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13. ABSTRACT (Maximum 200 Approximately, 1/10 women will develop breast cancer and of these, a large number will succumb to their disease. Although there are many reasons for treatment failure, it is partly due to the inability of current clinical methods to detect disease progression. Since the production of several enzymes including the type IV collagenases and heparanase partly contribute to breast cancer invasion and metastases, we hypothesized that their measurement may provide a means of detecting the early onset of disease progression. This would be useful in two settings. First, if metastatic lesions could be detected by measuring the levels of these enzymes prior to the onset of clinical manifestations which mark this phenomenon, it may be possible to aggressively treat these patients. Second, by measuring these enzymes we hope to detect the relapse of patients being treated with chemotherapeutic drugs/anti-estrogens again prior to the onset of clinical manifestations. Such patients could be rapidly switched over to alternate therapeutic strategies with the objective of controlling disease progression. Accordingly, we undertook studies to develop assays to measure type IV collagenases and heparanase and used these assays to measure these enzymes in serial serum samples from breast cancer patients. We report that while an assay for the 92 kDa type IV collagenase proved reproducible and highly sensitive in detecting this enzyme, we found no evidence that the amount of this serum enzyme was of utility in predicting breast cancer progression.				
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FOREWORD

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A. INTRODUCTION

There is now ample evidence implicating proteases in the invasive and metastatic phenotype of a variety of malignancies including breast cancer (Tryggvason *et al.* 1987). These enzymes work by hydrolyzing surrounding basement membranes and extracellular matrix thereby allowing access of the tumor cells to the vasculature and the lymphatics where they can be transported to distant sites.

A number of proteases have been implicated in this process including the 92 and 72 kDa type IV collagenases (MMP-9 and MMP-2 respectively) and endoglycosidases such as heparanases (Moses *et al.* 1998; Nakajima *et al.* 1992; Tryggvason *et al.* 1987). These enzymes target different basement membrane and extracellular matrix components and thus act in concert to facilitate the destruction of the surrounding tissue.

The majority of breast cancer patients die from disease which has spread to distant sites. Accordingly, we hypothesized that the measurement of these enzymes in breast cancer patients may allow for the identification of patients whose disease is progressing. If the rise in one or more of these enzymes preceded the onset of clinical manifestations of disease progression, then this could provide a means for altering the treatment strategies for that patient. As an extension of this argument, we also proposed that an elevation in protease amounts might be predictive of treatment failure when using common drugs employed in breast cancer.

Accordingly, we proposed to generate antibodies to the collagenases and heparanase and to develop assays for measuring these enzymes in breast cancer patients. Then, we would employ these assays to measure enzyme levels in serial plasma collections from breast cancer patients.

BODY

B.1. Develop prognostic tests for breast cancer invasion and metastasis-associated degradative enzymes,

Preparation of immunological reagents.

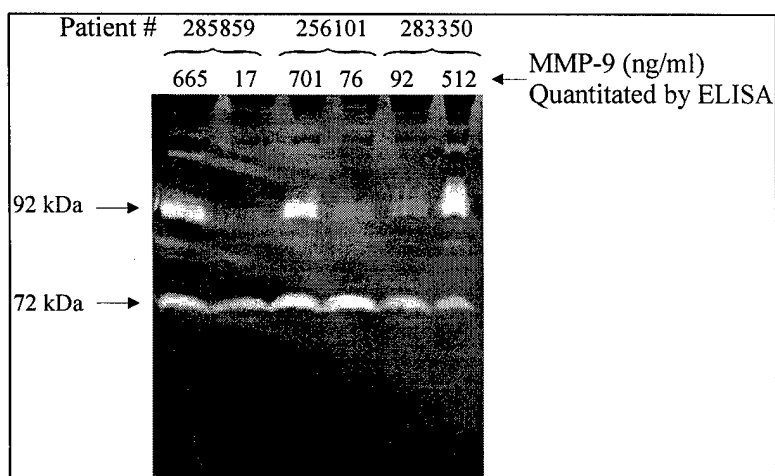
Polyclonal antibodies against purified synthetic peptides corresponding to the type IV collagenolytic (gelatinases) of $M_r \sim 72,000$ (MMP-2) and $\sim 92,000$ (MMP-9) enzymes as well as carbohydrate moieties of heparanases were produced using goats, rabbits and sheep. We synthesized peptides against specific hydrophilic sequences found in metalloproteinases and their cyanogen bromide cleavage products in order to produce highly sensitive and specific antibodies.

B.2. Develop assays to monitor the appearance of metastatic disease in breast cancer patients by detecting the appearance of metastasis-associated degradative enzymes in blood.

