Regulation of Stromal Versican Expression by Breast Cancer Cells and Importance to Relapse-free Survival in Patients with Node-negative Primary Breast Cancer1

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ABSTRACT

Purpose: Determination of meaningful prognostic indices remains a high priority for women diagnosed with node-negative primary breast cancer. Currently, 30% of these women relapse, and there is no reliable means of predicting this group of patients. This study investigates whether the level of expression of versican, an antical adhesive proteoglycan, in the peritumoral stromal tissue of women with node-negative, primary breast cancer predicts relapse-free survival. This study also examines whether breast cancer cells regulate the secretion of versican by mammary fibroblasts.

Experimental Design: Immunoreactive versican was measured in breast cancer tissue sections of 58 node-negative patients by video image analysis. Primary isolates of mammary fibroblasts were cultured in medium conditioned by the breast cancer cell lines ZR-75-1, MCF-7, BT-20, and MB231. Changes in versican secretion were measured by immunoblotting and enhanced chemiluminescence.

Results: Cox analyses indicated that peritumoral versican level was the only predictor of relapse-free survival. The relapse rate in patients with low versican levels was lower than in patients with high versican levels (Kaplan-Meier: 83% relapse free at 5 years for versican mean integrated absorbance <14 versus 33% for ≥14, P = 0.0006). Accumulation of versican in medium of mammary fibroblasts was increased after culture in conditioned medium from breast cancer cell lines.

Conclusions: Relapse in women with node-negative breast cancer is related to the level of versican deposited in peritumoral stroma by mammary fibroblasts. Versican secretion appears to be regulated by breast cancer cell mediators. Neoplastic remodeling of extracellular matrix through increased versican deposition may facilitate local invasion and metastasis.

INTRODUCTION

Predicting the clinical course of disease in breast cancer patients remains a particularly challenging problem. Through public education and mammographic screening programs, the number of patients who are diagnosed with early stage breast cancer has increased, the reduction in tumor size and number of involved lymph nodes at diagnosis having a favorable impact on patient outcome. However, although tumor size and lymph node involvement are the strongest prognostic indicators available to date, clinicians are not able to discriminate those women with small tumors without detectable axillary metastases who will develop metastatic disease from those who are potentially cured (1–5).

Previous studies from this laboratory have demonstrated that an increase in immunoreactive versican in the peritumoral stromal tissue of early stage prostate cancers is associated with an elevated risk of prostate-specific antigen-assessed tumor relapse (6, 7). Versican, a large CS proteoglycan synthesized by stromal cells, is a recognized antical adhesive molecule involved in regulating cell motility on ECM components (8–10). Recently, we reported that versican secretion by prostatic fibroblasts is regulated by prostate cancer cell mediators, principally TGFβ1 (11). Consequently, an increased level of versican in the peritumoral matrix of the prostate may facilitate local cancer cell invasion and formation of distant metastases by decreasing cell-matrix adhesion sufficient to promote tumor cell migration through the ECM. The focus of this study was to determine whether the level of versican deposition in the peritumoral

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3 The abbreviations used are: CS, chondroitin sulfate; ECM, extracellular matrix; CM, cancer cell-conditioned medium; cRPMI, complete RPMI cell culture medium; DAB, 3,3′-diaminobenzidine; FBS, fetal bovine serum; IOD, integrated absorbance; ITS, insulin, transferrin, and sodium selenite; MIOD, mean integrated absorbance; RFS, relapse-free survival; ABC, avidin-biotin complex method; MTT, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide; TGF, transforming growth factor.
stroma of breast tissue might be a reliable predictor of outcome for women with node-negative breast cancer and whether the production of stromal versican was controlled by breast cancer cell mediators.

**MATERIALS AND METHODS**

**Cohort.** Patients \((n = 65)\) were selected for this study from a cohort of 428 node-negative, primary breast cancer patients, studied previously for several potential prognosticators, *i.e.*, \(p53\) gene abnormalities, amplified HER2/neu oncoprotein expression, estrogen receptor concentration, and the tumor cell proliferation marker MIB-1 \((5, 12)\). The original study was performed on a cohort of patients assembled by 60 surgeons at six institutions, using the resources of the South Australian Breast Cancer Study Group. The current study is limited to the patients supplied to the original study by three surgeons at Flinders Medical Center. Collection of paraffin-blocked tissue from the Department of Anatomical Pathology archives and clinical data from the Flinders Breast Clinic were authorized by the Flinders Medical Center Clinical Investigations Committee. The clinical characteristics and treatment details for the cohort have been described \((12)\). In brief, all patients had an axillary node clearance to determine the extent of tumor involvement, and none of the node-negative patients received adjuvant chemo or hormonal therapy. Five patients were lost to clinical follow-up. The median follow-up for the remaining 60 patients was 61 (range 5–116) months. The median age for the patient subgroup in this study was 62 (range 27–82) years. Of this cohort, 14 of 60 patients relapsed, and 8 died. Tumor grading was performed on 58 tissues using the Elston and Ellis modification of the Bloom and Richardson method \((13)\). Eleven tissues were judged as grade 1, 27 as grade 2, and 20 as grade 3. Tumor size was available for 58 patients, and estrogen receptor concentration was available for 60 patients. Sufficient tissue was available for quantitative versican immunostaining in the paraffin blocks of 58 patients.

