Expression of Syndecan-1 in Paired Samples of Normal and Malignant Breast Tissue from Postmenopausal Women

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Abstract. *Background: The mammary stroma is important for modulating epithelial breast cell response to sex steroid hormones. Proteoglycans, such as syndecan-1, promote the integration of cellular signals. Materials and Methods: The immunohistochemical expression of syndecan-1 and of the androgen receptor (AR) was analyzed in paired samples of cancer and adjacent normal tissue from postmenopausal women. Results: Normal and cancer tissue showed dramatic differences in the expression of syndecan-1. In malignant breast stroma, mean values were more than 10-fold higher than in normal tissue ($p<0.001$). There was also a marked redistribution from the epithelium to the stroma. The expression of AR was on average 2-fold higher in cancerous than in normal tissue ($p<0.01$).Conclusion: Breast cancer patients have very different prognoses. Syndecan-1 and the AR may be new molecular markers relevant to clinical outcome. The redistribution from the epithelium and the dramatic increase of syndecan-1 in cancerous stroma may be related to the natural history of the disease.*

There is accumulating evidence that the mammary stroma has an important role in modulating the epithelial breast cell response to sex steroid hormones (1). Breast stroma accounts for more than 80% of the resting breast volume (2). This supportive platform for the epithelial cells is composed of collagen, fibroblasts, endothelial cells, adipocytes and a macro molecular network of proteoglycans.

Proteoglycans may be regarded as multireceptors which promote the integration of cellular signals (3-4). Syndecan-1 is a cell surface heparan sulphate proteoglycan which participates in cell proliferation, cell migration and cell–matrix interactions (5-7). The biological effects of syndecans are thought to be mediated through the binding of various growth factors. Using a cDNA micro array technique it has been demonstrated that the gene expression of syndecan-1 in the rat uterus is clearly estrogen dependent (8). An increased expression of syndecan-1 has been demonstrated in the stroma of invasive breast cancer (9-11). Syndecan-1 has also been found to promote proliferation of human breast cancer cells *in vitro* (10). Expression of syndecan-1 may be a predictor of a poor prognosis in breast cancer (11).

The regulation of breast cell proliferation in response to sex steroid hormones is complex and incompletely understood. Sex steroid action in target tissues is mediated partially *via* specific nuclear receptors. In addition to the estrogen (ER) and progesterone (PR) receptors the androgen receptor (AR) is a third member of the nuclear receptor super family (12). Information on AR content in normal and malignant breast tissue is limited. Divergent effects concerning androgens and ARs in the regulation of proliferation in breast epithelial cells and breast cancer have been reported from both *in vitro* and *in vivo* studies (13, 14). There are reports that low or absent levels of AR are characteristic of malignant breast tumor tissue (15-16).

It is unclear how the expression of growth factors and receptors is regulated and how it may change between normal and cancerous tissue (17-18). In particular, there is a lack of human data. Most of our knowledge so far comes from cell culture experiments and studies in rodents and other animal models (19). An increase of syndecan-1 and a suppression of AR have been suggested as characteristics of malignant breast tumor tissue. Therefore in the present study the immunohistochemical expression of syndecan-1 and AR in pairwise samples of normal and malignant breast tissue collected during surgery from postmenopausal women with primary breast cancer was analyzed.

Materials and Methods

Breast tissue sampling. Consecutive breast tissue samples were collected from postmenopausal women undergoing surgery for

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invasive primary ductal breast cancer at St Görans hospital, Stockholm Sweden. Data on age, tumor size, Elston grade and nodal status were collected from the medical records. ER content in tumor tissue was quantified by enzyme immunoassay (ER-EIA®, Abbot Laboratories, Abbot Park, IL, USA) (20). Tumors with ER >0.05 fmol/μg DNA were classified as positive. Women were defined as hormone users if they had been on systemic treatment with estrogen only (estradiol or conjugated estrogens) or estrogen in combination with progestogen (levonorgestrel, norethisterone or medroxyprogesterone acetate) until at least one month before diagnosis. For each patient, fresh tissue blocks containing the tumor and also adjacent apparently normal glandular structures at the periphery of lesions were collected and frozen at –70˚C until analysis. In addition to routine pathological analysis, all samples were subject to a blinded central reanalysis by an experienced pathologist to define invasive ductal carcinoma and histologically normal tissue. The study was approved by the local ethics committee of the Karolinska Institutet (98-173) and all the women gave their informed consent to participate.