We used the polyclonal antibodies to develop quantitative competition ELISA assays to monitor the amounts of the two collagenases in the blood plasma of patients with breast cancer. We found that the anti-peptide reagents reacted well with denatured enzyme but did not react well with native enzyme found in plasma. Subsequently, a sandwich fluorescence immunosorbent assay was examined for its ability to quantitate the 2 collagenases. Although many variations of the capturing or secondary antibody (to the peptide or whole molecule) were tried, none proved workable due to lack of sensitivity or specificity.

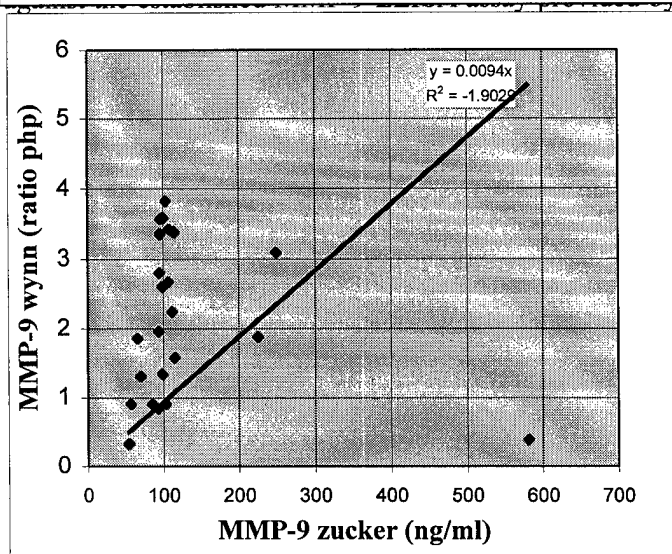
The assay was then modified to a substrate capture fluorescence immunosorbent assay. In this assay, gelatin (denatured collagen) was used as the capture substrate and the bound molecules were denatured. These bound and denatured molecules were recognized by the goat anti-peptide MMP-9 and rabbit anti-peptide MMP-2 antibodies. Subsequently, an alkaline phosphatase-conjugated secondary antibody was added followed by the addition of 4 methyl umbelliferyl phosphate as fluorophore. Fluorescence was finally read using 360 and 460 nm as activation and emission wavelengths respectively. To check the validity of this assay, our assay was authenticated against an established ELISA

Figure 1 Validation of the Calibration Assay



as described elsewhere (Zucker et al. 1993). First, however, the calibration ELISA was checked by zymography to confirm its accuracy in detecting MMP-9. As can be seen from Figure 1, there was a tight relationship between the amount of MMP-9 quantitated by the calibration ELISA and by zymography. Thus, the varying amount of this collagenase in 6 serum samples was accurately detected using the ELISA.

Figure 2 Authentication of Developed MMP-9 Assay



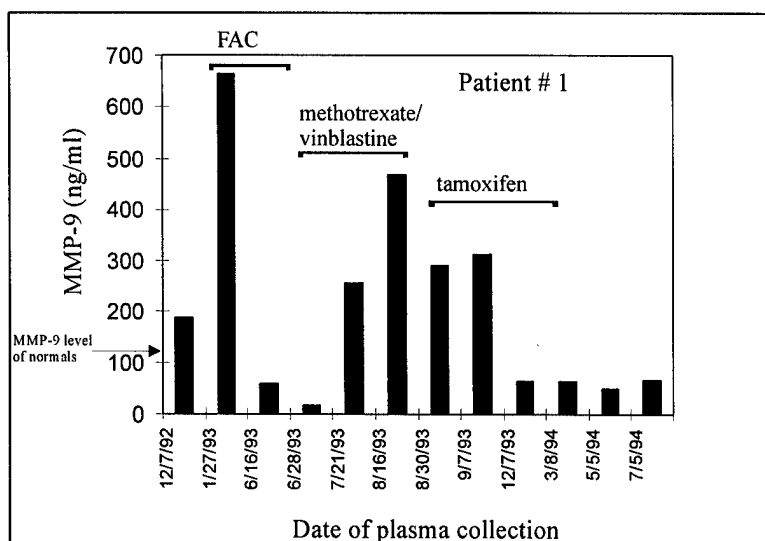
Dr. Stanley Zucker. Towards this end, 25 serum samples were run in parallel in both assays and the data correlated by linear regression (Figure 2). An extremely poor correlation was observed between the MMP-9 values determined by our developed assay and that of the ELISA. A correlation coefficient of 0.0355 was observed between both assays. Thus, we concluded that the fluorescence immunosorbent assay developed in our laboratory was detecting non-MMP-9 molecules in the serum from these breast cancer patients. Accordingly, we elected to employ, as a collaborative effort, the ELISA developed by Dr. Zucker for further quantitation of MMP-9 levels.

