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Molecular characterization of the tumor microenvironment in breast cancer

Min Hu and Kornelia Polyak

Department of Medical Oncology, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts, USA

Abstract

The tumor microenvironment plays important roles in cancer initiation, growth, progression, invasion, and metastasis yet the molecular basis underlying these tumor-promoting effects are not fully delineated. Recent advances in gene expression, genetic, and epigenetic profiling of stromal cells have improved our understanding of how mesenchymal-epithelial cell interactions may create a permissive microenvironment for malignancy and identified potential targets for cancer prevention and treatment including chemokine and cytokine networks. However, translating these findings into clinical practice may be difficult due to the complexity and redundancy of the interactions and the inherent ability of tumor epithelial cells to evolve and thrive in diverse environmental conditions.

Keywords

tumor microenvironment; epithelial-stromal cell interaction; paracrine signaling

Introduction

Tumor epithelial cells are surrounded by the microenvironment composed of extracellular matrix (ECM) and various non-transformed cells residing in the organ in which the tumor grows (e.g., myoepithelial and endothelial cells, fibroblasts, myofibroblasts, and leukocytes). Large amount of data suggest that the tumor microenvironment can modify the proliferation, survival, polarity, differentiation, invasive and metastatic capacity of cancer cells (1-4). However, the molecular mechanisms underlying these effects are poorly understood. This is mainly due to the fact that most genetic or gene expression profiles were performed using tumor epithelial cells or bulk tissue samples representing a mixture of multiple cell types. The purification and comprehensive characterization of each cell type comprising normal and cancerous tissue are likely to improve the understanding of the role these cells play in tumorigenesis and identify new molecular targets for cancer prevention and treatment. Several recent studies have begun to address the molecular mechanisms by which the tumor microenvironment may contribute to cancer initiation, progression, and metastasis. In this

Address correspondence to: Kornelia Polyak, Department of Medical Oncology, Dana-Farber Cancer Institute, 44 Binney Street, D740C, Boston, Massachusetts 02115, USA. Phone: (617) 632-2106; Fax: (617) 582-8490; E-mail: kornelia_polyak@dfci.harvard.edu.

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review, we focus on the phenotypic and molecular alterations in the breast tumor microenvironment, the dynamic interactions between the microenvironment and tumor epithelial cells, and the importance of paracrine signaling in the regulation of the proliferative, invasive, angiogenic, and metastatic behavior of cancer cells.

Changes in the microenvironment during tumor progression

Pathologists have long noticed that the tissue microenvironment dramatically changes during tumor formation, as evident by the increased number of fibroblast and myofibroblasts, lymphocytic infiltration, angiogenesis, and ECM remodeling adjacent to cancer cells. Numerous studies have analyzed the expression of selected candidate genes in primary human tumor tissue samples and found upregulation of invasion and angiogenesis related genes (e.g., MMPs and TIMPs), and growth factors in tumor-associated fibroblasts, endothelial cells, and myofibroblasts.

The first comprehensive gene expression portrait of all major cell types composing normal and neoplastic breast tissue came from the studies by Allinen *et al.* (5). The authors utilized cell-type specific cell surface markers and magnetic beads to sequentially enrich for distinct cell populations (epithelial, myoepithelial, and endothelial cells, myofibroblasts, fibroblasts, and leukocytes) from normal human breast tissue, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinomas (IDC), followed by comprehensive gene expression profiling using SAGE (Serial Analysis of Gene Expression). Dramatic gene expression changes were detected in all cell types during cancer progression, with the most pronounced differences at the normal-to-DCIS transition, implicating that stromal alterations play a role not only in progression to invasion and metastasis, but also in the early stages of malignancy. The findings of Allinen *et al.* were confirmed by an independent study comparing the gene expression profiles of stromal fibroblasts derived from invasive breast cancer and benign breast disorders using cDNA microarray analysis (6).