Immunohistochemistry. A standard immunohistochemical technique (avidin-biotin-peroxidase) was used to visualize syndecan-1 and AR immunostaining distribution. Monoclonal mouse anti-human antibodies were used for detection of syndecan-1 (CD-138, M7228, DakoCytomation, Glostrup, Denmark) and AR (M3562, DakoCytomation).

The tissue sections were dewaxed and rehydrated in descending concentrations of ethanol. Sections were then pretreated in 0.01 M sodium citrate buffer (pH 6.0), in a microwave oven at high power for 10 min and allowed to cool for a further 20 min. Following washing in buffer, 0.1 M phosphate-buffered saline (PBS) pH 7.4, non-specific endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 (Merck Darmstadt, Germany) in methanol for 10 min at 20˚C. Following a 10 min wash in buffer, sections were exposed to a 30 min non-immuno block using normal horse serum (Vector Laboratories Inc, CA, USA) in PBS in a humidified chamber at 20˚C. The tissue sections were then incubated with the primary antibody. The syndecan-1 antibody was diluted 1:50 and the AR antibody 1:100 in PBS and incubated on sections at 4˚C overnight. Negative controls were obtained by replacing the primary antibody with mouse IgG of the equivalent concentration. Following primary antibody binding, the sections were incubated for 30 min at 20˚C with the second antibody, a biotinylated horse anti-mouse IgG antibody (Vector Laboratories Inc) diluted in normal horse serum.

The tissue sections were then incubated for 30 min at 20˚C with horseradish peroxidase-avidin biotin complex (Vectastain Elite, Vector, CA, USA). The site of the bound enzyme was visualized by the application of 3,3'-diaminobenzidine (DAB kit, DakoCytomation), a chromogen which produces a brown, insoluble precipitate when incubated with enzyme. Thereafter the sections were counterstained with haematoxylin and dehydrated before mounting in Pertex (Histolab, Gothenburg, Sweden).

Visual scoring. Visual scoring of syndecan-1 expression in the stroma and the epithelium was performed. Two observers (LL, LSa), blinded to the identity of the slides, performed all the assessments. The syndecan-1 staining was evaluated using a grading system. The staining intensity was graded on a scale of no staining (0), faint staining (1), moderate staining (2) and strong staining (3).

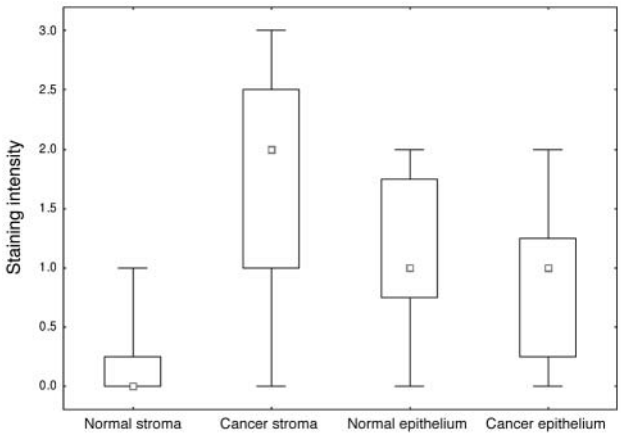

Figure 1. *Semiquantitaive assessment of staining intensity (0-3) for syndecan-1 in paired samples of cancerous and adjacent histologically normal breast stroma (n=26) and epithelium (n=27) collected in postmenopausal women undergoing surgery for ductal breast cancer. Box-and-whisker plots represent the median value with 50% of all data falling within the box. The "whiskers" represent the range.*

Image analysis. A Leica microscope connected to a computer using Colorvision software (Leica Imaging System Ltd. Cambridge, U.K.) was used to assess AR immunostaining quantitatively by a computer image analysis system. Quantification of immunostaining was performed on the digitized images of systematic randomly selected mammary gland samples, from which the stromal elements had been interactively removed. The whole sample, or 10 fields, were measured separately for each tissue section. By using color discrimination software, the total area of positively stained nuclei (brown reaction product) was measured, and expressed as a ratio of the total area of cell nuclei (brown reaction product + blue haematoxylin).

Statistical analysis. Differences between paired normal and malignant tissues were evaluated by the Wilcoxon signed rank test and differences between groups were assessed by the Mann-Whitney *U*-test.