**Immunohistochemical Staining of Tissue Sections for Versican.** Tissue sections \((4 \mu m)\) of paraffin-embedded primary breast cancers were dewaxed, and endogenous peroxidase activity was blocked by immersion in hydrogen peroxide \((0.3\% H_2O_2 in PBS)\) for 5 min. All incubation steps, except where stated, were performed at room temperature. To facilitate immunostaining, the CS side chains of versican were subjected to enzymatic cleavage to unmask core protein epitopes using chondroitinase ABC \([\text{Sigma Chemical Co. (St. Louis, MO)}; 0.5\ units/ml in 0.1\% \text{Tris-Acetate buffer (pH 7.8)}, 1\% \text{BSA}, 90\ min]\). After blocking of nonspecific binding sites with 10% normal goat serum, sections were incubated with rabbit antibody to antihuman recombinant versican \((1:1500 in 10\% normal goat serum)\) overnight at 4°C \((14)\). Biotinylated secondary antibody was goat antirabbit IgG \((1:500 in 10\% normal goat serum, 30\ min)\), and antibody binding was visualized by a standard streptavidin immunoperoxidase reaction \([\text{Vector Laboratories, Burlingame, CA)}\) using dianaminobenzidine tetrahydrochloride as the chromagen. A light nuclear counterstain was applied with weak Lillie Mayers’ hematoxylin \((1:10, 10\ s)\). Immunostaining was accomplished in three batches. To reduce staining variation between batches to a minimum, the immunostaining protocol was strictly adhered to, and known positive control sections were included in each run to monitor any variation.

**Image Analysis of Immunohistochemically stained Tissue Sections.** The area and absorbance \((\text{optical density})\) of DAB deposition were measured using an automated image analysis system \([\text{VideoPro 32; Leading Edge P/L, Marion, South Australia)}\) as described previously \((6, 7)\). Color images were collected at a magnification of \(\times 100\). Twenty contiguous fields were captured for each breast cancer sample, beginning adjacent to a randomly chosen cancer focus. Captured fields included both epithelial and stromal tissue areas. Because of the exclusively stromal localization of versican in breast tissue \((Fig. 1; Ref. 15)\), for video image measurement, all cancer cell foci were manually edited from the images. The IOD of DAB in the stroma was determined for each image \((\text{absorbance of DAB deposit integrated over the immunopositive pixel area})\), and the amount of specific antibody staining was expressed as the MIOD per unit area of stromal tissue \((\text{IOD divided by total stromal area)}\) averaged over the 20 images. The level of versican in the tissue was therefore expressed as the MIOD of staining in arbitrary density units per pixel. Staining intensity between runs varied by <10%, as determined by image analysis of identical areas within control tissue sections, and no adjustments to individual values were made in this study.

**Statistical Analysis.** The relative risk of relapse associated with patient subgroups, stratified according to defined tumor features, was determined using Cox’s univariate analysis \([\text{SPSS package}; \text{SPSS, Inc., Chicago, IL)}\). Rates of relapse after diagnosis for different patient subgroups were compared using the Kaplan-Meier product-limit method and Log-rank test. Disease-free survival was calculated from the date of diagnosis to the date of relapse or the date of last follow-up if disease free. Patients who died from causes other than breast cancer were censored on the date of their death. Association of versican levels with tumor grade was analyzed by the Kruskal-Wallis test. Statistical significance was set at \(P < 0.05\).