In light of the dramatic alterations of gene expression patterns in all cell types during tumor progression and prior reports describing somatic genetic alterations in stromal fibroblasts of breast tumors (7-9), Allinen *et al.* also performed aCGH (array comparative genomic hybridization) and SNP (Single Nucleotide Polymorphism) array analyses to assess clonally selected genomic alterations. Using these approaches clonally selected genetic aberrances such as amplifications, and homozygous and heterozygous deletions (LOH-loss of heterozygosity) were only found in tumor epithelial cells and not in any of the non-transformed stromal cells (5). Correlating with these findings, recent results indicate that somatic copy number alterations detectable by 500K SNP arrays are exceedingly rare in breast and ovarian carcinoma-associated fibroblasts (Qiu *et al.*, unpublished). The studies that reported somatic genetic alterations, including gene copy number changes, microsatellite instability (MSI), and point mutations in tumor suppressor genes and oncogenes, in breast tumor stroma (7-13), utilized PCR analyses of small amounts of DNA isolated from LCM (laser dissection capture microscopy) dissected FFPE (formalin-fixed paraffin embedded) tissue samples. Thus, their findings most likely are due to technical issues associated with such approach (14).

It is well-documented that the phenotypic alterations of cancer-associated stromal cells are maintained for a long time even in the absence of malignant epithelial cells (e.g., in tissue culture). Other than genetic abnormalities, epigenetic changes including DNA methylation and chromatin modification can be alternative mechanisms underlying the relative stability of these abnormal stromal cell phenotypes. Prior DNA methylation studies have focused on tumor epithelial cells and only three genes were analyzed for methylation in the stroma of colorectal carcinomas (15-17). To investigate the possibility that DNA methylation plays a role in regulating the phenotypic abnormalities present in the tumor microenvironment, Hu *et al.*

developed a novel genome-wide unbiased sequence-based DNA methylation profiling method, MSDK (Methylation-Specific Digital Karyotyping), and determined the comprehensive DNA methylation profiles of these cells (18). DNA methylation changes were detected in epithelial and myoepithelial cells, and fibroblasts isolated from DCIS and invasive tumors, compared to their normal counterparts. Furthermore, quantitative RT-PCR analysis of selected genes indicated that DNA methylation was consistently associated with changes in mRNA expression levels, but the effect of methylation was positive or negative depending on the location of the modified CpGs relative to the transcription start sites. Therefore, epigenetic modifications are at least in part responsible for the phenotypic alterations observed in tumor stromal cells. Studies in HER2+ breast cancer (19) and prostate tumors (20) also demonstrated differential methylation status of selected genes in tumor epithelial as well as in surrounding stromal cells. Overall, tumor stromal cells are phenotypically and epigenetically abnormal, while the presence of clonally selected somatic genetic changes needs further investigation.

Dynamic reciprocal regulation between the microenvironment and tumor epithelial cells

Numerous studies have described that the cross-talk between the stroma and epithelium is bidirectional. As a consequence of these interactions, changes in gene expression patterns (21) and in the activity of various enzymes (e.g., serine hydrolases and metabolic enzymes) (22) were detected both in tumor epithelial and in neighboring host stromal cells during tumor growth and metastasis in xenograft models of MDA-MB-435 derivatives and MDA-MB-231 cells. Some of these interactions can be reproduced in cell culture. For example, co-culturing of pancreatic cancer cells with fibroblasts lead to the upregulation of COX2 expression in both cell types, and increased the invasive capacity of tumor cells (23). Downregulation of Cox2 activity in tumor epithelial cells using shRNA or COX2 inhibitors abrogated the growth and invasion promoting effects of the fibroblasts (23). Cox2 has been implicated to play a role in the initiating steps of breast tumorigenesis, regulation of epithelial cell immortalization and proliferation, and epithelial-stromal cell communications (24-27). Human epidemiologic data also demonstrated that users of NSAIDs have decreased risk of breast cancer (28). Thus, despite the recent cardiovascular complications associated with specific Cox2 inhibitors, the prostaglandin pathway remains a promising target that could potentially be exploited for cancer prevention and treatment.