Correlations were calculated using Spearman's rank correlation test. A p-value of <0.05 was considered as significant.

Results

Samples were obtained from 37 postmenopausal women with a mean age (±) SD of 70±10 years and a mean tumor size of 19.3±9.3 mm. Thirty-three (89%) of the tumors were ER-positive and 13 of the patients were node-positive. There were no distant metastases. The distribution according to Elston grade was I: 4 patients; II: 19 patients and III: 14 patients. At the time of diagnosis, 14 women were on menopausal hormone therapy. In some cases pairwise assessment could not be performed due to low amounts of relevant epithelial tissue.

As illustrated in Figure 1 and Table I the immunostaining intensity for syndecan-1 in breast stroma was markedly higher in the cancerous compared to the normal tissue. In pairwise

samples as obtained from 26 women, the mean value (±SD) for syndecan-1 expression in cancerous stroma of 1.78 (±0.97) was more than 10-fold higher than the corresponding value of 0.16 (±0.26) in normal stroma ($p<0.001$). In contrast, in the cancerous epithelium values for syndecan-1 expression (0.90±0.59) were lower than in the normal epithelium (1.14±0.60; n=27, $p=0.05$). In the normal stroma, the staining intensity was very low and syndecan-1 was much more abundant in the epithelium ($p<0.001$). On the other hand, in cancerous tissue there was an apparent redistribution of the proteoglycan, with higher values in the stroma than in the epithelial compartment ($p<0.001$). The marked difference in syndecan-1 staining between normal and malignant tissue in a 60-year-old woman with a strongly ER-positive primary ductal breast cancer of 12 mm is illustrated in Figure 2. No significant differences between women with or without hormonal treatment at the time of diagnosis were found and there were no apparent correlations with age, tumor size, nodal status or Elston grade.

The AR was distinctly expressed in the nuclei of both normal and malignant breast epithelium whereas staining in the stroma was faint or absent (Figure 3). The expression of AR as quantified by image analysis in primary ductal carcinoma and normal tissue is illustrated in Figure 4. Irrespective of hormonal treatment, cancer tissue showed on average almost 2-fold higher values for the percentage of nuclei positive for the androgen receptor than normal tissue in the same individuals ($p<0.01$).

Discussion

In the present limited series of pairwise samples of normal and malignant breast tissue, a remarkable difference in stromal staining for syndecan-1 was evident. The values for the expression of this cell surface heparan sulphate proteoglycan in tumor tissue were on average more than ten-fold higher than in histologically normal stroma. In previous studies on the normal breast, syndecan-1 was found to be highly expressed at the surface of epithelial cells whereas levels in the stroma were very low (6, 21). Our data are in agreement with the results from two small series of paired samples of tumors and normal tissue. These immunohistochemical findings of an overexpression of syndecan-1 in tumor tissue were supported by additional mRNA analysis (5, 22).

No significant correlations between syndecan-1 and clinical tumor characteristics were found in the present study. Such a correlation has, however, been described previously and during long-term follow-up of a larger clinical group in which syndecan-1 was associated with a poor prognosis (11). Previously we have shown estradiol to induce syndecan-1 gene expression in the uterus of oophorectomized rats (8). However in the present clinical material of women with breast cancer there was no apparent influence of estrogen treatment on protein expression of syndecan-1. Irrespective of hormonal treatment at the time of diagnosis, the same redistribution of syndecan-1 from epithelium to stroma was observed in malignant tissue.

A reactive stromal response seems to be a characteristic of infiltrating carcinomas. There is evidence that stromal fibroblasts promote tumor development and growth. syndecan-1 is believed to act as a co-receptor for growth factors and extracellular matrix interactions. Previously an increase of syndecan-1 expression has been demonstrated in reactive stromal cells (10). Alterations in syndecan expression may have a dramatic effect on breast epithelial cells. Accumulation of syndecan-1 within the stroma has been suggested to enhance angiogenesis and stimulate epithelial proliferation (9).

The fact that sex steroid hormones and their receptors act in concert has stimulated interest in the role of the androgen receptor in women with breast cancer. Expression of AR has been reported in 35-75% of breast carcinomas. The values seem to correlate with the estrogen receptor values (23). In addition there is a strong correlation with the expression of progesterone receptors. It is noteworthy that breast cancer patients with AR-positive tumors may be more responsive to endocrine treatment and have a better survival than those with AR-negative tumors (23).

