Myofibroblastic stromal reaction in breast carcinoma correlates with lymph node status and may result from the transformation of fibrocytes by the TGF-β1/TGF-β R1 pathway

Xavier Catteau¹,², Philippe Simon²,³, Jean-Christophe Noël²,⁴

¹ Institute of Pathology and Genetics, Gosselies, Belgium

² Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium

³ Gynecology Unit, Erasme University Hospital-Université libre de Bruxelles, Brussels, Belgium

⁴ Gynecopathology Unit, Pathology Department, Erasme University Hospital-Université Libre de Bruxelles, Brussels, Belgium

Corresponding author:

Dr. Xavier Catteau,

Department of Pathology, Institute of Pathology and Genetics

25, Avenue Georges Lemaître, 6041 Gosselies, Belgium

Phone: +32 71473047; Fax: +71471520; Email: xavier.catteau06@gmail.com
ABSTRACT

Introduction: The microenvironment modulates tissue specificity in the normal breast and in breast cancer. The stromal loss of CD34 expression and acquisition of smooth muscle actin myofibroblastic features may constitute a prerequisite for tumor invasiveness in breast carcinoma. The aim of the present study is to examine the stromal expression of CD34 and SMA in cases of invasive ductal carcinoma and to try to understand the underlying mechanism responsible for this particular myofibroblastic reaction, particularly via the TGF-ß pathway.

Methods: We carried out an immunohistochemical study of CD34, SMA, TGF-ß and TGF-ß R1 on a series of 155 patients with invasive ductal carcinoma. We also treated a breast fibrocytes cell line with TGF-ß1.

Results: We found a loss of stromal expression of CD34 with the appearance of a myofibroblastic reaction in almost 100% cases of invasive ductal carcinoma. The strong stromal expression of SMA correlates with the presence of lymph node metastases. We were also able to show a greater expression of TGF-ß in the tumor cells as well as a higher expression of TGF-ß R1 in the tumor stroma compared to normal breast tissue cells. Finally, we demonstrated the transformation of breast fibrocytes into SMA positive myofibroblasts after being treated with TGF-ß1.

Conclusions: Our study demonstrated that a significant tumor myofibroblastic reaction is correlated with the presence of lymph node metastases and that this myofibroblastic reaction can be induced by TGF-ß1. Future research on fibrocytes, myofibroblasts, TGF-ß and stromal changes mechanisms is essential in the future and may potentially lead to new treatment approaches.
Keywords: Breast carcinoma, Tumor microenvironment, Fibrocytes, Myofibroblasts, SMA, CD34, TGF-β, Metastasis.
Introduction

Breast cancer is the most common cancer in women in the world [1]. The microenvironment modulates normal breast tissue, as well as the growth, survival, polarity, and invasive behavior of breast cancer cells [2, 3]. The stromal loss of CD34 expression and acquisition of smooth muscle actin (SMA) myofibroblastic features may constitute a prerequisite for tumor invasiveness in breast carcinoma [4, 5]. The origin of myofibroblasts is not yet clear and multiple hypotheses have been proposed. Myofibroblasts modulate the stroma in physiology and pathology through direct cell-to-cell contact and through secretion of different proteinases, extracellular matrix (ECM) components, growth factors and cytokines. In cancer, myofibroblasts may induce the production of proinvasive proteinases [6]. We already carried out a study on the stromal expression of CD34 and SMA in ductal carcinoma in situ (DCIS) [7]. The present study aims to investigate this phenomenon in cases of invasive ductal carcinoma (IDC) and to try to understand the underlying mechanism responsible for this myofibroblastic reaction, especially the role played by Transforming Growth-Beta (TGF-ß).

Materials and Methods

Study population

Breast tissue from cancer patients and normal controls (reduction mammoplasty) was collected from consecutive patients who were identified through the Pathology and Genetics Institute (IPG) resulting in 165 consecutive patients diagnosed between January 2010 and December 2012. Tissue was collected from 155 patients with breast cancer and from 10 patients who underwent reduction mammoplasty. All patients were female. 83 resection specimens and 82 biopsies were obtained. Informed consent meeting all federal, state and
institutional guidelines was obtained from all subjects. Final pathological tumor stage was
determined using the TNM staging system (AJCC Cancer Staging Manual, 7th edition, 2007)
and graded using the Nottingham system [8]. In addition, age, tumor size, tumor shape,
estrogen receptor (ER), progesterone receptor (PR), HER2/Neu status and KI-67 index were
assessed in each tumor. Table 1 summarizes the clinical characteristics of the breast cancer
patient population.

