collagen was inhibited by antibodies directed against the β1 integrin subunit and by DGEA peptide, providing evidence for the role of the α2β1 integrin in ovarian carcinoma cell motility. The effect of adhesion to collagen on the production of matrix degrading proteinases was also assessed. Culturing ovarian carcinoma cells on type I collagen gels led to a significant increase in conversion of the 72 kDa matrix metalloproteinase-2 zymogen (proMMP-2) to the 66 kDa active form. Similar results were obtained upon ligation of the α2β1 integrin with anti-integrin subunit specific monoclonal antibodies. These data suggest that α2β1 integrin-mediated interaction of ovarian carcinoma cells with type-I collagen, a protein prevalent both in the mesothelial extracellular matrix and in the peritoneal cavity of ovarian carcinoma patients, may function on multiple levels to promote metastatic dissemination of ovarian carcinoma cells.

PB6.11

Osteopontin upregulation is associated with increased cellular invasiveness and urokinase expression of human mammary epithelial cell lines 21PT and 21NT

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We assessed the effect of osteopontin (OPN) on cellular invasiveness of two human mammary epithelial cell lines: 21PT (non-tumorigenic) and 21NT (tumorigenic). Both cell lines expressed low basal levels of OPN. In transwell assay, both 21PT and 21NT showed increased invasiveness through Matrigel in response to human recombinant OPN. Stable transfectants were generated by transfection with either control vector (pcDNA3), or the same plasmid with full-length human OPN downstream of the constitutive CMV promoter. Pooled and cloned transfectant populations were isolated and assessed for OPN protein levels. The highest OPN-expressing pool and clone of both 21PT and 21NT were examined in transwell assays for invasion through Matrigel, with fibronectin as chemoattractant. All OPN-overexpressing transfectants showed increased invasiveness, with 21NT-derived cells more invasive than those from 21PT. The OPN-transfected 21NT clone expressing the highest level of OPN showed a degree of invasive ability approaching that of highly malignant MDA-MB-435 human mammary carcinoma cells.

(and over)

Assay of mRNA from transfected cells for a number of secreted proteases showed a close association between level of urokinase-type plasminogen activator (uPA) expression, OPN expression, and cellular invasiveness. These data provide evidence that OPN may play a role in invasiveness of human mammary carcinoma, and that the invasive response may be accompanied by upregulation of uPA expression. Supported by: The U.S. Army Medical Research and Material Command under DAMD17-96-1-6075 ("Career Development" Award to Dr Tuck) and the Canadian Breast Cancer Research Initiative (#8426)

PB6.12
Mammary epithelium-induced motility and invasion of breast carcinoma cells
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Invasion and metastasis requires tumor cell adhesion to the basement membrane, elaboration of proteolytic enzymes, and migration through the membrane into the surrounding stroma. We examined whether normal human mammary epithelial cells (HMEC) interact with breast cancer cells to enhance their invasive characteristics. HMEC and 184 A1, an immortalized but non-tumorigenic HMEC clone, produce one or more proteins which induce dose-dependent motility in MCF-7 breast carcinoma cells. Motility was demonstrated with scattering assays, Boyden chamber assays, and time-lapse video microscopy. Conditioned medium of 184 A1 cells also increased the motility of BT-20 and SKBR-3 breast carcinoma cells. Adhesion of MCF-7 cells to reconstituted Engelbreth-Holm-Swarm basement membrane (Matrigel) was increased by 50% in the presence of 184 A1 conditioned medium. To determine if the 184 A1 conditioned medium could promote invasion, 100,000 carcinoma cells were placed in Boyden chambers with a layer of 250 $\mu g/cm^2$ Matrigel over the membrane surface. Cells were incubated in the presence or absence of conditioned medium, and the invading cells were counted after three days. The conditioned medium encouraged the invasion of BT-20 cells, although little or no invasion was induced in MCF-7 cells. Efforts to purify and sequence the proteins responsible for these effects are currently underway. These data suggest that in some patients, substances elaborated by the normal mammary epithelium may contribute to the invasive properties of their breast cancers. These findings may also help explain previous observations of greater invasive and metastatic potential of mammary carcinomas implanted in orthotopic sites rather than in the subcutis. This work has been funded by the University of California Cancer Research Co-ordinating Committee.

