Stroma as an Active Player in the Development of the Tumor Microenvironment

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Abstract The stroma is a considerable part of the tumor microenvironment. Because of its complexity, it can influence both cancer and immune cells in their behavior and cross-talk. Aside from soluble products released by non-cancer and cancer cells, extracellular matrix components have been increasingly recognized as more than just minor players in the constitution, development and regulation of the tumor microenvironment. The variations in the connective scaffold architecture, induced by transforming growth factor beta, lysyl oxidase and metalloproteinase activity, create different conditions of ECM density and stiffness. They exert broad effects on immune cells (e.g. physical barriers, modulation by release of stored TGF- β 1), mesenchymal cells (transition to myofibroblasts), epithelial cells (epithelial-to-mesenchymal transition), cancer cells (progression to metastatic phenotype) and stem cells (activation of differentiation addressed by the microenvironment characteristics). Physiological mechanisms of the wound healing process, as well as mechanisms of fibrosis in some chronic pathologies, closely recall aspects of cancer deregulated biology. Their elucidation can provide a better understanding of tumor microenvironment immunobiology. In the following short review, we will focus on some aspects of the fibrous stroma to highlight its active participation in the tumor microenvironment constitution, tumor progression and the local immunological network.

Keywords Tumor stroma · Tumor microenvironment · Fibrosis · TGF- β · LOX · Stiffness

Introduction

The stroma is an important and variably developed component of the tumor mass [1]. Broadly defined, the tumor stroma includes all cellular and structural components of tumors other than cancer cells. This includes blood and lymphatic vessels, fibroblasts and extracellular matrix (ECM) components, nerves, immune cells infiltrating the tumor [1, 2] and normal tissue cells within which a developing cancer cell clone can expand. In addition, both normal and tumor cells release soluble products that affect both local, as well as systemic conditions within a cancer-bearing subject [3]. These soluble products include cytokines, chemokines, ROS, growth factors, enzymes and other products. The highly dynamic interactions between cells, soluble products and components of the extracellular matrix (ECM) all influence the tumor microenvironment. Such interactions are further modified by the capability of ECM to store soluble and insoluble components. This has been shown for molecules such as fibronectin, latent transforming growth factor-beta1 and matrikines. Thus, cell motility, as well as local immunity, can be modulated by the ECM [4–8]. In recent years, particular attention has been given to the role of extracellular matrix components (i.e. collagens) in the development and regulation of the tumor microenvironment. In this context, it has been recognized that the mechanisms involved in the wound healing process, as well as those participating in fibrosis, are reminiscent of the deregulation observed in cancer. In the following review, we will focus on the possibility that the fibrous stroma is an important component of the tumor microenvironment affecting the local immunological network, tumor microenvironment and tumor progression. We will support this hypothesis

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by examining the biological and immunological aspects of aberrant tissue repair, as it can occur in wound healing and in some diseases affecting the fibrous component of tissues.

A Model of Aberrant Tissue Repair

Both cancer and non-cancer cells occupy spaces created by the stromal architecture. The same cells contribute to determining this architecture, as well as its progressive remodeling. Both ECM components (including several distinct families of molecules including glycosaminoglycans and proteoglycans, collagens and non-collagenous glycoproteins), as well as vessels and nerves, comprise a complex scaffold. Within the spaces of this structure and along the fibers and elements composing the scaffold, cells can migrate, meet and directly interact [9–13]. Consequently, it is important to consider the physical characteristics of the tumor microenvironment structure. It can be influenced by the type of stroma, its constitution, its three-dimensional organization and its density/stiffness, with consequences on cell behavior and function, including the immune response [7, 9–11]. This is especially true for effector cells of the immune system which require direct intercellular contact for immune priming, as well as for cytotoxic responses. Accordingly, tissue remodeling and changes in the architecture and density of the fibrous stroma may either help or hamper anticancer immune responses in the tumor microenvironment [12, 13]. Insights from the study of ECM in wound repair and connective tissue illnesses show the value of a “stromal” approach to immunobiology of the tumor microenvironment. Previous reports have already suggested similarities between tumor tissue remodeling and altered wound repair, pointing out chronic inflammation as a common background [14, 15].

