Involvement of extracellular matrix constituents in breast cancer

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It has recently been established that the extracellular matrix is required for normal functional differentiation of mammary epithelia not only in culture, but also in vivo. The mechanisms by which extracellular matrix affects differentiation, as well as the nature of extracellular matrix constituents which have major impacts on mammary gland function, have only now begun to be dissected. The intricate variety of extracellular matrix-mediated events and the remarkable degree of plasticity of extracellular matrix structure and composition at virtually all times during ontogeny, make such studies difficult. Similarly, during carcinogenesis, the extracellular matrix undergoes gross alterations, the consequences of which are not yet precisely understood. Nevertheless, an increasing amount of data suggests that the extracellular matrix and extracellular matrix-receptors might participate in the control of most, if not all, of the successive stages of breast tumors, from appearance to progression and metastasis.

Key words

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ABOVE AND BEYOND providing the physico-mechanical and geometric scaffolding of a tissue, the extracellular matrix (ECM) encodes a large variety of specific signals which directly influence growth, migration and differentiation of cells. In recent years it has become clear that ECM components are composed of structural modules whose amino acid and sugar moieties encode information which is interpreted by the cell through interaction with specific plasma membrane receptors.[1] For example, the multimeric laminin molecule comprises several domains harboring different oligopeptide motives responsible for such diverse effects as cell adhesion, cell proliferation, cell migration and neurite outgrowth.[2] In some cases, one motif fulfills more than one function; in other cases, more than one motif fulfill the same function. Consistent with such multifunctionality, there is, for each ECM molecule, a battery of receptors, most of which belong to the integrin family.[3] In addition, different ECM components can compete for the same receptor potentiating the number of possible ECM–ECM receptor interactions.[3] The precise mode of ECM action remains elusive, but available evidence suggests that ECM influences cellular behavior by both changes of the three-dimensional organization of the cytoskeleton and activation of second messenger and protein kinase pathways.[4-6] It is in fact conceivable that some of the known oncogenes and tumor suppressor genes interfere with intracellular pathways conveying ECM signals.[7-9]

When normal mammary epithelial cells or cell lines from rodents or humans are placed in culture and maintained in the absence of ECM, they spread, proliferate and grow as a poorly polarized monolayer. By contrast, when they are cultured on top, or inside type I collagen gels or basement membrane gels derived from Engelbreth-Holm-Swarm (EHS) tumors, they form organized colonies with sharply delineated boundaries, either in the form of spheres, or as branching tubes.[9-12] These alveolar- and duct-like structures exhibit features of typical polarized epithelia, such as basolateral distribution of cadherins and integrins, apical expression of sialomucin and deposition of an endogenous basement membrane.[9,13] Synthesis and vectorial secretion of milk proteins so far has been achieved only with rodent cells. Consistent with the general model in which tumor cells escape the normal growth and differentiation control, breast tumor cell lines and cells cultured from biopsy samples exhibit aberrant responses to exposure to ECM. They grow continuously in the absence of epithelial polarity, and fail to deposit basement membranes.[7,9,14] In addition, several cell lines from breast tumors degrade and invade ECM gels, mimicking metastatic behavior in culture.[15,16]

The objective of this review is to draw attention to the profound changes in ECM composition and function associated with tissue remodeling during carcinogenesis of the breast. We provide evidence that the ECM constitutes a structure impinging on most, if not all, master switches associated with cancer progression from a benign lesion to an aggressive, invasive and thus life-threatening phenotype.

Expression of ECM constituents in normal and malignant breast tissues

The structural and molecular composition of ECM changes during preadult development, estrous cycles and pregnancy, lactation and involution. ECM remodeling occurs in the basement membrane, which separates basal and luminal epithelial cells from the surrounding mesenchyme, as well as in the interstitial stroma which is made up of fibroblastic cells and adipocytes. The

major changes in ECM composition which occur during carcinogenesis in the breast are summarized in Table 1.