As for AR, there are reports that a loss of ERβ expression may relate to tumor progression (15, 24). Previously we reported ERβ to be significantly reduced in breast carcinomas compared to normal tissue. Moreover, there was a decrease in the ERβ/ERα ratio (17).

In the present work using a monoclonal antibody, the AR was readily detected by immunostaining and was found in all tumor and normal tissue samples except for one. However, the values displayed wide variation and cut-off limits for AR-positive and -negative samples were not defined (Figure 3). Rather than a decline, as previously reported, the mean values for the percentage of AR-positive cells were about 2-fold higher in malignant than in normal tissue ($p<0.005$).

It is well established that sex steroid hormones are important for normal breast development as well as in tumor progression and growth. Estrogen has received most attention but progesterone and androgens are also involved. However, the relative importance of the two latter hormones is still controversial (13, 25, 26).

The expression of ER and PR in breast tissue is influenced by hormonal therapy. Thus, there is a suppression of ERα and a stimulation of PR expression during estrogen/progestogen, as well as tamoxifen treatment (17, 27, 28). However no differences in AR expression between women using or not using hormones at the time of diagnosis could be observed in the present study.

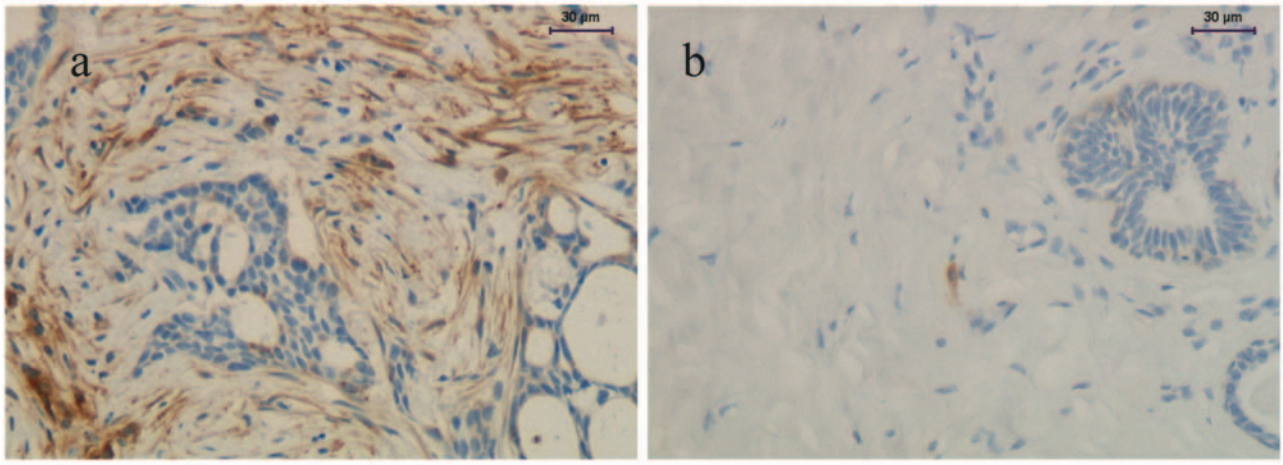

Figure 2. *Immunostaining for syndecan-1 in ductal breast carcinoma (a) and adjacent histologically normal breast tissue (b) from a 60-year-old woman.*