Cell line cultures.

Human mammary fibrocytes P10893 was purchased from Innoprot® and maintained in
Innoprot-recommended media and conditions. Media was changed every two days. When
cells reached confluence they were passaged to a 25 cm² flask (Corning® Plasticware Cell
Culture, Corning, NY, USA) by treating with 0.25% trypsin-25 mM EDTA (Gibco®
Invitrogen Corporation) and agitating until cells began to detach from the surface of the flask
(passage 1; p1). P2 cells were moved to a 75 cm² flask and then passed 1:4. All
experiments were performed on fibrocytes that had been cultured for 3–10 passages.

Cells were phenotypically characterized by immunostaining. Cells positive for vimentin and
negative for cytokeratin staining were considered fibroblasts. Cells were plated in six well
chamber slides (Corning®), and grown to confluence. Cells were washed with PBS and fixed
with 4% buffered formalin and immunostained according to manufacturer’s protocols

Assessment of fibrocytes activation into myofibroblasts.

Cells were plated and grown to confluence in six-chamber slides in BM. Media was aspirated
from the cultures and cells were washed twice with PBS and then incubated for 24 h in serum
free media with 0 or 2.5 ng/ml TGF- β1 (Peprotech®) for 48h with a change in the culture
medium after 24h. After 48 h, cells were fixed, and incubated with SMA and CD34 antibodies.
The percentage of myofibroblasts was assessed by counting at least 1,000 total cells and determining the proportion stained positively for SMA in three fields at 200X in duplicate preparations.

**Immunohistochemistry**

The specimens were fixed in histology-grade 4% buffered formalin. Paraffin sections were stained with hematoxylin and eosin and immunohistochemical detection was performed according to the manufacturer’s protocols (Table 2). We used a fully automated immunohistochemical system (Autostainer Link 48 from Dako®).

**Semi-quantitative assessment of Immunohistochemistry**

We analyzed the stromal distribution of CD34 and SMA in the tumor. Immunohistochemical expression of TGF-β and TGF-β R1 was evaluated in normal breast tissue (glands and stroma) in tumor stroma and tumor cells. Ten cases of interesting breast reduction from normal breast tissue were selected to compare the expression of the two antibodies between the tumor and normal breast tissue. The immunoreactivity of CD34, SMA, TGF-β and TGF-β R1 was assessed semi-quantitatively. The percentage of stromal cells expressing CD34 and SMA was graded as ‘‘0’’, ‘‘+’’, ‘‘++’’, ‘‘+++’’, ‘‘++++’’ when up to 5%, more than 5% and up to 25%, more than 25% and up to 50%, more than 50% and up to 75% or more than 75% of stromal cells, disclosed immunoreactivity, respectively. Percentages were assessed by two independent observers, assuming that a high-power microscopic field (objective x40, microscopic magnification: x400) harbored 100 stromal cells (range: 75–150). We also evaluated the presence or absence of expression of TGF-β and TGF-β R1 in glands and stroma of normal and tumor tissue. Staining intensity for the TGF-β et TGF-β R1 antibodies was assessed in a semiquantitative manner by two of us using the H scoring system as described by McCarty et al. [9]. Briefly, scores are generated by adding together 3×%
strongly staining, 2×% moderately staining, and 1×% weakly staining, giving a possible range of 0 to 300. An H-score >50 was considered as positive. An assessment of total percentage of cells showing positive staining was also carried out. When disagreements occurred between the two observers they were resolved using a double-headed microscope.

Statistical analysis

The relationship between the staining patterns of SMA and different clinical and histological features - age, tumor size, shape of tumor (stellate or nodular), grade of invasive carcinoma, lymph node status, luminal classification, and KI-67 index - was compared using a Chi-squared test. A Student’s t-test was used to compare H-score and percentage positivity. A p-value <0.05 was considered statistically significant. All analyses were performed using Statistica®.