Common basement membrane components

Laminin, entactin, type IV collagen and heparin sulfate proteoglycan (perlecan) are found in all basement membranes.[17] Thus, in the mammary gland, these molecules are synthesized and deposited when active growth, characterized by elongation and dichotomous branching of mammary ducts, takes place during development.[18] The most conspicuous alterations in basement membrane structure occur during involution when ECM degrading proteases break down the basement membrane with accompanying apoptosis and loss of milk secretory function.[19-21] However, even in the resting mammary gland, laminin, type IV collagen and heparan sulfate proteoglycan display estrous cycle-dependent regulation.[22] It has been noted that entactin is most abundantly expressed in the gland from lactating animals compared to mammary tissue from virgin animals.[23] In the latter case, entactin is associated with interstitial ECM, but not with the basement membrane.[23]

A dramatic loss of basement membrane occurs only during involution and in invasive carcinomas of the breast, and it has been shown that mammary carcinomas exhibit a pronounced down-regulation of laminin expression.[24-26] Similarly, at least some invasive breast carcinoma cells lose their expression of type IV collagen.[27] However, non-basement membrane type IV collagen is increased in elastotic tumor tissues of the breast.[28]

Other collagens

The major mesenchymal collagen, type I collagen, is present in the mammary gland at all times but is increased in the stroma of neoplastic mammary tissue, as is type III collagen.[29] Interestingly, type I-trimer collagen otherwise only found in preadult breast tissues, is re-expressed in ductal infiltrating carcinomas.[30] Type V collagen appears to be mainly associated with basement membranes from glands of lactating animals,[18] although it can be detected in the human mammary glands before pregnancy where its expression is subject to regulation during menstrual cycles.[22] In cases of ductal infiltrating carcinomas, this molecule is overexpressed.[31] Localization of type VII collagen is restricted to the basement membrane of mammary epithelia.[32] Extensive type VII collagen expression is also detected around some breast carcinomas *in situ*.[32] Recently, a new form of collagen, designated OF/LB collagen (onco-fetal, laminin-binding collagen), has been identified which is predominantly expressed in embryonic and malignant mammary breast tissues.[33]

Other ECM glycoproteins

Fibronectin is mainly a component of the mammary mesenchymal compartment where it can be detected during all times of embryonic development and in the postnatal breast. In basement membranes, it is most prominent during lactation.[18,34,35] Isoforms containing the ectodomain D of fibronectin and oncofetal fibronectin are expressed in the normal gland during prenatal, but not postnatal normal development.[36] In tumor tissues of the breast, fibronectin accumulates in

the stroma as a result of desmoplasia,[29,35,37] as does vitronectin.[37] Remarkably, fibronectin synthesis in tumors is characterized by re-expression of ectodomain D of fibronectin and oncofetal fibronectin.[36] However, at the invasive edge of mammary carcinomas, fibronectin is lost in a majority of cases.[34] Elastin, which is the main component of elastotic tissue, is commonly associated with breast tumors, but basically absent in the normal breast.[38]

Thrombospondin is expressed in the dense mesenchyme surrounding budding mammary epithelia during fetal development.[39] Later in development, it is located in the basement membrane at the epithelialstromal junction of mammary ducts.[39,40] During lactation, however, thrombospondin disappears from the basement membrane and is instead expressed at the apical surface of secretory mammary epithelia.[39] Strong thrombospondin immunoreactivity is found in basement membranes surrounding in-situ carcinomas of the breast and in the desmoplastic stroma of invasive ductal carcinomas.[40,41] Intriguingly, thrombospondin is virtually absent from most ductal invasive carcinomas but synthesized by a majority of lobular carcinomas. In the later case, its expression appears to be related to the secretory activity found in these cells.[40,41]