The cells of the innate immunity system play an important role in carcinogenesis, as well as in the development of the tumor microenvironment. Macrophages and myeloid-derived cells (MDCs) are both involved in acute inflammation responses. In physiological conditions, these cells eliminate pathologic elements and collaborate in reconstructive processes of altered tissue. Macrophages can physiologically express different polarizations, i.e. M1 and M2, in relation to the microenvironment in which they operate (e.g. M1 are activated in infective foci; M2 are found during wound healing). M1 macrophages predominantly produce NO, TNF, IL-12, and IL-23, while M2 macrophages produce mainly ornithine, IL-10 and IL-1RA. [16]. In addition, M1 macrophages contribute to pathogen/tumor antigen presentation for adaptive immunity priming and response. In non-tumor conditions, both M1 and M2 macrophages accumulate at the site of damaged tissue and clear cellular and scaffold debris. While M1 macrophages help eliminate the pathologic insult, M2 macrophages allow tissue recovery from acute inflammatory responses and

reconstruction of tissue integrity by ECM remodeling (e.g. pro-angiogenic activities, cytokine and enzyme release) [16–19].

In the tumor microenvironment, the immunological network elicited by the cross-talk between immune cells and cancer cells produces a paradoxical inhibitory effect impeding antitumor immunity [20, 21]. Moreover, the involved cells of innate immunity negatively interfere with the adaptive immune response (i.e. specific cytotoxic aggression against the tumor cells). This occurs not via some newly acquired function but by exerting physiological functions that in normal conditions maintain tissue homeostasis. Paradoxically, regulative and reparative immune responses can be activated in the wrong place and/or wrong time in response to the particular tissue environment progressively created by the carcinogenetic process. Thus, in cancer, there is a deregulation of physiological activities sustaining recruitment of myeloid cells for tissue clearing, promotion of neo-angiogenesis, activation of (myo) fibroblasts for restoration of the tissue scaffold and replacement of lost tissue by controlled cell replication [22–26]. To underscore this point, some authors have noted the similarities between wound repair processes (with a focus on the non-healing wound) and the immunologically modulated tissue plasticity of the tumor microenvironment. According to the previously cited parallelism between tumor constitution and altered wound repair, we suggest that a developing cancer produces something that we can call a “phantom wound” in a tissue. The uncontrolled and destructive proliferation of cancer cells inside a tissue refurbishes pro-inflammatory signals, but also elicits healing mechanisms for preserving the homeostatic integrity of the tissue (i.e. termination of inflammation and tissue remodeling) [14, 27]. Under these conditions, the remodeling functions elicited in the tumor microenvironment lead to the establishment of a permissive niche sustaining implantation, proliferation and invasion of tumor cells in advance of an impeded immune response [28, 29].

TGF- β and Lox Regulate Tissue Structure and Stiffness

Among the many molecules which are produced and released during the evolution of a tumor, particularly crucial are interleukin-1 (IL-1) and transforming growth factor-beta (TGF- β), together with collagenases. Both cytokines exert a multitude of effects. IL-1 coordinates the pro-inflammatory response (with the support of TNF- α), while TGF- β works as a major regulatory/inhibitory molecule of immune cell functions. Both cytokines modulate not only immune cells but also stromal cells (fibroblasts, endothelial cells). The effects of these cytokines on the composition and structure of the tumor microenvironment (i.e. neo-angiogenesis, fibrous scaffold) are also exerted by regulation of matrix metalloproteinases – MMPs expression (IL-1 α induces MMP-1 and -13, IL-1 β

induces MMP-9, TGF- β increases MMP-2 and -9 levels and induces MMP-13) [30–36]. IL-1 and TGF- β cytokines are produced not only by specific immune cells but also by cancer cells and by a variety of normal cells (macrophages, monocytes, dendritic cells, B lymphocytes, NK cells, fibroblasts, epithelial cells, osteoblasts and endothelial cells). Similarly, MMPs are produced by leukocytes (mainly macrophages), as well as connective cells and cancer cells.