PB6.13
Role of Tiam1 and Rac1 in invasion and migration of human renal cell carcinoma
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Tiam1 has been identified as an invasion and metastasis-inducing gene in murine T-lymphoma cell line and activates the Rho-like GTPase Rac 1. Recent studies, however, indicate that Tiam1–Rac1 signalling affects invasion in different ways depending on the cell type studied. In the present study, a human renal cell carcinoma (RCC) cell line lacking endogenous Tiam1 expression was stably transfected with active C1199-Tiam1, which comprises the C-terminal 1199 amino acids and is known to give similar effects as full-length-Tiam1. Morphologically, C1199-Tiam1 induced membrane ruffling and a polygonal epithelial phenotype, whereas mock transfected control cells were much more spindle-shaped. In the matrigel invasion assay C1199-Tiam1 transfected tumor cells were significantly less invasive than control cells ($p = 0.006$) and in a migration assay motility of C1199-Tiam1 transfected cells was significantly decreased ($p = 0.001$). These effects of C1199-Tiam1 were at least partly dependent of the N-terminal pleckstrin homology (PHn) domain of Tiam1, since expression of C580-Tiam1, which lacks the PHn domain, exhibited a markedly less pronounced, but still significant inhibitory effect on invasion ($p = 0.02$), while cell motility and cell morphology were not affected. Similar to C1199-Tiam1, transfection of constitutively active V12 Rac1 induced a polygonal epithelial phenotype and resulted in a significant inhibition of invasion ($p = 0.018$), but the effects were less pronounced. In contrast to Ras-transformed MDCK cells, in which Tiam1 and Rac1 promote E-cadherin-mediated cell–cell adhesion, the observed effects in our RCC cell lines are rather mediated by other cadherins, since E-cadherin is absent. In conclusion, C1199-Tiam1 inhibits invasion and migration of a human RCC cell line. The inhibitory effects partly depend on the PHn domain of Tiam1 and are at least in part mediated by Rac1.

PB6.14
The roles of heparin-binding epidermal growth factor-like growth factor in thyroid cancer cells
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Growth factors are known to play many roles in cell growth, differentiation and the development and progression of cancer, and can also modulate a number of integrin-dependent functions, including cell adhesion, cell migration and cytoskeletal organization. However, the mechanisms underlying these phenomena are less clear. The purpose of this study is to evaluate the possible roles of heparin-binding epidermal growth factor-like growth factor (HB-EGF), which is a member of the EGF family, in thyroid cancer cells. It was first demonstrated that HB-EGF was not only a potent mitogen but also chemotactic factor for thyroid cancer cells, 8305c cell line, as previously described for fibroblasts, smooth muscle cells and keratinocytes. HB-EGF induced phosphorylation of EGF receptor (EGF-R, ErbB-1), but not of ErbB-4 in the cancer cells. Chemotaxis was inhibited by tryphostin AG1478 (a specific inhibitor of EGF receptor tyrosine kinase), suggesting a possible role for EGF-R in mediating HB-EGF-stimulated chemotaxis. Moreover, HB-EGF also induced the decreased expression of integrin alpha 5 beta 1 in 8305c cells and reduced the cancer cell adhesion to fibronectin. On the other hand, in immunohistochemical study HB-EGF protein and its receptors, ErbB-1 and ErbB-4 were observed in 8305c cells and tissue samples of thyroid papillary adenocarcinoma. These results suggest that HB-EGF might be a potent chemotactic factor, which could modulate integrin-dependent functions in the development and progression of thyroid cancer cells.