Tenascin, like thrombospondin, is detectable in the early embryonic mammary gland anlage in the dense mesenchyme. Later, tenascin levels decrease in the mammary gland as the animal develops from juvenile to adult.[25,42] During involution the amount of detectable tenascin mRNA and protein increases again.[43] Tenascin is re-expressed and concentrated in the mesenchyme surrounding mammary carcinomas.[42] Furthermore, it appears that the amount of tenascin expression is correlated with the stage of malignant progression of a tumor.[25,26,36] The strongest tenascin immunoreactivity is seen at the invasive edge of mammary carcinomas.[36]

Glycosaminoglycans

In the developing pre-adult mammary gland, sulfated glycosaminoglycans, i.e. chondroitin sulfate, dermatan sulfate and heparan sulfate, are localized to the basement membrane of subtending ducts, whereas the end buds are rich in hyaluronan.[44] Chondroitin sulfate is prevalent in mammary tissues of virgin and pregnant animals and dermatan sulfate in tissues of lactating animals.[45] It has been found that the composition and quantity of glycosaminoglycans is changed in breast neoplasia as well. Thus, the level of chondroitin sulfate and hyaluronan increase significantly with the onset of cancer, whereas the amount of dermatan and heparan sulfates decrease.[46-48]

ECM and tumor development

On the brink of progressing from an in-situ to a metastatic phenotype, tumor cells acquire some properties of unicellular organisms.[49] They break up their tight connections with neighboring cells, become motile, and subsequently force their path through surrounding obstacles such as basement membranes and dense interstitial mesenchyme. This process, which is associated with changes in the expression of cytoskeletal proteins, cell adhesion molecules of the cadherin family, matrix-degrading proteases and integrins involves a crosstalk between cancer cells and tumor stroma.[50,51] Transplantation experiments with mammary tumor tissues have shown that tumor growth takes place preferentially at orthotopic, rather than ectopic, sites.[52,53] Interestingly, in these types of experiments, the coinjection of tumor cells with EHS matrix dramatically

increases the incidence of tumor growth and metastatic behavior,[52] which might be due to angiogenesis induced by EHS.[54] A similar tumorpromoting role of ECM is suggested by the reexpression of embryonic traits, such as oncofetal fibronectin, type I-triple collagen, OF/LB collagen and tenascin (see above), which are hallmarks of the stromal fibrotic response to tumor cells. Conceptually, reexpression of embryonic features should facilitate proliferation and migration of cells and thus promote tumor growth and metastasis. Despite the clear effects of EHS matrix on tumor and metastasis formation *in vivo*, and the coincidental reappearance of embryonic forms of ECM constituents, the role of ECM components deposited in the tumor environment *in situ* remains elusive. Most recently, it has been shown that stromelysin-1 overexpressing mice[55] exhibit a dramatic incidence of mammary tumors,[56] suggesting that a proper ECM has a protective, rather than a tumor promoting function, at least for inception of tumors. Similarly, high fibronectin and elastin expression have been correlated with favorable prognosis and reduced incidence of metastasis in breast cancer patients. As further outlined below and summarized in Figure 1, expression of ECM molecules in tumor tissues conforms with both alternatives, and thus tumor growth and progression can be viewed as the result of the imbalance between tumor suppressing and tumor promoting microenvironmental cues.

Proliferation

Escape from cell cycle and unrestrained proliferation is one crucial sign of malignant transformation *in vivo* Once again, the molecular mechanisms that prevent normal cells from proliferation and which are overcome by tumor cells are poorly understood. Although it is likely that autocrine, paracrine and systemic growth factors and cytokines are the major determinators of growth, ECM molecules can also be influential.