Results

Table 1 summarizes the clinical and histological features of the study population.

Morphologically, most of the time a fibrous stroma, hyaline, of sometimes eosinophilic appearance around tumor cells is highlighted. Stromal cells are fusiform and have spindle-shaped nuclei. Stromal cells do not show nuclear-cytoplasmic atypia and only have very rare mitoses. 65.2% (101/155) of tumors had a stellar pattern, 21.3% (33/155) of tumors a nodular pattern and 14.2% (22/155) of tumors showed a mixed pattern. When the cells are organized in nodular pattern, the stroma between the cells is less visible but is nevertheless present.

Stromal CD34 and SMA in vivo expression.

In all cases investigated (100%), the stroma of invasive carcinomas showed a complete loss of CD34 fibrocytes except around the vessels, while the surrounding mammary tumor-free tissue, when seen in histological sections, disclosed a normal distribution of this cell population (Fig.
1A and 1B). 97.4% (151/155) of invasive ductal carcinomas revealed SMA myofibroblasts forming accumulations to a varying extent. 78.7% (122/155) showed a significant to very significant myofibroblastic reaction (+++, ++++) while 18.7% (29/155) showed low to moderate expression (+, ++). The cancer-associated stromal cells were SMA positive, vimentin positive and cytokeratin negative, confirming their identity as myofibroblasts. Myofibroblasts are found intimately surrounding tumoral cells (Fig. 1C to 1E). We correlated the level of stromal expression of SMA to the following clinical and histological parameters: age, tumor size, shape of tumor, grade of invasive carcinoma, luminal classification, KI-67 index and nodal status. Only the presence of lymph node metastasis is correlated with stromal expression of SMA. Indeed, strong stromal SMA expression is correlated significantly with the presence of lymph node metastasis (Table 3).

*TGF-β and TGF-β R1 in vivo expression.*

We showed expression of TGF-β in normal breast ducts (Fig. 2A). This expression of TGF-β was higher in tumor cells compared to normal ducts \( (p = 0.02) \). On the other hand, we found no expression of TGF-β in normal and tumor stroma. We showed expression of TGF-β R1 in normal ducts and tumor cells with no statistically significant difference \( (p = 0.4) \) (Figure 2B and 2F). On the other hand, the myofibroblastic stroma of the tumor expressed more TGF-β R1 than the normal stroma \( (p = 0.001) \) (Figure 2E and 2F). This expression of TGF-β -R1 in the tumor stroma was present regardless of the level of expression of TGF-β by the tumor (Table 4).

*In vitro transformation of fibrocytes into myofibroblasts by TGF-β1 in mammary cell line fibrocytes.*

The proportion of myofibroblasts in each culture treated with 0 or 2.5 ng/ml TGF- β1 was determined by counting the number of cells immunostained for SMA expression. The
percentage of myofibroblast varied from 0 to 70% among the normal and cultures with TGF-β1 treatment causing a noticeable shift in the percentage of activated myofibroblasts in many normal and treated cultures (Fig. 3). TGF-β1 treatment significantly increased the mean percentage of myofibroblasts in cultures (p < 0.05). It should be noted that the fibrocytes of this cell line do not express CD34.

Discussion

The importance of changes in the microenvironment during tumor progression has been increasingly recognized [3, 10, 11]. We have just demonstrated in this work the appearance of a myofibroblastic reaction accompanied by a loss of fibrocytes in invasive ductal carcinoma. This reaction is present in almost 100% of cases, irrespective of clinical and histological parameters (age, tumor size, shape of tumor, grade of invasive carcinoma, luminal classification, KI-67 index). This phenomenon is therefore almost constant and more than likely plays an important tumoral role, particularly in the invasion process. Furthermore and importantly, this pro-invasive action seems to be confirmed by the fact that an intense expression of SMA myofibroblasts was correlated with the presence of lymph node metastasis. To our knowledge, this is the first time a statistically significant difference between the expression of SMA and nodal status has been demonstrated across all organs.