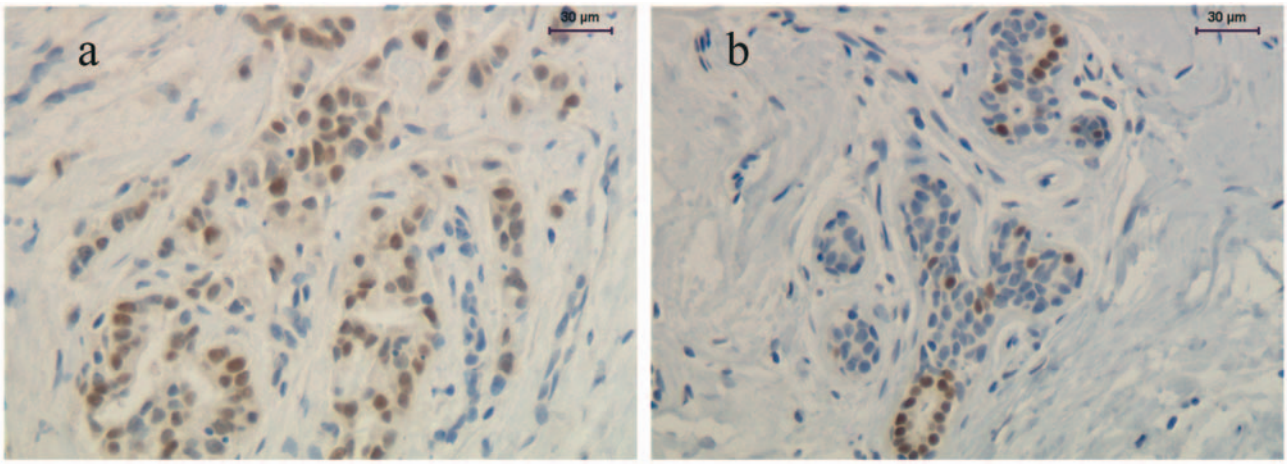

Figure 3. *Immunostaining for the androgen receptor in ductal breast carcinoma (a) and adjacent histologically normal breast tissue (b) from a 63-year-old woman.*

Data from *in vitro* studies suggest that androgens may have two different effects on mammary tumor cells. Firstly, in the absence of estrogen and after aromatase conversion they elicit an ER-mediated stimulation. This effect can be blocked by treatment with antiestrogen. Secondly, in the presence of estrogen, androgens will act as antiestrogens and inhibit the estrogenic stimulation of growth. This effect is exerted through the AR and can be blocked by antiandrogens (26, 29).

Women affected by breast cancer may have very different clinical outcomes and efforts should be made to define characteristics and identify markers to predict prognosis and response to therapy in the individual patient. Syndecan-1 may be such a new molecular marker. The redistribution from the epithelium and the dramatic increase of this proteoglycan in cancerous stroma may also be related to the

Table I. *Semiquantitative assessement of staining intensity (0-3) for syndecan-1 in cancerous and histologically normal breast tissue collected from postmenopausal women undergoing surgery for ductal breast cancer.*

	Cancer (n=34)	Normal (n=29)	Cancer *vs.* normal
Epithelium			
mean±SD	0.90±0.54	1.12±0.58	$p=0.05$
range	0-2	0-2	
Stroma			
mean±SD	1.73±0.94	0.15±0.25	$p<0.001$
range	0-3	0-1	
Epithelium *vs.* stroma	$p<0.001$	$p<0.001$	

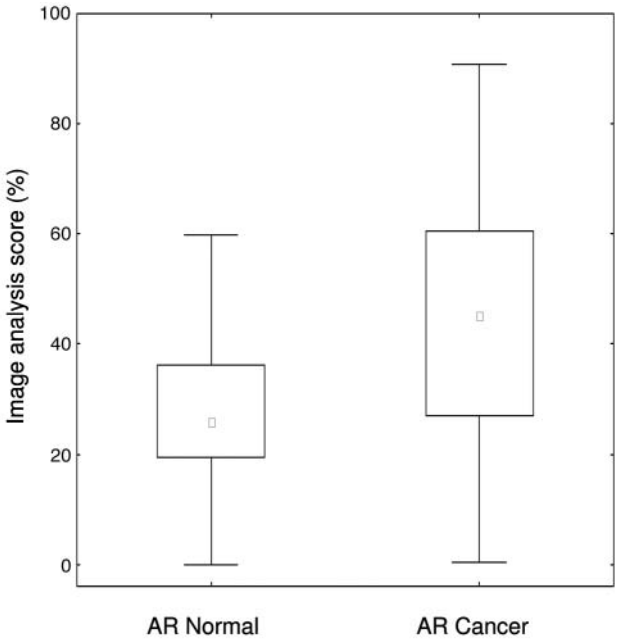

Figure 4. *Image analysis score for the expression of the androgen receptor (AR) (n=27). Percentage area of cell nuclei positive for the AR in pairwise samples of normal breast tissue and ductal breast carcinoma obtained from postmenopausal women. Box-and-whisker plots represent the median value with 50% of all data falling within the box. The "whiskers" represent the range.*

natural history of the disease. There is emerging evidence that the hormonal regulation of breast cell proliferation is a paracrine event mediated by hormone-sensitive stromal cells (30, 31). Improved knowledge of the function and role of the AR has a potential to provide additional therapeutic modalities for breast cancer.

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