The responsiveness of a mammary carcinoma cell line to bFGF and PDGF was dependent on the ECM substrate used for culturing the cells, with higher proliferative activity obtained on fibronectin than on type I collagen.[59] Furthermore, ECM molecules bind growth factors and thereby limit their diffusion.[60,61] While some growth factors are still active when bound to ECM, others are not, and have to be released by proteolysis of their matrix ligand in order to become activated. Thus, the matrix environment determines where, whether and with what kinetics growth factors can act on cells. In addition, the ECM influences the synthesis of growth factors and might thus lead to accumulation of a specific growth factor set.[60] For example, it has been shown that TGF-β synthesis is down-regulated in mammary epithelial cells when they are exposed to EHS matrix or allowed to make their own basement membrane.[62]

When mammary epithelial cells are plated in a malleable matrix gel, they remain rounded and stop proliferating; on a substrate permissive for cell spreading, they continue to grow.[9] Similarly, type V collagen, a non-permissive substrate for cell spreading, inhibits proliferation of a mammary cancer cell line.[30] Thus, the effect of ECM on cell adhesion and shape might result in cytoskeletal/nuclear skeletal configurations which do not allow proliferation.[5,63] On the other hand, other ECM molecules encode signals which can modulate proliferation in a growth factor-like manner in the absence of gross changes in cellular morphology. [64-67] It is tempting to speculate that when the basement membrane is broken down by the proteolysis in tumors, the released fragments may feed back on the behavior of the cells both locally and through circulation. In the stromelysin-1 transgenic mice discussed above, the animals develop both

mammary tumors and lymphomas (see also Sympson *et al*, this issue).[56] Since it is known that particular motifs of ECM molecules can be cryptic, and that the conformation of ECM molecules is important for the interaction with their ligands,[68-70] basement membrane and interstitial matrix are reservoirs for hidden, potentially mitogenic or otherwise effective sites. Thus degradation of ECM in these areas might expose a variety of new stimuli and increase the concentration of already existing ones. Since the major component of basement membrane is laminin, and laminin contains motifs of potential mitogenic activity[66,67] stimulation of tumor growth in the wake of basement membrane degradation appears plausible, although there is yet no experimental evidence for this hypothesis.

Angiogenesis

Growth of new blood vessels in the vicinity of the tumor tissue is absolutely required for the tumor to grow and to metastasize.[71] Besides the numerous growth factors and cytokines implicated in angiogenesis, ECM molecules have direct effects on the formation of new blood vessels.[72] Not surprisingly, ECM components can facilitate angiogenesis as well as inhibit it. Chondroitin sulfate and hyaluronan increased tube formation of endothelial cells embedded in type I collagen gels.[73] Fibronectin has been reported to both promote angiogenesis[74] and to inhibit it.[75] Thrombospondin, commonly regarded as antiangiogenic factor,[76] has recently been shown to stimulate angiogenesis indirectly by promoting the proliferation of myofibroblastic cells which in turn stimulate the proliferation of endothelial cells.[77] Thus thrombospondin which otherwise inhibits angiogenesis, might promote it when in the course of carcinogenesis the stroma responds with increased appearance of myofibroblasts.[26] Laminin and its binding protein entactin exhibit similar dual roles in angiogenesis.[78,79] Here, however, some insight is gained by the concentration dependence of laminin–entactin complexes on vessel formation.[79] When laminin-entactin is offered to the cells at low concentrations, angiogenesis is facilitated, whereas application of lamininentactin at high concentrations inhibits angiogenesis.

Metastasis

A simplistic tale of sequences of events leading to dissemination of tumor cells would begin with the escape of cells from their local environment. For this to occur, the first step in the life of a tumor cell which has just become motile is to break all stable physical contacts with neighboring cells. Thereupon, cells move towards and through the surrounding basement membrane and towards and through the blood vessels surrounding the basement membrane. Tumor cellsecreted proteases, the expression of which is influenced by ECM constituents,[80,81] are causative in the degradation of basement membranes around epithelial cells. They are also involved in the penetration of other physical barriers, such as dense mesenchyme. Infiltration of newly formed blood vessels, on the other hand, might not require basement membrane degradation since the latter are frequently fenestrated. Tumor cell migration is a complex process involving formation of stable substrate connections at the leading edge to exert tensional forces for directional movement, and loosening of substrate attachment points to allow displacement of the cell body. A balance of adhesive and antiadhesive matrix properties is likely to function as promoter or inhibitor of migration depending on the exact composition of ECM.[82] Adhesive and anti-adhesive matrix features are attributable to different ECM molecules: with laminin, fibronectin and collagens exhibiting adhesive, and chondroitin sulfate proteoglycans, tenascin and