PB6.15
Influence of exposure to carbon dioxide in cancer cell growth and motility
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Introduction: The application of laparoscopic surgery for malignant disease is still controversial. Above all port site recurrence is a big problem, but the mechanism for the formation of port site metastasis is unclear. We suppose 100% concentration of carbon dioxide (CO_2) plays some roles for port site metastasis. Aim: The purpose of this study is to investigate cancer cell growth and motility after exposure to 100% CO_2 as is usually used in laparoscopic surgery in order to know the influence of 100% CO_2 with the association of port site metastasis. Methods: Several human colon cancer cell lines were incubated for 6 hours in 100% or 5% (for control) CO_2. The effect of CO_2 concentration on the cell growth after 24, 48 and 72 hours was examined by MTT assay.

US and Canadian Academy of Pathology, 88th Annual Meeting
San Francisco, CA Abstract #165 March 20-26, 1999

161 **Syndecan-1 Expression Is Induced in the Stroma of Infiltrating Breast Carcinoma**

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Loss of expression of the heparan-sulfate proteoglycan Syndecan-1 leads to decreased cell adhesion, increased invasive potential and dysregulated growth of mammary epithelial cells *in-vitro*. Therefore, we compared Syndecan-1 expression in malignant and nonmalignant breast tissues by immunohistochemistry using monoclonal antibody B-B4. Staining for Syndecan-1 is greatly diminished in malignant cells of infiltrating ductal carcinoma (IDC; n = 20) as compared to ductal epithelium of both normal breast tissue and stromal-epithelial neoplasms (SEN = 10) which exhibit extensive basolateral staining. Surprisingly, the comparison between malignant and nonmalignant breast tissue also reveals a striking difference in expression of Syndecan-1 within the stromal compartment. In IDC, strong staining for Syndecan-1 is present both within the connective tissue and on stromal cell surfaces, while Syndecan-1 is absent in the stroma of both normal breast and SEN. Because Syndecan-1 interacts with the heparin-binding growth factor FGF-2, accumulation of Syndecan-1 may contribute to the extensive angiogenesis and stromal proliferation that are characteristic of IDC. Moreover, the induction of Syndecan-1 within the stroma coupled with the loss of Syndecan-1 from malignant epithelial cells suggests that changes in expression play a critical role in promoting the metastatic phenotype of IDC.

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Surgical biopsy findings in patients with lobular carcinoma *in situ* (LCIS) or atypical lobular hyperplasia (ALH) at percutaneous breast biopsy.

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Background: There are at present no guidelines for the management of patients with LCIS or ALH diagnosed by percutaneous breast biopsy. The purpose of this study was to evaluate surgical biopsy findings in lesions yielding LCIS or ALH at percutaneous breast biopsy.

Methods: We identified 23 lesions in which percutaneous biopsy revealed LCIS or ALH from 1993-1998. Subsequent surgical excision was performed in 18 lesions. The percutaneous biopsy and surgical breast specimens in these 18 lesions were reviewed.

Results: Of 15 lesions yielding LCIS at percutaneous biopsy, surgical specimens showed LCIS in 7 (47%), benign findings in 4 (27%), invasive lobular carcinoma in 2 (13%), and ductal carcinoma *in situ* (DCIS) in 2 (13%). In 2 of the 4 lesions yielding DCIS or invasive carcinoma at surgery, the LCIS in the percutaneous biopsy had morphological features that overlapped with those of DCIS; in 1, percutaneous biopsy showed LCIS and a radial scar; and in 1, the lesion was evident on the mammogram as an area of architectural distortion. Of 3 lesions yielding ALH at percutaneous biopsy, surgery revealed LCIS in 2 and benign findings in 1.

Conclusion: Lesions yielding LCIS at percutaneous biopsy may warrant surgical excision if a) the LCIS in the percutaneous biopsy material has features that overlap with those of DCIS; b) there is an associated lesion warranting excision, such as a radial scar; or c) there is discordance between histologic and imaging findings. No DCIS or invasive carcinoma was identified at surgery in lesions yielding ALH at percutaneous biopsy, but our findings should be confirmed in larger series.

163 EXPRESSION OF THREE PROTEASES BY REACTIVE STROMAL CELLS AND BREAST CANCER PROGNOSIS

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Background: Current literature supports the concept that proteases produced by reactive stromal cells act in a cascade manner to mediate dissolution of the extracellular matrix (ECM) and favor cancer progression. We and others recently identified cathepsin D (CD), stromelysin-3 (ST3) and urokinase plasminogen activator (uPA) expression by reactive stromal cells as significant factors of poor prognosis in breast cancer. We tested the hypothesis that cancer aggressiveness is associated with the number of proteases involved.