**Primary Cultures of Stromal Cells from Breast Cancer Tissue.** Primary cultures of stromal cells were isolated from human breast cancer tissues using a technique based on that described by van Roozendaal *et al.* \((16)\) for the isolation of mammary fibroblasts. Fresh tissues were obtained from patients undergoing surgery for primary breast cancer at Flinders Medical Center, with informed consent. The resected specimen was minced in ice until diagnostic pathology cut up commenced, and fragments of malignant and nonmalignant tissue processed aseptically into tissue culture, usually in ≤3 h of surgery. The tissue fragments were rinsed in sterile calcium- and magnesium-free Hank’s medium, and one-third of each specimen was fixed in phosphate-buffered formalin and blocked in paraffin wax for histological analysis. The residual portion of each specimen \((150–200\ mg)\) was minced finely using a razor blade. The minced tissue was digested overnight with rocking motion at 37°C, in 10 ml of 0.1% collagenase type II \([\text{Sigma Chemical Co.}]\), 0.1% hyaluronidase type I \([\text{Sigma Chemical Co.}]\), cRPMI \([\text{Life Technologies, Inc., Grand Island, NY;} \ i.e., \text{RPMI containing 4 mm L-glutamine and antibiotics, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml amphotericin B},\) in the presence of 5% FBS \([\text{Trace Scientific, Melbourne, Victoria, Australia)}\). The resulting cell suspension was centrifuged at
2000 rpm (Hettich, Tuttlingen, Germany), and the pellet resuspended in 5 ml of cRPMI containing 10% FBS. Cell suspensions containing $\sim 8 \times 10^5$ cells were derived per 500 mg of wet weight of breast cancer tissue. The viability of the cells released was generally 60–70%. The cell suspension was divided into two 25-cm$^2$ tissue culture flasks (Nunc, Roskilde, Denmark), coated previously with neat FBS for 1 h and then drained to maximize cell attachment. The cells were cultured at 37°C in cRPMI with 5% FBS, with medium being replaced at 24 h and then every 2–3 days. Confluent monolayer cultures were observed after 10 days. After three to four passages, the stromal cells were cryopreserved in RPMI containing 10% FBS, 10% DMSO over liquid nitrogen.

The stromal cell population was characterized immunocytochemically as 95% fibroblasts, using specific antibodies to pan-cytokeratins (Signet Laboratories, Dedham, MA), desmin (DAKO, Botany, New South Wales, Australia), and vimentin (Biogenex Laboratories, San Ramon, CA). No cytokeratin immunostaining was observed in the primary cell cultures at the second passage, and vimentin staining was observed in 100% of cells, confirming their mesenchymal origin. Staining with the antidesmin antibody at the third passage indicated that the cell cultures contained $\sim 5\%$ smooth muscle cells.

**Collection of Breast CM.** Breast cancer cell lines BT20, MDA-MB231, ZR75-1, and MCF-7 (American Type Culture Collection, Bethesda, MD) were cultured in cRPMI medium containing 5% FBS for 4 days. The medium was then changed to cRPMI containing 0.5% FBS for 24 h and finally to serum-free cRPMI containing 1% ITS (Sigma Chemical Co.) supplement. Serum-free CM was then collected from each cell line at 72 h and frozen at $-70^\circ$C.

**Treatment of Fibroblast Cultures with Breast CM.** Normal and cancer-associated mammary fibroblasts (two cultures of each) were grown to confluence under standard conditions, trypsinized, and replated in 80-cm$^2$ flasks at a density of $1 \times 10^4$ cells/cm$^2$. Fibroblasts were grown for 4 days in cRPMI containing 5% FBS, and the medium was then replaced for 24 h with 10 ml of cRPMI containing 0.5% FBS. The medium was finally changed to either 10 ml of cRPMI containing 1% ITS (control) or 10 ml of filter-sterilized (0.22 μm) CM from the breast cancer cell lines BT20, ZR75-1, MCF-7, and MDA-MB231, diluted 1:1 in cRPMI containing 1% ITS. Fibroblast culture medium was then harvested at 24, 48, and 72 h. To inhibit proteolytic degradation of versican, one protease inhibitor tablet (Boehringer Mannheim, Mannheim, Germany) was added per 50 ml of harvested medium. Samples of medium collected from fibroblast cultures at 24, 48 and 72 h in the presence or absence of cancer cell CM were concentrated 25-fold using Centrisart I centrifuge tubes (Sartorius, Goettingen, Germany) with a molecular weight cutoff of $M_r$ 300,000 at 4°C for 4 h at 2,000 × g. The concentrated samples were stored at $-70^\circ$C.