Our in vitro experiments showed a transformation of fibrocytes into myofibroblasts by TGF-β1, which is one of the main agents involved in this fibro-myofibroblastic transformation. Indeed, different studies have shown that TGF-β upregulates SMA expression in fibrocytes and transdifferentiates them into myofibroblasts [12, 13]. This in vitro study also showed, for the first time, that fibrocytes not expressing CD34 are also capable of transforming into...
myofibroblasts under the action of TGF-β1. Indeed, in a previous study, we thought that only periductal fibrocytes expressing CD34 were able to transform into SMA myofibroblasts [7]. In our opinion, the loss of fibrocytes and myofibroblast activation does not appear to be just a passive reaction. We believe they are an integral part of the process by facilitating tumor progression and tumor invasion. Besides their role in wound healing, myofibroblasts provide pro-invasive signals that in combination affect invasion of the cancer cells [14, 15]. The cross-talk between cancer cells and stromal cells may be mediated through direct heterotypic cell-to-cell contact or through secreted molecules, comprising growth factors, cytokines, chemokines, extracellular matrix proteins, proteinases, proteinase inhibitors, and lipid products [16]. The mechanism leading to the loss of fibrocytes and the appearance of SMA myofibroblasts in the stroma of invasive carcinomas is complex and far from being understood. Breast cancer cells have been shown to be capable of factor secretion [17]. Therefore, we speculate that loss of CD34 fibrocytes and gain of SMA myofibroblasts might be initiated by a soluble factor secreted by tumor cells and especially TGF-β. We have shown that medium conditioned with TGF-β1 induces SMA expression in mammary fibrocytes stromal cells lines. Moreover, some research has already found that fibrocytes acquire SMA expression when exposed to transforming growth factor-β [12, 18]. Considering the results of the present study, it appears to be more likely that mammary fibrocytes acquire SMA after having been treated by TGF-β1. Indeed, our in vivo study of the immunohistochemical expression of TGF-β and TGF-β R1 allowed us to show that:

1) Tumor cells secrete TGF-β and normal fibrocytes have TGF-β receptors. As demonstrated in vitro, tumor cells are therefore able to transform fibrocytes into SMA myofibroblasts.

2) Tumor cells may have an autocrine effect on their growth because they have TGF-β1 and express TFG-β.
3) Since the stroma does not express TGF-β, it therefore seems unlikely that the stroma may trigger the process of tumorigenesis via the TGF-β pathway in any case.

We believe that several mechanisms may explain the promotion of tumor invasion in breast tissue induced by the loss of CD34 fibrocytes and the gain of SMA myofibroblasts.

What are the mechanisms involved in the pro-invasive capacity of fibrocytes?

1) CD34 fibrocytes are potent antigen-presenting cells and might be involved in specific immune surveillance [19, 20].

2) CD34 fibrocytes are involved in the remodeling of stromal tissue damage not only through tissue contractility via TGF-beta, collagen I and III synthesis and SMA, but also in terms of migration factors within the injured tissue via CCR7, CXCR4, SLC, and CXCL12.

3) CD34 fibrocytes also play a role in angiogenesis via bFGF, VEGF, PDGF-a, IL-8, and MMP-9.

What are the mechanisms involved in the pro-invasive capacity of myofibroblasts?

1) The increase in myofibroblasts in breast cancer could result from transdifferentiation of resident interstitial expressing or not of CD34 fibrocytes into myofibroblasts or from the recruitment of CD34 fibrocytes originating from myeloid precursors in the bone marrow. They were considered as a source of myofibroblasts in cancer of the colon [21], pancreas, [22], and breast [4]. Another intriguing possibility is that epithelial cells are precursors of myofibroblasts also called the epithelial-mesenchymal transition [23].

2) Orimo et al. [15] demonstrated that carcinoma-associated fibroblasts (CAF), represented to a large degree by myofibroblasts, promote tumor growth and increase tumor angiogenesis by secretion of stromal derived factor (SDF)-1/CXCL12, which acts in a paracrine fashion to
increase tumor cell proliferation via CXCR4. Hepatocyte growth factor (HGF) is another CAF-derived factor that has been implicated in promoting tumor progression and metastasis. The paracrine activation of c-Met on tumor cells by HGF increases invasion of experimental DCIS lesions in xenografts, for example [24]. Interestingly, co-culture of normal mammary fibroblasts with breast cancer cells can ‘educate’ the fibroblasts to secrete HGF and increase their tumor-promoting activities [25].