Design: Protease expression was analyzed by immunohistochemistry (CD) and in situ hybridization (ST3 and uPA using antisense RNA probes) on formalin-fixed paraffin-embedded specimens from 557 breast cancers without distant metastasis at diagnosis and with an average of 10 years of follow-up.

Results: Of the 557 breast cancers, 80 expressed all three proteases and 134 expressed none of them. The expression of all three proteases strongly correlated with each other ($p<0.001$). An adjusted Cox model revealed that distant-metastasis-free survival (DMFS) was significantly worse for patients whose tumor expressed all three proteases ($p=0.0001$). The DMFS of patients whose tumor lacked at least one of the three proteases was similar to that of patients without any protease expression, irrespective of the type or number of proteases missing.

Conclusion: This study suggests that proteases act in a stepwise manner and that a breach in the protease pathway may impair breast cancer progression.

Grants: Cancer Research Society Inc and FRSQ-Hydro-Québec program.

164 **Reactive spindle cell pseudotumor: association with trauma and infarct to papillary and sclerosing lesions of the breast.** G Tse*', H Gobbi', D L Page'. *Department of Anatomical and Cellular Pathology, Chinese University of Hong Kong, Shatin, Hong Kong and 'Department of Pathology, Vanderbilt University, Nashville.

Background: Reactive spindle cell proliferation in the breast in association with papillary lesions or complex sclerosing lesions (CSL) are uncommon. The histologic features can be worrisome and may lead to confusion with malignant lesions. Awareness of this entity can reduce potential over-diagnosis of malignancy.

Design: Histologic features of 14 such cases are evaluated by light microscopy including size, margin, nuclear pleomorphism, mitotic activity and the associated lesions.

Results: Eight cases arise in association with papillary lesions including 6 papillomas and 2 encysted papillary carcinomas; 5 cases arise with CSL and 1 case with both papilloma and CSL. Of the 8 papillomas, 3 show infarction and 3 have a previous history of biopsy at the same site. The sizes of the lesions ranged from 1.5 to 5mm in greatest dimension. Histologically the spindle cell proliferation can be nodular (2 cases) or show an infiltrative margin (14 cases), hence the term spindle cell pseudotumor (SCP) is coined. There is nuclear pleomorphism but mitotic count is low (less than 5 per 10 high power fields). The morphology of the spindle cells correspond to myofibroblasts, and SCP may be regarded as related to inflammatory myofibroblastic pseudotumor. Lymphocytic infiltrate is present in all cases, together with plasma cells in 4 and eosinophils in 3; in 1 case, rare multinucleated giant cells are seen. Necrosis is absent.

Conclusion: The association of SCP with CSLs, which are known to possess myofibroblastic activity in their formative stages and with papillary lesions with infarction or post traumatic history suggest the possibility of an exuberant reparative etiology. The importance of recognizing SCP as reactive is to avoid over-diagnosis of malignant spindle cell tumors of the breast.

165 INDUCTION OF MAMMARY EPITHELIAL CELL (MEC) MOTILITY BY OSTEOPONTIN (OPN) IS INTEGRIN-DEPENDENT AND IS ASSOCIATED WITH INCREASED HEPATOCYTE GROWTH FACTOR RECEPTOR (Met) ACTIVITY

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Background: OPN is a secreted glycophosphoprotein which induces motility of some mammary carcinoma cells, and has been implicated in malignancy of breast carcinoma. Hepatocyte growth factor (HGF) induces cell migration of several MEC lines. The mechanism of OPN-induced cell migration is here addressed, with respect to involvement of cell surface integrins and induction of the HGF/Met pathway.

Design: Cell migration of a series of MEC lines of differing malignancy was examined in transwell assay, using human recombinant OPN as the chemoattractant. Cell migration response to OPN was also tested in the presence or absence of blocking antibodies to integrins known to bind OPN. Migration response to HGF was also tested, alone and in combination with OPN. The effect of OPN on expression and activity of Met kinase was also examined.