**Measurement of Versican in Fibroblast Culture Medium by Western Blotting.** To permit electrophoretic migration of versican ($M_r \sim 900,000$) through 5% polyacrylamide gel, the CS side-chains were cleaved enzymatically from the core protein ($M_r \sim 400,000$) using chondroitinase ABC. An aliquot (36 μl) of each concentrated sample was digested with chondroitinase ABC. The CS side-chains were then cleaved enzymatically from the core protein ($M_r \sim 400,000$) using chondroitinase ABC. An aliquot (36 μl) of each concentrated sample was digested with chondroitinase ABC.
droitinase ABC (20 units, 2 μl; Sigma Chemical Co.) in ovalbumin (400 ng, 2 μl; Sigma Chemical Co.) and Tris buffer [250 mM, 10 μl (pH 8.0)] for 3 h at 37°C. Electrophoresis was performed on a gel, which was then stained with Coomassie Blue R250. One milliliter of 5% aqueous MTT (Sigma Chemical Co.) and Tris buffer was added to each well and incubated at 37°C for 4 h. This was followed by the addition of 100 μl of 20% SDS in 0.02 M HCl and the cells left to solubilize overnight in a dark environment at room temperature. The plates were then read in a microplate reader (Bio-Rad 450) using a dual wavelength setting of 570 and 655 nm, and cell numbers were calculated from a standard plot.

**RESULTS**

**Versican Immunolocalizes to Peritumoral Stromal Tissue of Breast Cancer.** Prominent immunostaining for versican was observed in stroma associated with malignant areas of sectioned breast tissue, whereas negligible deposits of versican were identified in stroma surrounding nonmalignant glands (Fig. 1). In well-differentiated invasive ductal carcinomas (grade 1), small neoplastic glands were associated with a stromal matrix that generally showed weak to moderate immunoreactivity for versican (Fig. 1C). The stroma surrounding foci of grade 2 invasive ductal carcinomas generally stained with a moderate to strong intensity (Fig. 1D), whereas grade 3 invasive ductal carcinomas generally demonstrated the most intense staining for versican in the bands of peritumoral stroma (Fig. 1B). Despite this visual trend however, video image measurements indicated that the level of versican immunoreactivity in breast cancers was not significantly associated with tumor grade, Kruskal-Wallis test P = 0.963. 

**Increased Expression of Versican in the Peritumoral Stromal Breast Tissue of Women with Node-negative Breast Cancer Is Associated with Decreased RFS.** Cox regression analyses (Table 1) indicated that elevated expression of peritumoral versican is an important determinant of breast cancer relapse. Stromal versican levels, when examined as a continuous variable, contributed a 13% increase in relative risk for each unit increase in versican concentration. When analyzed as a dichotomous variable, patients with high peritumoral versican concentration were 6-fold more likely to relapse than patients with low versican levels (P = 0.003). Tumor size and grade and cytosolic estrogen receptor concentration were not associated with risk of relapse in this cohort. Kaplan-Meier product-limit curves comparing RFS in patient groups with versican-poor or versican-rich peritumoral stroma supported the Cox regression analyses (Fig. 2). Patients with a low concentration of stromal versican (MIOD < 14) had a significantly reduced incidence of relapse.
relapse compared with patients with high versican concentrations (MIOD ≥ 14; 83 versus 33% relapse free at 5 years, respectively, \( P = 0.0006 \), Log-rank statistic = 11.84). The overall relapse rate for patients with MIOD < 14 was 19 (10 of 52) versus 67% (4 of 6) for MIOD ≥ 14.

**Fibroblastic Synthesis of Versican Is Regulated by Cancer Cell Mediators.** Immunoblot analysis of 25-fold concentrated aliquots of medium, harvested from cancer-derived mammary fibroblasts, indicated that versican levels were increased after culture in conditioned medium from ZR-75-1, BT-20, MCF7, and MB231 breast cancer cells (Fig. 3). Versican levels were between 230 and 350% of the level in control fibroblast cultures at the 48-h time point and between 130 and 230% of control level at the 72-h time point. There was no apparent difference in the basal level of versican synthesis, or the degree of stimulation observed, between the two normal breast tissue-derived and the two cancer tissue-derived mammary fibroblast cultures studied (data not shown). Confirmation that pipetting error was negligible, during quantification of versican in concentrated fibroblast culture medium, was obtained by immunoblotting of the transferrin supplied in the ITS medium (Fig. 3, E and F). In some immunoblots of culture medium from mammary fibroblast culture was loaded onto the gels, blot for BT20 CM shown (E and F). Representative data from one of four experiments are presented in the figure. In toto, two cultures of normal and two of cancer-derived mammary fibroblasts were studied.