thrombospondin exhibiting anti-adhesive properties. [2,83-85] However, it should be kept in mind that, depending on the experimental setup, tenascin and thrombospondin can exert adhesive effects and laminin and collagens can have anti-adhesive functions.[83,86,87]

Tumor cell locomotion is also influenced by migratory signals. Among the ECM molecules which can promote tumor cell migration and/or chemo- and haptotaxis are hyaluronan, laminin, fibronectin, type IV collagen, and type I-trimer collagen but not normal type I collagen.[30,88-90] As discussed above, degradation of basement membranes results in the solubilization of ECM fragments which then might have chemotactic effects. This is particularly important in the light of the observation that separate domains of thrombospondin have chemo- and haptotactic activities, respectively.[91] Tenascin has been reported to both inhibit and promote cell migration. Here, again, concentration dependence, as well as presentation as soluble versus substrate (matrix)-bound might play an important role.[92,93]

The vast majority of tumor cells found in the blood do not contribute to metastasis formation. The few exceptions must overcome the obstacle of the adhesion to, and penetration of, blood vessel basement membranes. ECM receptors are crucial for this extravasation. The latter notion is underlined by experiments in which synthetic peptides containing the RGD motif, which is part of the peptide sequence recognized by some integrins, inhibited tumor cell invasion in assays in culture,[94] an effect which is probably conveyed by the α5β1 fibronectin receptor.[95] In contrast, ligands for the αvβ3 vitronectin receptor increased tumor cell invasiveness of basement membranes in tissue culture.[96] Furthermore, synthetic RGD and YIGSR peptides, the latter competing for laminin binding to its receptors, successfully interfered with dissemination of intravenously injected tumor cells and with metastasis formation.[97-100] However, tumor metastases were increased when cells were co-injected with laminin into blood vessels.[101-103] Interestingly, the active site responsible for the increase in metastatic potential is located in a region of the laminin molecule different from the inhibition site YIGSR.[102]

Perspectives

As briefly summarized in this review, ECM and ECM-receptor status are altered in breast tumors with profound functional implications which are of value for cancer diagnosis, prognosis and therapy. It is obvious that an understanding of normal mammary gland function with respect to ECM is an indispensable prerequisite to gain insight into the significance of ECM in carcinogenesis.[104] On the other hand, the large number of different types of breast tumors, and the underlying variability of ECM-associated changes prevent a clear-cut view of which ECM component fulfills which role. This situation is further complicated by the multitude of functions buried in one molecule. Often, these functions are even contrary, as outlined above for effects of some ECM molecules on proliferation, angiogenesis, cell-substratum adhesion and cell migration (see Figure 1). In addition, the picture drawn is largely reductionist, since one ECM function is studied by largely neglecting the overall tissue function in the context of other important signal molecules such as hormones, cytokines, cell–cell adhesion, ECM molecules and lipids. Despite these caveats, the above mentioned interrelations suggest that there might be decipherable ECM signals which can exert dominant, tumor suppressing functions. Identification of these signals might in turn lead to the development of therapeutic approaches for tumor treatment. As mentioned above, small synthetic peptides, homologous to sequences found in ECM molecules,