The importance of the dynamic reciprocal communication between tumor epithelial and stromal cells in tumorigenesis is clearly demonstrated by studies of Moses and colleagues. The authors generated mice with conditional deletion of the TGF-beta type II receptor gene in fibroblasts (Tgfr2fspKO) (29-31). Loss of TGF- β signaling in stromal fibroblasts induced malignant tumors of the prostate and forestomach (29). Co-transplantation of Tgfr2fspKO fibroblasts with mammary carcinoma cells into the mammary fat pad of wild-type mice promoted tumor growth, invasion and metastasis (30,31). Tgfr2(fspKO) fibroblasts displayed increased secretion of TGF- α , MSP (macrophage-stimulating protein), and HGF (hepatocyte growth factor) compared to wild-type cells, which resulted in increased phosphorylation of receptors erbB1, erbB2, RON, and c-Met, and downstream mediators Stat3 and p42/44 MAPK in epithelial cells. Inhibition of TGF- α and HGF/Met signaling using enzyme inhibitors, neutralizing antibodies, or siRNA, blocked tumor progression and metastasis suggesting that these pathways play a key role in the tumor promoting effects of the Tgfr2(fspKO) fibroblasts (30,31). These studies demonstrated a significant role for stromal TGF- β signaling in mammary tumorigenesis via paracrine regulation of multiple signaling pathways in tumor epithelial cells. The same group also generated mice with selective deletion of the TGF-beta type II receptor in mammary epithelial cells (32). Loss of TGF- β signaling in mammary epithelial cells alone resulted in minimal phenotypic changes. However, overexpression of oncogenes or

inactivation of tumor suppressor genes in mutant mice resulted in accelerated invasive and metastatic carcinoma development. Specifically, deletion of the TGF-beta type II receptor in PyVmT driven mammary carcinomas resulted in decreased tumor latency and increased pulmonary metastases (32,33). To dissect the potential mechanisms underlying these effects, the authors analyzed the histology of the resulting tumors and found increased recruitment of myeloid immune suppressor cells (MISCs) to the invading edge of the tumors due to increased secretion of CXCL12 and CXCL5 chemokines by the Tgfr2 null mammary epithelial cells (33). The infiltrating MISCs have high expression of TGF- β and multiple MMPs that enhanced tumor growth and metastasis (34). Thus, using TGF- β signaling as an example, these studies demonstrated the bidirectionality of stromal-epithelial interactions and the impact of this on tumor initiation, progression, and metastasis.

The importance of paracrine interactions

Stromal and epithelial cells may interact with each other through direct cell-cell contact or via paracrine signaling. Both epithelial and stromal cells secrete various ECM proteins, chemokines, cytokines, growth factors, proteases, and protease inhibitors that act as signal transducers (Figure 1). Tumor cells recruit endothelial cells by expressing angiogenic factors including VEGF (vascular endothelial growth factor) to build up neovasculature (35). Co-injection of lethally irradiated fibroblasts or fibroblast-conditioned medium with tumor epithelial cells had similar tumor-promoting effects as that of viable fibroblasts cells supporting a role for soluble factors released by fibroblasts (36,37).

The importance of autocrine/paracrine signaling in tumorigenesis was also indicated by the observation that a large fraction of genes abnormally expressed in breast tumor epithelial and stromal cells encode for secreted proteins and cell surface receptors (5). Among others, the CXCL12 and CXCL14 (CXC motif chemokine ligand 12 and 14) chemokines, overexpressed by myofibroblasts of invasive breast tumors and DCIS-associated myoepithelial cells, respectively, increased the proliferative, migratory, invasive, angiogenic, and metastatic capacity of tumor epithelial cells (5,38,39). On the other hand, inhibition of CXCR4, one of the signaling receptors for CXCL12, effectively inhibited both primary tumor growth and metastasis (40,41). Similarly, in a lung adenocarcinoma (A549 cells) xenograft model, interactions between cancer cells and the host was illustrated to regulate the expression of genes involved in extracellular interaction and growth factor signaling in both cell types (42). VCAN (versican, also CSPG2-chondroitin sulfate proteoglycan 2), which was selectively induced *in vivo* and overexpressed in human lung adenocarcinoma, was essential for tumor growth *in vivo* but not cell proliferation *in vitro*.