**DISCUSSION**

Women diagnosed with stage 1 breast cancer (i.e., carcinoma that has not spread to axillary lymph nodes) have the
lowest risk of relapse and death from their disease. Early surgical treatment affords these women a chance of cure. However, ~30% of women with stage 1 disease will develop a local or distant recurrence, and, unfortunately, there is no reliable means to determine those women at greatest risk of relapse who might benefit from more aggressive treatment. Our recent studies of early stage prostate cancer demonstrated that increased peritumoral expression of versican, an anticell adhesive molecule, is associated with relapse in men treated with radical surgery (6, 7). In addition, secretion of versican by prostate-derived fibroblasts into culture medium is regulated by prostate cancer cell mediators (11). In this study, we investigated whether peritumoral versican expression was predictive of disease outcome for early stage, i.e., node-negative breast cancer in women, and whether secretion of versican by mammary fibroblasts was regulated by breast cancer cell mediators.

Immunostaining of breast cancer tissue sections demonstrated negligible deposition of versican in nonmalignant periglandular stroma, whereas prominent staining for versican was present in peritumoral stroma. In no instances were cancer cells stained for versican, confirming that versican is a product of stromal cells. The visual impression of an increase in intensity of stromal staining with increasing tumor grade suggested that versican deposition may be associated with histological grade or degree of differentiation of breast cancer, as was observed for early stage prostate cancer (6). This trend, however, was not confirmed by statistical analysis of video image measurements.

Cox regression analyses indicated that elevated expression of peritumoral versican, examined as either a continuous or a dichotomized variable, is an important determinant of breast cancer relapse. Kaplan-Meier product-limit curves supported the Cox regression analyses, indicating that patients with tumors containing versican-rich peritumoral stroma experienced shorter relapse-free intervals than patients with versican-poor tumors. Lymph node status, followed by tumor size, grade, and hormone receptor status, are accepted as the strongest determinants of outcome in primary breast cancer. Although size, grade, and receptor status were not associated with increased risk of relapse in this cohort of 60 node-negative patients, they were associated with outcome in the larger cohort from which the test cohort was extracted at random (5, 11). These results suggest that measurement of peritumoral versican staining may be a stronger predictor of relapse for patients with node-negative breast cancer than traditional markers. Consequently, elevated expression of versican in peritumoral stroma appears to be a reliable predictor of early relapse for both breast and prostate cancers.

The peritumoral location of versican demonstrated by us and by Nara et al. (15), and its implied involvement in tumor spread in breast and prostate cancers (6), is indicative of the production of soluble mediators by more aggressive cancer cells, which control fibroblastic cell synthesis and deposition of versican into ECM. In a previous publication (10), we reported that conditioned media from LNCaP, PC3, and DU145 prostate adenocarcinoma cell lines stimulated increased accumulation of the V0 and V1 isoforms of versican in culture medium from prostatic fibroblasts. In this study, we demonstrate that conditioned media from several breast cancer cell lines (ZR-75-1, MCF-7, BT-20, and MB231) stimulated a 2–3-fold increased secretion of versican into culture medium by mammary fibro-
blasts isolated from normal and breast cancer tissues. There appeared to be no difference between estrogen receptor-positive or -negative cell lines. In addition, basal or induced levels of versican secretion by fibroblasts derived from tissue fragments taken adjacent to fragments containing either malignant or non-malignant pathology appeared to be similar. Bands representing the versican isoforms V0 and V1, respectively, were observed by Western immunoblotting of concentrated culture medium from mammary fibroblasts, as with prostatic fibroblasts (10). To date, the V2 and V3 isoforms of versican appear to have been detected predominantly in neural tissues, although a recent report documents the presence of V3 in cultured rat vascular smooth muscle cells (18, 19). Supporting the stromal localization of versican in breast cancer tissue sections, the conditioned medium from breast cancer cells cultured in vitro was unreactive immunologically for versican. The induction of versican secretion by mammary fibroblasts after treatment with breast CM was not related to any increase in cell proliferation of the mammary fibroblasts. This agrees with our study published on prostate fibroblasts stimulated with prostate cancer mediators (11). Those studies suggested that the cancer cell mediator in prostate cancer tissue inducing elevated versican secretion by prostatic fibroblasts might be TGFβ1. Whether TGFβ1 is the active principal for mammary fibroblasts remains to be determined.

In conclusion, it appears likely that breast cancer cells with the most aggressive behavior in vivo secrete a soluble mediator leading to elevated deposition of versican by tissue fibroblasts during neoplastic remodeling of the ECM. The versican- and hyaluronan-containing pericellular sheath has been shown to be a prime determinant of smooth muscle cell locomotion (10), and versican is considered to promote motility by destabilizing focal cell contacts in fibroblasts (9). Consequently, because breast and prostate cancer cells appear to produce little or no versican, mechanisms to increase versican levels in peritumoral stroma may be an important prelude to local invasion and metastasis. Because this study suggests that versican is related to disease relapse in early breast cancer, additional studies on molecular regulators of breast cancer cell attachment and motility, including versican, are warranted.

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