Consequently, the various cells involved in chronically established inflammation and in the resulting regulatory/suppressive response can lead to progressive remodeling of the tissue structure. This is achieved by modifying the architecture of the fibrous scaffold and the characteristics of the ECM [3, 28, 36]. Increased TGF- β 1 was shown to inhibit the proliferation and function of lymphatic endothelial cells [37]. TGF- β may exert either activating or inhibitory effects on tumor, immune and stromal cells, as dictated by conditions of different microenvironments and the timing of TGF- β production [38–40]. An example of this is seen in lymphangiogenesis. Depending on the environmental balance, TGF- β can act either as a promoter [41] or an inhibitor of lymphatic vessel development, influencing both wound healing and the development of fibrosis [42]. Reduction in the number of lymphatic vessels and induction of fibrosis can modify the flow and pressure of extracellular fluid inside cancer tissue, contributing to the stiffening of the tumor tissue [43]. Moreover, TGF- β produces immune suppressive effects on cytotoxic (CD8⁺T lymphocytes and NK cells) and Th1 (CD4⁺ T lymphocytes, producing IL-2 and IFN- γ) cells and also stimulates the maturation of regulatory T cells (Treg, CD4⁺CD25^{high} Foxp3⁺). Myeloid derived suppressor cells [(MDSCs) Gr-1⁺ CD11b⁺] actively participate in this network, favoring the clonal expansion of Tregs by secreting TGF- β and IL-10 [44, 45]. Thus, the inhibitory effects and promotion of fibrosis create functional and physical impediments to the immune cells that are unable to contact and react against tumor cells, or to interact with each other.

Furthermore, various enzymes expressed and delivered by activated tissues in the tumor microenvironment (e.g. matrix metalloproteinases – MMPs, serine proteases, lysyl oxidase - LOX) contribute to these functional and physical impediments. In some tumors, kallikrein-related peptidases, from the serine protease family, were found to increase the levels of TGF- β 1 via its proteolytic maturation. As previously stated, TGF- β can sustain the hyper-expression of ECM-modifying enzymes in the tumor microenvironment but these enzymes may increase local TGF- β levels by acting on ECM-associated large latent TGF- β complex (LLC). The LLC is composed of latent transforming growth factor- β (TGF- β)1 binding protein (LTBP-1), TGF- β and TGF- β propeptide (or latency-associated protein - LAP). Since TGF- β cannot bind to its receptor if linked to LAP, the amount of the cleaved molecule can work as a regulator for TGF- β signaling activity.

Kallikrein-related peptidases can digest LAP; however, the activation of latent TGF- β during tissue repair and in cancer-associated tissue remodeling can also be performed by other proteases (e.g. MMP-2, -9 and BMP-1). Additionally, changes in the ECM in the tumor microenvironment can induce an indirect activation of TGF- β by the release of a truncated LLC, which is then processed into an active form [4, 7, 46–48].

Many enzymes that can modify the ECM, including matrix metalloproteinases (MMPs) and LOX family oxidases, are abnormally expressed during malignant transformation, progression and metastasis. LOX family oxidases (LOX and its family members, LOX-like proteins 1–4) modify the mechanical properties of the tumor microenvironment [49]. Since these copper-dependent amine oxidases have collagen and elastin as their substrate, they are important in the regulation of tensile strength and structural integrity of collagen and elastin fibers through oxidation of their lysine residues. The result is a covalent crosslinking and stabilization of these ECM structural components with effects on connective tissue homeostasis. Consequently, over-expression of these enzymes can increase tissue tension and ECM rigidity and be involved in tissue fibrosis [49, 50]. An example of LOX family deregulated activity has been documented for LOXL2. Remodeling of ECM promoted by LOXL2 was found to be associated with regulation of epithelial-to-mesenchymal transition, epithelial cell polarity and differentiation mediated by transcriptional repression mechanisms [51], sprouting angiogenesis by modulation of type IV collagen assembly in endothelial basement membrane [52] and fibroblast activation [53]. LOX and LOXL2 resulted in being regulated by hypoxia, with a relevant role in hypoxia-induced metastases [54]. Moreover, Pickup et al. (2013) demonstrated in a murine mammary carcinoma model (polyoma middle T-induced mammary carcinomas lacking the type II TGF- β receptor) that the alterations and stiffening of ECM in these mice was secondary to elevated production of LOX by activated fibroblasts, with enhancing effects on malignant transformation of the epithelium and metastasis formation [55]. Increased tissue rigidity and stiffness has been found to augment the release of active TGF- β 1 from ECM-associated LTBP-1. The mechanism appears related to interaction of integrins expressed by myofibroblasts present in the stiffened tissue with LTBP-1. According to Wipff et al. (2008), involvement of integrins α v β 3, α v β 5, α v β 6, and α v β 8 are required for changing the conformation of the latent TGF- β 1 complex by transmitting cell traction forces that are exerted on a mechanically resistant ECM [7]. Consequently, the release of active TGF- β 1 from ECM-associated LTBP-1 can be the result of a process independent from proteolysis and only dependent on mechanical stress. More recently, an in vivo study showed that the α v β 1 integrin is probably the major factor responsible for LTBP-1/TGF- β 1 activation by myofibroblasts [56]. Interestingly, in a