Results: OPN induced cell migration of all of the MEC lines tested. OPN-induced cell migration of 21NT (tumorigenic, non-metastatic) and 21PT (non-tumorigenic) cells was $\alpha v\beta 5$ and $\beta 1$ integrin-dependent, $\alpha v\beta 3$-independent, while that of MDA-MB-435 (metastatic) cells was $\alpha v\beta 3$-dependent. HGF also induced migration of all three cell lines, and a synergistic response was seen to HGF and OPN together. The increased migration response to OPN was found to be associated with an initial increase in Met kinase activity (within 30 minutes), followed by an increase in Met mRNA and protein.

Conclusions: OPN-induced cell migration is mediated by different cell surface integrins in MEC lines representing different stages of progression. Integrin-dependent induction of cell migration by OPN may be modulated at least in part by activation of Met.

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166 EXTENT OF MARGIN INVOLVEMENT AND RISK OF LOCAL RECURRENCE (LR) IN PATIENTS WITH INVASIVE BREAST CANCER TREATED WITH CONSERVATIVE SURGERY AND RADIATION THERAPY (CS+RT). M Tulecke, J Connolly, B Silver, A Recht, J Harris, S Schnitt. Beth Israel Deaconess Medical Center, Brigham and Women's Hospital, Dana-Farber Cancer Institute, Joint Center for Radiation Therapy, and Harvard Med. School, Boston, MA.

BACKGROUND: The presence of cancer at the microscopic margins of excision is the most important prognostic factor for LR in women with invasive breast cancer treated with CS+RT. However, the relationship between the extent of margin involvement and LR risk has not been studied in detail.

DESIGN: We reviewed slides from 120 women with invasive breast cancer treated with CS+RT who were found to have invasive cancer and/or ductal carcinoma in situ (DCIS) at the microscopic margin of their final excision specimen. The extent of margin involvement was quantified in each case by measuring the total number of millimeters (mm) of margin involved by invasive cancer and DCIS. Median follow-up for surviving patients was 129 months. Results are presented as 8-year crude LR rates.

RESULTS: Among the 120 patients with positive margins, the total extent of margin involvement (invasive cancer + DCIS) ranged from 0.1mm to 22.0mm (median 1.5mm, mean 2.9mm). Sixteen patients (13%) developed a LR at or near the primary tumor site at 8 years. The likelihood of LR was related to the number of mm of margin involvement. Four of 51 patients (7.8%) with ≤1.0mm of the margin involved by carcinoma had a LR at 8 years compared with 12 of 69 patients (17.4%) with >1.0mm of margin involvement. Although this difference was not statistically significant, the analysis is limited by the small number of LR. The low rate of LR in patients with ≤1mm of margin involvement is similar to the 8 year LR rate of 7% among 204 patients with negative margins treated with CS+RT at our institution during the same time period.

CONCLUSIONS: The results of this study are the first to suggest that there is a relationship between the total number of mm of margin involvement and risk of LR in patients with invasive breast cancer treated with CS+RT. In particular, patients with positive margins who have very limited involvement of the microscopic margins (i.e., ≤1mm) have a low risk of LR and may be adequately treated with CS+RT.

CBCRI "Reasons for Hope"
June 17-19, 1999 Toronto, Ont.