To determine if MMP-9 levels was predictive of progression of patients to metastatic lesions, we assayed the serum from breast cancer patients. Patient # 1 (Figure 3) was a 59 year old female diagnosed with Blacks nuclear grade I, lymph node-positive breast cancer in November of

1992. The patient, was treated with combined 5-fluorouracil/ adriamycin / cytoxan (FAC) after surgery and this treatment phase was associated with a decline in MMP-9 levels to below that of the average of normal persons (110 ng/ml determined from 10 healthy individuals from whom at least 3 plasma collections were made). At the end of June 1993,

the patient completed the treatment course and was switched over to combined methotrexate/vinblastine. Interestingly, for the duration of this treatment, plasma MMP-9 levels continued to rise to a maximum concentration of 470 ng/ml. However, after a 2 month treatment period, this patient was deemed asymptomatic based on clinical criteria and had completed the methotrexate/vinblastine treatment. The increase in the amount of this collagenase observed during methotrexate/vinblastine treatment therefore may have been indicative of tumor progression in contrary to clinical findings. At this point the patient was put on tamoxifen and in response to the anti-estrogen, the plasma concentration of MMP-9 diminished over 66 %. However, a rising CEA level and a chest CT scan confirmed tumor progression in June of 1994. Thus, for this patient at least, it does not appear that plasma MMP-9 is indicative of tumor progression. The patient expired in December of that year (1994). Our findings of a lack of correlation between elevated MMP-9 levels and the establishment of metastatic disease were borne out with studies using serum from other breast cancer patients whose disease progressed.

Figure 3 Increased MMP-9 is not evident in Progressing Breast Cancer

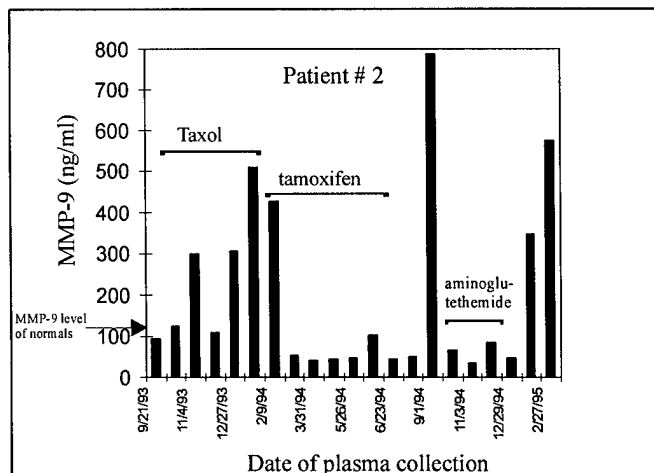


B.3. Develop assays to monitor the treatment of metastatic disease in breast cancer patients by following the levels of metastasis-associated degradative enzymes in blood.

We then determined whether MMP-9 levels could be used to predict the onset of drug-resistance before the manifestation of clinical markers. If this could be achieved, the clinician would have a means of detecting patient re-

lapse in response to a drug or drug combination. Consequently, a series of patients were selected for serum analysis. For example, patient # 2 (Figure 4) was a 57 year old woman diagnosed with invasive ductal breast cancer. A mastectomy was performed in August of 1992 and this indicated a lymph node-positive tumor of 4.3 cm in size. The patient was treated in September of 1993 with taxol. However, for the 4 month duration of treatment, MMP-9 levels steadily rose from a pretreatment level of about 100 ng/ml to a peak amount of 500 ng/ml. At this point, the patient was deemed by

Figure 4 Increased MMP-9 evident in patients relapsing drug therapy



standard clinical criteria (skull lytic lesions) to be progressing and thus switched over to tamoxifen. Like patient # 1, MMP-9 concentrations rapidly decreased to a level comparable to that of normal control patients (100 ng/ml). This level was maintained for the treatment duration although the disease was judged as progressing based on a bone scan. However, cessation of the anti-estrogen was accompanied by a sharp increase in the amount of this collagenase (790 ng/ml) in the plasma. Treatment with aminoglutethimide, which inhibits estrogen production, was associated with a return of MMP-9 levels to that of normal controls. Again, cessation of treatment, due to disease progression, was associated with a rise in the amount of this metalloproteinase. The patient expired in May 1995. These data were initially consistent with the notion that plasma MMP-9 levels are indicative of tumor progression at least in patients failing treatment with these three drugs. However, further analysis of the serum from a series of patients receiving this and other

regimens (e.g. taxol-18 patients; tamoxifen-9 patients) failed to reveal a consistent increase in MMP-9 levels in patients who failed therapy.

CONCLUSIONS

These studies failed to indicate MMP-9 levels as a marker of breast cancer progression. Further, it would not appear that this collagenase is of utility in predicting drug relapse in breast cancer patients. At the same time, we do not rule out the possibility that diurnal variations in MMP-9 levels were a confounding factor in these negative results. Further, since we did not examine MMP-2 or heparanase levels (due to the lack of reagents and time constraints), it remains to be determined whether one, or a combination, of these proteases is/are predictive of breast cancer progression.

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