study on wound healing, myofibroblasts showed enhanced activation of Smad2/3 (downstream targets of TGF- β 1 signaling) in stressed rather than in relaxed wound tissues, even though the levels of both TGF- β 1 and its receptor were similar in both conditions [57]. Thus, tissue mechanical stress can modify not only TGF- β 1 levels in the tissue microenvironment but also influence the cell sensitivity to this cytokine. Levental et al. (2009) found that the level of collagen crosslinking modulates tissue fibrosis and growth factor signaling, supporting malignant progression in breast cancer. Indeed, ECM stiffening, increased collagen crosslinking and forced integrin clustering were able to promote focal adhesions, enhance PI3 kinase (PI3K) activity and stimulate invasiveness of an oncogene-initiated epithelium in the breast [58].

TGF- β 1 is actively involved in the modulation of the tumor microenvironment, not only as an immune inhibitory cytokine but also as a promoter of fibrosis during chronic inflammation. The profibrotic activity includes activation of fibroblasts (tissue-resident cells or circulating cells invading the tumor), and their transition into proto-myofibroblasts (with formation of smooth muscle α -actin -SMA-negative stress fibers). Lastly, myofibroblasts achieve maturation [59, 60] with deposition and retraction of ECM components (collagen) [61, 62]. Rigidity of collagen structures, fibrosis, poor lymphatic vascularization and increased interstitial fluid pressure generate stiffening of the tumor tissue. This elicits stress reactions involving stretching of myofibroblasts and induction of cellular transduction of integrin tensile forces. All these mechanical factors create favorable conditions for maintaining TGF- β 1 up-regulation and its activation in the tumor or in the chronic inflammatory microenvironment [63–66].

Wound Healing and Other Non-tumor Conditions as Models to Elucidate the Role of Ecm in the Tumor Microenvironment

Fibroblasts are the principal mesenchymal cell type present in the connective tissue. These cells deposit collagen and elastic fibers and organize the structure of the ECM. Fibroblasts show a considerable functional diversity, even within a single tissue. It is still unclear if this is due to different stages of differentiation or to their adaptation to specific microenvironmental conditions. As an example, Driskell RR and al. (2013) found two distinct lineages of fibroblasts in murine skin. One lineage was present in the upper dermis and was related to dermal papilla that regulates hair growth and the erector pili muscle. The second lineage was present in the lower dermis. This second lineage includes reticular fibroblasts generating the fibrillar ECM. In wound repair, they have different functions and timing of activation. The second lineage initiates reparation and, subsequently, the first lineage sustains the re-epithelialization, as well as hair follicle formation, after activation by

epidermal β -catenin [67]. This demonstrates how multiple types of fibroblasts can collaborate in the maintenance and restoration of local structure and homeostasis in the context of a given tissue. Similarly, different types of fibroblasts (cancer associated fibroblasts –CAF) are present in the tumor microenvironment where they are involved in atypical tissue remodeling [26, 60, 68].