OSTEOPONTIN INDUCES INCREASED INVASIVENESS AND UROKINASE EXPRESSION OF HUMAN MAMMARY EPITHELIAL CELL LINES 21PT AND 21NT

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Osteopontin (OPN) has been associated with enhanced malignancy in breast cancer, but its functional role in this disease is poorly understood. We assessed the effect of OPN on cellular invasiveness of two human mammary epithelial cell lines: 21PT (non-tumorigenic) and 21NT (tumorigenic). Both cell lines expressed low basal levels of OPN. In transwell assay, both 21PT and 21NT showed increased invasiveness through Matrigel in response to human recombinant (hr)OPN. Populations of 21PT and 21NT cells stably transfected with an OPN expression vector were more invasive than control vector transfectants. 21NT-derived transfectants were more invasive than those derived from 21PT. The OPN-transfected 21NT clone expressing the highest level of OPN showed a degree of invasive ability approaching that of highly malignant MDA-MB-435 human mammary carcinoma cells. Assay of mRNA from transfected cells for a number of secreted proteases showed a close association between level of urokinase-type plasminogen activator (uPA) expression, OPN expression, and cellular invasiveness. Incubation of the parental 21PT and 21NT cells with hrOPN resulted in both increased secreted uPA activity, and increased uPA mRNA expression. These data provide evidence that OPN may play a functional role in invasiveness of human mammary carcinoma, and that the invasive response may be accompanied by upregulation of uPA expression.

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THE ROLE OF THE SECRETED PHOSPHOPROTEIN OSTEOPONTIN (OPN) IN METASTATIC BREAST CANCER: INTERIM REPORT

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OPN is an integrin-binding secreted protein associated with malignant transformation. Preliminary work suggested that elevated blood OPN levels in women with metastatic breast cancer were associated with shorter survival. In this study, serial plasma samples (q3-12 wks) for OPN levels are being collected from 150 patients (pt) with breast cancer (≤8 wks from diagnosis of metastases) at each clinic visit. Here we report progress in accrual and a preliminary analysis of the first 75 pt (50%) registered, focusing on feasibility issues and pt demographics.

Accrual started July 1997 and will be complete Sept 1999. About 75% eligible pt enter the study. Entry date range 4/7/97-16/7/98, median age 64 years (range 30-81). 17 pt had metastases (mets) at diagnosis, the rest developed mets at median 58 months (range 4-345) after treatment of primary. At diagnosis - hormone receptors measured in 66 pt, ER+ 40, PR+ 39. At metastasis - 12 pt pre/peri-menopausal, 63 post-menopausal. Median # metastatic sites/pt 1 (range 1-5), distribution bone 46, liver 21, lung 19, nodes 17, effusion/ascites 18, skin 8. First systemic treatment for mets - hormones 54 pt, chemotherapy 18 pt, none 3 pt. Median number blood samples/pt for OPN 4 (range 1-15), with ≥2 samples in 63 pt. 8 pt are off study and 29 have died from breast cancer. Progress to date confirms feasibility of obtaining multiple blood samples throughout treatment of pt for metastatic breast cancer. Association between OPN (ELISA) and # sites of disease, response to first systemic treatment (hormone or chemotherapy) and survival will be investigated.

Supported CBCRI grant # 8426

Abstract submitted: 22nd Annual San Antonio Breast Cancer Symposium, December 8-11, 1999.

OSTEOPONTIN (OPN)-INDUCED INCREASE IN HUMAN MAMMARY EPITHELIAL CELL (MEC) INVASIVENESS IS UROKINASE (UPA)-DEPENDENT. Tuck AB*, Hota C, and Chambers AF. London Health Sciences Centre, London Regional Cancer Centre, and University of Western Ontario, London, Ontario, Canada N6A 4G5.

We have recently shown that either exogenous (human recombinant) or endogenous (transfected) OPN induces both uPA expression and increased invasiveness of 21PT (non-tumorigenic) and 21NT (tumorigenic) human MECs (Tuck et al, Oncogene, in press). Here we asked whether uPA contributes functionally to the increased invasiveness of these cells. The most invasive OPN-transfected 21T series cells were assessed for migration through Matrigel in transwell assays in the presence or absence of various different blocking antibodies and uPA inhibitors. Both anti-uPA and anti-uPA receptor (uPAR) antibodies were shown to significantly inhibit cell invasion, as did the uPA inhibitors (plasminogen activator inhibitor-1 [PAI-1] and aprotinin). Both anti-uPA and anti-uPAR antibodies inhibited invasion to a level comparable to that of the untransfected parental cells. In contrast, non-specific IgG showed no anti-invasive effect. These data thus provide direct evidence that the OPN-induced increase in MEC invasiveness requires uPA.

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