Several recent studies have provided examples how paracrine signals emitted by stromal cells may play important roles in promoting breast cancer metastasis. Karnoub *et al.* demonstrated that mixing bone marrow-derived mesenchymal stem cells (MSCs) with weakly metastatic variant of human MDA-MB-231 breast cancer cells at subcutaneous sites in a xenograft model, dramatically enhanced the frequency of lung metastases (43). The interaction of MSCs with breast cancer cells stimulated the secretion of CCL5 by MSCs which then bound to its receptor, CCR5 present on the breast cancer cells and increased their motility, invasion, and metastatic spread. Neutralizing antibodies against CCL5 were able to block the MSC-induced lung metastases (43), demonstrating a critical role for CCL5-CCR5 signaling interaction in this process. The metastasis promoting effect of MSCs did not induce permanent changes in the tumor epithelial cells, since re-injection of the tumor cells from metastases did not produce metastatic tumors. Thus, this study provides an example for how the tumor microenvironment may promote metastasis by reversibly altering the phenotype of neoplastic cells. Massagué and colleagues have also been utilizing the MDA-MB-231 breast cancer cell line as a xenograft model of metastasis to investigate molecular mechanisms underlying organ preference for

metastatic colonization and growth (44,45). They isolated and characterized variants of MDA-MB-231 cells that preferentially colonized and grew in a particular organ such as bone or lung. Gene expression profiling of these cells identified distinct groups of secreted and membrane proteins that were associated with organ preference for metastasis, suggesting the involvement of chemokines/cytokines and their receptors in this process. Another study by Kaplan *et al.* proposed that VEGFR1+ bone marrow-derived cells (BMDCs) form pre-metastatic niches in distant organs that enhance the homing and growth of cancer cells at these sites (46). They found that primary tumor cells or their conditioned medium upregulated fibronectin expression at sites of future distant metastases, which facilitated the homing of integrin $\alpha_4\beta_1$ positive BMDCs to these sites prior to the arrival of tumor epithelial cells. These BMDCs formed cell clusters and created a permissive local microenvironment for circulating tumor cells. Blocking VEGFR1 signaling or removing VEGFR1+ cells from the bone marrow abolished the formation of these pre-metastatic clusters and prevented metastasis (46). Conditioned media collected from tumor cells with distinct organ preference altered the pattern of fibronectin expression and BMDC cluster formation and changed the sites of distant metastases (46). Thus, paracrine signaling between tumor epithelial cells and the host/organ microenvironment significantly affects tumor cell homing and growth during tumor metastatic process.

Potential clinical relevance of microenvironmental changes

Most of the studies analyzing the functional relevance of microenvironmental changes in tumorigenesis have been performed in mice and other model systems. However, numerous observations obtained in human patients also support the hypothesis that the host microenvironment plays an important role in tumorigenesis. For example, polymorphism in various genes encoding ECM proteins influences the risk of breast cancer metastasis (47). Disparities in cancer incidence and mortality in ethnic groups may also be in part explained by genetic polymorphisms in genes regulating the microenvironment. Based on the observation by Dvorak that “tumors are wounds that never heal” (48), an interesting hypothesis is that differences in wound healing responses among individuals may influence cancer risk or progression. African-Americans are especially prone to keloid formation potentially indicating over-reactive stromal responses to tissue injury (49). In light of the above-described observations on the role of TGF β and chemokine signaling in mammary carcinogenesis in animal models, it is particularly interesting that keloids show overactivation of TGF β and other growth factor signaling pathways (50,51). In addition, an “activated stroma” gene expression signature in breast tumors was shown to correlate with risk of distant metastasis and poor clinical outcome (52). Thus, the potential contribution of genetic differences in genes encoding proteins involved in epithelial-stromal cell interactions to breast cancer risk and mortality would be interesting to investigate.

Conclusions

It is now well-established that molecular and phenotypic alterations in cells composing the tumor microenvironment contribute to tumor initiation, progression, and metastasis. Although the initiating event triggering these changes is still poorly understood, targeting abnormal tumor promoting paracrine signals between tumor epithelial and stromal cells may be a feasible approach for cancer prevention and treatment.

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Figure 1. Examples of paracrine interactions between stromal and tumor epithelial cells
Chemokines-CXCL; MMPs-matrix metalloproteinases; HGF-hepatocyte growth factor; TGF- β ; VEGF, vascular endothelial growth factor. Interactions between tumor epithelial and stromal cells promote metastatic spread from the primary tumor. At the same time, bone-marrow derived cells promote growth at sites of distant metastases by preparing a "premetastatic niche". also