As seen above, dermal fibroblasts are required for homeostatic purposes, i.e. preserving the structural integrity of the skin and for hair follicle development. Uniform Wnt signaling activity is present in dermal fibroblast precursors preceding hair follicle initiation. Under dysregulation Wnt signaling can contribute to profibrotic evolution and thickening of connective tissue. The Wnt/ β -Catenin pathway is a well preserved signaling pathway found from flies to mammals that is involved in morphogenesis and controls multiple cellular processes, including proliferation, cell fate determination and differentiation. It was also found to play a role in cell migration, stem cell maintenance, tumor suppression and oncogenesis [69, 70]. In a mouse model, epidermal Wnt ligands are required for uniform dermal Wnt/ β -catenin signaling activity and this interaction regulates fibroblast cell proliferation and initiation of hair follicle placodes. The up-regulation of this signaling activity induces thickened dermis, enlarged epidermal placodes and dermal condensates, due to prematurely differentiated, enlarged hair follicles. These effects are the result of an alteration of cell proliferation and maturation [71]. The Wnt/ β -catenin signaling pathway was found to be involved in tissue sclerosis, both in mouse models of human systemic sclerosis – SSc - and in tissue samples from patients with this illness. SSc is an illness caused by pathologic activation of fibroblasts that promote fibrosis both in the skin and in internal organs of patients [72]. Increased nuclear β -catenin expression was found in biopsy specimens. By in vitro experiments, it was shown that Wnt-3a induced β -catenin activation promoted proliferation and migration of stimulated fibroblasts. This led to collagen gel contraction, myofibroblast differentiation and enhanced profibrotic gene expression, with involvement of autocrine TGF- β signaling via Smads. Therefore, Wnts resulted in potent profibrotic effects [73].

Other studies confirmed the role of increased nuclear levels of β -catenin in fibroblasts in SSc skin. In comparison to fibroblasts from the skin of healthy individuals, β -catenin accumulated in SSc fibroblasts and this alteration was due to increased expression of Wnt-1 and Wnt-10b [74].

The overexpression of various canonical Wnt ligands and/or mutations affecting the activating downstream components of the canonical pathway has been demonstrated in cancer, as well as in various human diseases. Furthermore, hyperactivation of β -catenin-mediated transcription was found to be involved in cancer development, especially in its early stages [75].

Kato S. et al. (2013) described Wnt- TGF- β cross-talk in several murine experimental models of cancer. These authors found that cancer cells induced by TGF- β to epithelial-mesenchymal transition (EMT) were able to release Wnt3 and Wnt5B. By releasing these ligands, epithelial-mesenchymal transitioned cancer cells could induce neighboring epithelial cancer cells towards a more invasive behavior and a secondary EMT-phenotype [76]. Wnts involved in SSc fibrotic evolution were found to be present also in human cancers. In head and neck squamous cell carcinomas, Wnt-1 was rarely expressed in contrast to Wnt-5A and Wnt-10B. Expression levels of Wnt-10B correlated with a higher grade of poor differentiation [77]. In breast cancer, however, Wnt-1, together with Wnt-6, was found to be over-expressed and Wnt10B was associated with the most malignant phenotype [78, 79]. In the breast cancer microenvironment, both CAFs and adipocytes are induced to modify their phenotype. The adipocyte-derived fibroblasts (ADFs) are particular fibroblast-like cells characterized by increased secretion of fibronectin and collagen I, enhanced migratory/invasive abilities and higher expression of the CAF marker FSP-1 but not α -SMA. Generation of the ADF phenotype resulted in dependence on the Wnt/ β -catenin pathway, reactivated in response to Wnt3a secreted by tumor cells [80].

Interestingly, TGF- β 1 was also found to induce proliferation of human adipose-derived stem cells and stimulate their ability to differentiate to acquire the phenotype of myofibroblasts, including cell motility and the ability for collagen gel contraction. This kind of differentiation was suggested as a possible mechanism to assist wound healing [81].

Non-cancer illnesses that have chronic inflammation as a common background evolve toward TGF- β -related fibrosis and increased carcinogenetic risk. In chronic obstructive pulmonary disease, new collagen formation by TGF- β -activated fibroblasts strongly correlated with gene expression of Wnt-1 inducible signaling pathway protein 1. This gene was up-regulated together with others that are involved in the induction of new collagen production during TGF β 1-induced differentiation of fibroblasts into myofibroblasts [82].

