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.

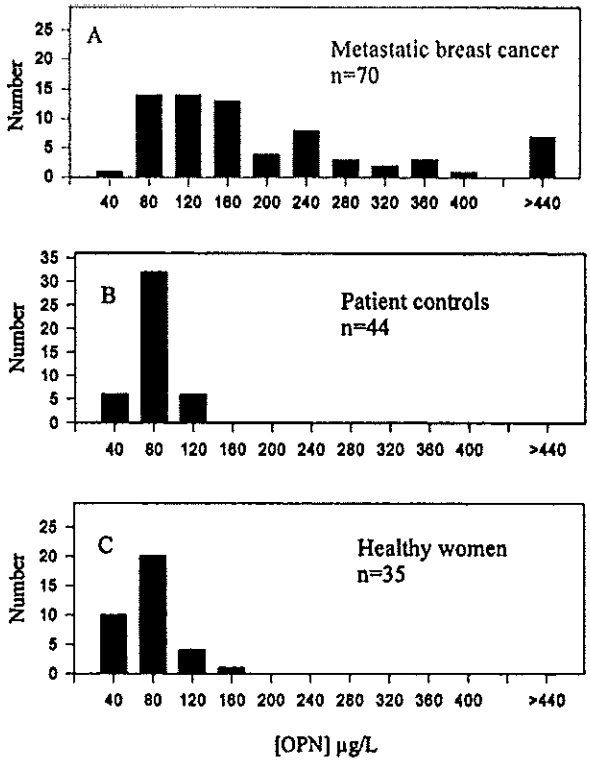

Fig. 1 Frequency distribution of plasma OPN in women diagnosed with metastatic breast cancer, treated for primary breast cancer, and normal controls. The average OPN level was calculated from plasma samples using the immunoassay described in "Materials and Methods" and displayed as a histogram with the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

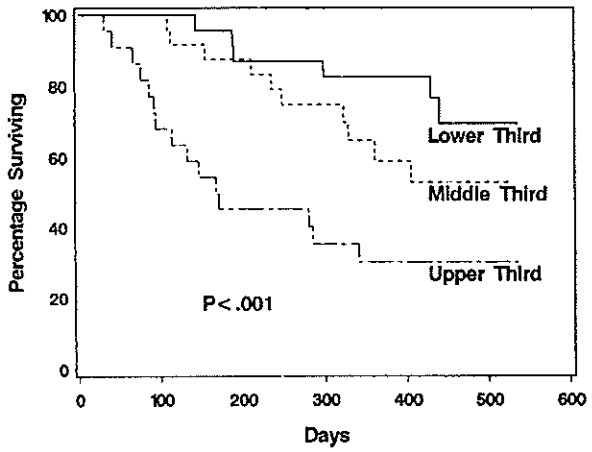

Fig. 2 Survival of patients with metastatic breast cancer grouped into tercile plasma OPN levels. The Kaplan-Meier adjusted percentage surviving is shown as a function of time (days) after OPN plasma levels were determined for patients (n = 69, one lost to follow-up). Patients were grouped into three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

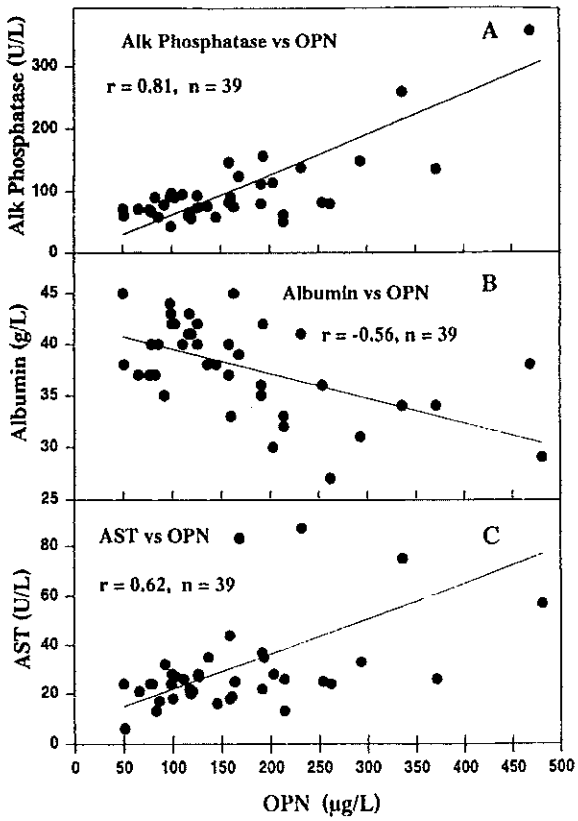

Fig. 3 Correlations between OPN levels and biochemical markers. Correlations between OPN levels and various biochemical markers are shown for alkaline phosphatase (*A*: $r = 0.81$, $P < 3 \times 10^{-10}$, $n = 39$), albumin (*B*: $r = -0.56$, $P < 2 \times 10^{-4}$, $n = 39$), and AST (*C*: $r = 0.62$,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

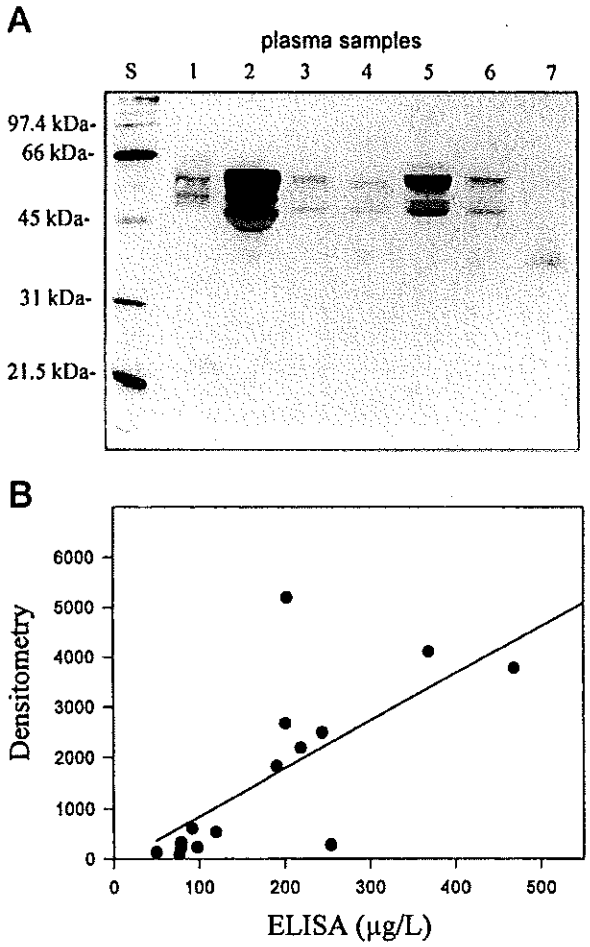

Fig. 4 Western blot analysis of plasma OPN and comparison between ELISA and densitometry for quantification of plasma OPN levels. Plasma OPN was immunoadsorbed to mAb53-conjugated Sepharose, eluted, and fractionated in 12% SDS-PAGE as described in "Materials and Methods." *A*, representative plasma samples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