3) Myofibroblasts have been observed in cancer of the colon [26], the breast [27], the liver [28], the lung [29], the prostate [30], and the pancreas [31]. The causal role of myofibroblasts in the transition from the non-invasive towards the invasive phenotype is suggested by the finding that the appearance of myofibroblasts precedes the invasive stage of cancer. This hypothesis seems to be confirmed in one of our previous studies in which we demonstrated the appearance of myofibroblasts around the lesions of ductal carcinoma in situ. This expression was more intense around the high-grade lesions (pre-invasive lesions) [7].

4) Associated myofibroblasts prevent physical contact between cancer cells and immune cells, an essential phenomenon for cancer cell destruction. Histology of different types of tumors indicates that, in those tumors in which the myofibroblastic network is poorly developed, inflammatory cells infiltrate the tumors and are in close contact with the cancer cells. In contrast, the presence of myofibroblasts around progressive tumors is associated with the absence of immune and inflammatory cells within tumors [32].

5) In contrast to wound healing, myofibroblasts in the tumor microenvironment do not disappear by apoptosis, indicating that cancer is a wound that does not heal [33].

6) The stromal reaction induced by carcinomatous lesions leads to acquisition of SMA expression and in turn to stabilization of the lesion (wound contraction) that helps prevent the spread of tissue damage [34]. This may reflect a defense mechanism against “stromal
invasion” that induces a phenomenon of stromal healing and stabilization. However, the phenotypic transformation or suppression of (CD34) fibrocytes into SMA myofibroblasts could also cause the loss of most essential functions (including immunity, cell adhesion, motility, stromal remodeling, and angiogenesis inhibition), and in a paradoxical manner promote tumorigenesis, thus facilitating invasion and metastatic dissemination of tumor cells.

**Conclusions**

It is in any case difficult to prove the origin of myofibroblasts but what seems important is that the tumor cells more than likely need the microenvironment to continue to live and grow. Furthermore, our study showed that the microenvironment may facilitate metastasis. This makes these myofibroblasts an extremely interesting therapeutic target. In this context, an attractive target in anti-stroma therapy is most notably TGF-β1. Therefore, future research on both fibrocytes, myofibroblasts, TGF-β and stromal changes mechanisms is essential and may potentially lead to new treatment approaches.
Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

Xavier Catteau, Philippe Simon, Jean-Christophe Noël conceived the study and participated in its design. Xavier Catteau and Jean-Christophe Noël provided formalin-fixed, paraffin-embedded archived patient materials for the study. Isabelle Fayt and Benedicte Culot performed immunostaining. Xavier Catteau and Jean-Christophe Noël conducted pathologic reviews and clinical data evaluations. Xavier Catteau performed statistical analyses. Xavier Catteau and Cécile Dupond cultured cell lines. Xavier Catteau and Jean-Christophe Noël conducted in vivo experiments. Xavier Catteau and Cécile Dupond conducted in vitro experiments. Xavier Catteau and Jean-Christophe Noël drafted the manuscript. All authors read, edited and approved the final manuscript.

Acknowledgments

We thank Isabelle Fayt, Benedicte Culot, Cécile Dupond for their help with processing histological specimens and cell-lines cultures. This study was supported by IRSPG (Institut de Recherche Scientifique de Pathologie et de Génétique).
References


17. Liang X, Huuskonen J, Hajivandi M, Manzanedo R, Predki P, Amshey JR, Pope RM:


Figure legends

Figure 1: Stromal CD34 and SMA expression in invasive ductal carcinoma


Figure 2: TGF-ß and TGF-ß R1 expression in normal breast tissue and invasive ductal carcinoma


Figure 3: CD34 and SMA expression in breast fibrocytes cell line before and after treatment by TGF-ß1

Additional files provided with this submission:

Additional file 1: Table 1.docx, 27K
http://breast-cancer-research.com/imedia/6463809171168653/supp1.docx
Additional file 2: Table 2.docx, 28K
http://breast-cancer-research.com/imedia/174339698116865/supp2.docx
Additional file 3: Table 3.docx, 35K
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