Inflammatory bowel diseases (IBD), i.e. ulcerative colitis (UC) and Crohn's disease, are characterized by chronic inflammation, fibrosis and increased cancer risk. Wnt signaling activation, evidenced by intracellular β -catenin accumulation, was evaluated in patients with UC. In the affected colon mucosa, areas of dysplasia were found to display Wnt signaling activation together with enhanced accumulation of nuclear protein cyclin D1 and reduction of membranous E-cadherin. Deregulation of the Wnt pathway, oncogene cyclin D1 and tumor suppressor E-cadherin may support the malignant degeneration of dysplastic foci in the mucosa of UC patients [83]. Another study of IBD demonstrated that TGF- β , together with IL-1 and TNF- α , can induce EMT of microvascular endothelial cells. The acquisition of de novo collagen

synthesis capacity by these cells was interpreted to be a part of other profibrotic events accompanying IBD [84]. Deregulation of Wnt, as well as TGF- β associated with EMT and fibrosis, are processes that are similar to those described above, taking place in the tumor microenvironment, as well as in SSCs. Modifications of connective tissue density and stiffening of the tissues appear to be constant events in all described conditions. In a stiffer context, mesenchymal stem cells can be activated by increased kinase activity of ROCK, FAK, and ERK1/2, apparently mediated by α_2 -integrin up-regulation [85, 86].

Epithelial stem cells can also respond to stiffness. They can address their differentiation in different directions according to local conditions, as described in a recent report. Human mammary progenitor cells were cultivated under different mechanical stress conditions. The mechanical properties of the microenvironment were sensed through the small GTPase RhoA protein (which modulates cytoskeletal tension through its effectors, including Rho-associated kinase – ROCK, similar to mesenchymal stem cells) and by cytoskeletal contractility. The differentiation of mammary progenitor cells was found to be modulated by the microenvironment in which they were cultivated. In a stiffer microenvironment, the cells differentiated toward myoepithelial cells, while in a softer matrix, they differentiated to luminal epithelial cells [87]. Involvement of ROCK in the tumor promoting activities of CAFs has been recently described by Calvo et al. (2013). These authors have shown that the appearance and maintenance of the CAF phenotype is dependent on mechano-transduction and Yes-associated protein (YAP)-dependent matrix remodeling. It was found that a signature feature of CAFs is the activation of the YAP transcription factor, a protein regulated by the Hippo pathway (involved in mammalian organ size regulation and cell proliferation) during tissue development and homeostasis. YAP activation is required for CAFs to promote matrix stiffening, cancer cell invasion and angiogenesis. YAP modulates the expression levels of several cytoskeletal regulators (such as ANLN and DIAPH3), as well as protein levels of MYL9 (MLC2). Matrix stiffening further enhances YAP activation through involvement of actomyosin contractility and Src function. The established vicious circle that sustains the CAF phenotype can be interrupted by transient ROCK inhibition with reversion to the CAF phenotype [88]. It is interesting to note that ROCK is increased by TGF- β and, as previously described, matrix stiffening can promote the increase of TGF- β levels in the tissue.

Conclusion

The tissue scaffold represents an important modulatory structure influencing the immunobiology of the tumor microenvironment. Its composition and mechanical characteristics can

deeply influence the development and progression of tumors (gene expression and stem cell activation, stimulation to ETM, limitation of immune cell trafficking, release of the immune suppressor TGF- β 1, promotion of a metastatic phenotype). TGF- β 1 appears to be a central player that exerts multiple effects on both the immunology and morphology of the tumor microenvironment, via cross talk with the Wnt/ β -catenin pathway. Wnt, β -catenin, TGF- β 1 and LOX appear to be interesting targets for anti-fibrotic treatment and anticancer interventions. The possibility to prevent stiffness and rigidity in tissues and to maintain or recover a regular scaffold structure may allow better modulation of tumor biology and anticancer immunity, with a possible impact on the responsiveness to treatments. The evaluation of pro-fibrotic events in a tissue may also offer possibilities for preventive interventions to reduce the risk of cancer.

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