can be used to successfully interfere with metastasis formation in animal models. More recently, short peptides derived from the thrombospondin sequence have been intravenously injected into nude mice with a corresponding repression of mammary tumor growth.[105] Alternatively, peptides might be directly injected into breast connective tissue or into a localized carcinoma *in situ*. Similarly, it is anticipated that the use of antisense oligonucleotide might efficiently block synthesis of tumor-promoting ECM molecules or components of still to be identified intracellular signal transduction cascades. This strategy has already yielded partial success in a number of tumor models and depends largely on the design of appropriate, long-lived oligonucleotides along with the development of effective uptake mechanisms.[106] We have briefly mentioned that the stroma and the ECM shapes epithelial cell behavior and that ECM components, both stromal and epithelial, are likely to contribute to tumor formation or its inhibition. Whether this phenomenon can also be exploited, for example by genetic manipulation and reintroduction into the breast of stromal fibroblasts isolated from biopsy material, remains to be determined.

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Figures and Tables

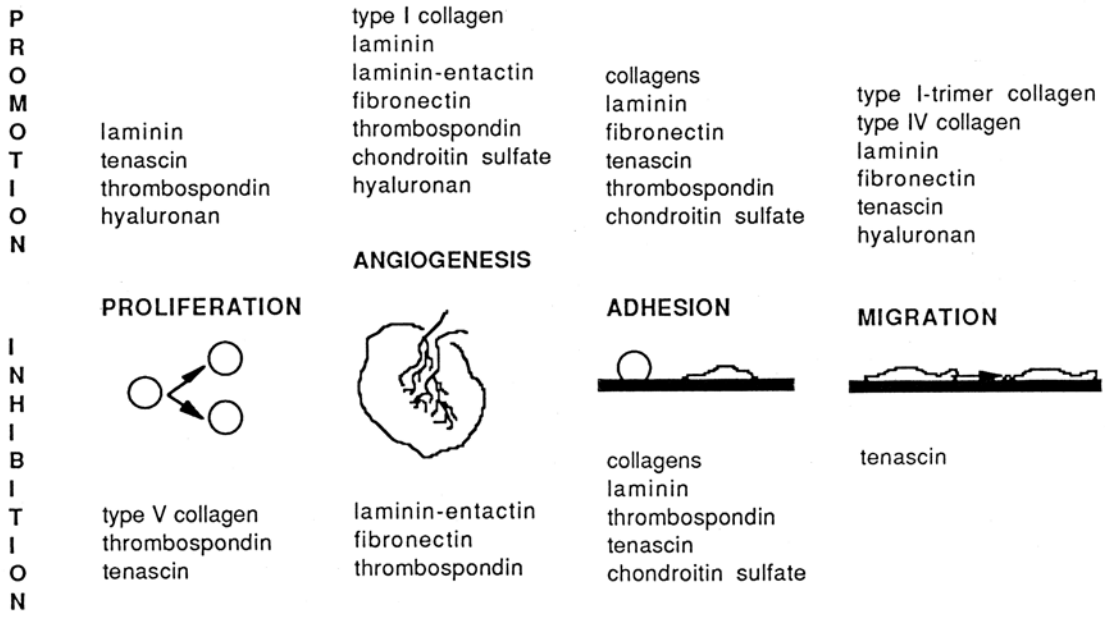
TABLE 1

Table 1. ECM constituents with altered expression in breast tumors and carcinoma cells

Collagens		Glycoproteins		Glycosaminoglycans	
Type I collagen	+	Laminin	–	Hyaluronan	+
Type I-trimer collagen	+	Fibronectin	+	Chondroitin sulfate	+
Type III collagen	+	Vitronectin	+	Dermatan sulfate	–
Type IV collagen	–	Elastin	+	Heparan sulfate	–
Type V collagen	+	Thrombospondin	+		
OF/LB collagen	+	Tenascin	+		

Summary of ECM constituents which are expressed more (+) or less (–) abundantly in tumor tissues or tumor cell lines of the breast than in normal breast tissues or cell lines.

FIGURE 1



Summary of extracellular matrix components which have positive (upper part) or negative (lower part) effects on cell proliferation, angiogenesis, cell-substratum adhesion and cell migration.