REVIEW ARTICLE

Hidden keys in stroma: Unlocking the tumor progression

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ABSTRACT

Malignancy is considered as a pathological imbalance of tissue-cell societies, a state that emerges from tumor-host microenvironment in which host participates in induction, selection and expansion of the neoplastic cells. Invasion of these malignancies can be viewed as a derangement in the proper sorting of cell populations, causing a violation of normal tissue boundaries. This violation is carried out by certain stromal cells like carcinoma associated fibroblasts (CAFs), tumor associated macrophage (TAMs), endothelial cells (ECs) leucocytes, bone marrow derived cells, etc. Tumor cells may alter the surrounding stroma and in turn, stromal cells may promote cancer progression and invasion. Thus, this review compares the role of CAFs, TAMs and ECs in tumor microenvironment towards tumor progression. This compilation aims to collate existing literature on stromal cell with particular emphasis on their role in tumor invasiveness and summarize experimental studies, trials and literature of last 10 years collected from pubmed central indexed journals.

Key words: **Carcinoma associated fibroblasts, matrix remodelling, tumor associated macrophage, tumor microenvironment**

INTRODUCTION

In the recent years, various studies and facts collected from pubmed central indexed journals, prove that a significant amount of attention has been drawn to the concept of the tumor microenvironment in an effort to better describe and predict the phenotypic characteristics of cancer.[1-3] The tumor microenvironment is an evolving concept that defines the behavior of cancer not by the genetics of the tumor cells alone, but by the surrounding milieu that the tumor cells need for survival, growth, proliferation and metastasis.[4] The tumor microenvironment is a dynamic network that includes the cancer cells, stromal tissue (immune cells, fibroblasts, myofibroblasts (MFs), cytokines, and vascular tissue), as well as the extracellular matrix (ECM) that surrounds it all.[2] The foundation for this theory was laid by Paget, when he described his "seed and soil theory" in 1880's.[3] Beside structural environmental components as ECM, stromal cells as tumor associated macrophages (TAMs), endothelial cells (EC), and cancer-associated fibroblasts (CAFs) play a definite and importantrole in cancer progression.[5]

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Studies have suggested that TAMs, CAFs and ECs play diverse and often conflicting role in regulation of tumor growth. Yet, there is still no worldwide consensus about this. To elucidate their role, these components of stroma will be reviewed individually in this paper, with final emphasis on therapeutic strategies.

CARCINOMA ASSOCIATED FIBROBLASTS

Fibroblasts are the most abundant cell type in connective tissues and form the structural framework of tissues through their secretion of ECM components.[6] Quiescent fibroblasts undergo activation and become myofibroblasts during wound healing and fibrosis where both conditions share the requirement for tissue remodeling, as originally described by Giulio Gabbiani in 1971.[6] MFs acquire contractile stress fibers, de novo express a-smooth muscle actin (α-SMA) and the extra domain-A (ED-A) splice variant of fibronectin and form cell to cell contacts through gap junctions.[7] Upon completion of the wound healing process, activated fibroblasts undergo a particular type of programmed cell death, called emosis, and are removed by the granulation tissue.[8] Considering that "tumors are wounds that do not heal",[8] CAFs share some similarities with MFs, including expression of SMA and ED-A fibronectin, but greatly differ in their lifespan (that they are not removed by apoptosis) and their activation is irreversible. Whereas, according to Isaiah G. Schaue CAFs are a group of activated fibroblasts and MFs,[9] which can be differentiated from normal fibroblasts and

MFs by firstly, an activated phenotype and can be identified by specific markers (cofilin, Fibroblast specific protein 1, a-SMA). Secondly when compared to transformed tumor cells, CAFs are more genetically homogeneous and thirdly CAFs communicate among themselves as well as with cancer cells, inflammatory and immune cells directly through cell contact and indirectly through paracrine exocrine signaling, proteases, leading to modulation of the ECM.[10]

ORIGIN OF CAFs

Paolo Cirri and Paola Chiarugi classified the line of evidence about CAFs origin as:[8]

Resident CAFs originate primarily by activation of local fibroblasts by cancer-derived growth factor like transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and basic fibroblastic growth factor (bFGF) that activate stromal cells including resting fibroblasts, as well as smooth muscle cells, pericytes, adipocytes or inflammatory cells. This trans-differentiation mesenchymal-mesenchymal transition process is accompanied by the expression of CAF-specific genes in fibroblasts such as α-SMA, matrix metalloproteinases (MMP-1, MMP-3), collagens etc.[8,11]

A second kind of CAF source is represented by bone marrow derived mesenchymal stem cell (MSC). MSCs are able to differentiate into bone, fat, cartilage and muscle cells in many physiological and pathological processes[8,12] and is mediated by many cytokines and growth factor produced by tumor cells or by activated stroma such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), bFGF, PDGF and chemokine ligand 2 (CCL2).[8,13]

The third proposed source of CAF origin is epithelial cells, which through an epithelial mesenchymal transition (EMT) process, achieve mesenchymal characteristic and transform into fibroblasts.[14] This hypothesis arises from the evidence that epithelial cells exposed to MMP-driven oxidative stress undergo DNA oxidation and experience mutations, thereby undergoing a specialized EMT in which they trans-differentiate into activated MFs.[14,15] In addition CAF's may arise from carcinoma cells through EMT.[14]

ROLE OF CAFs IN CANCER PROGRESSION

Two closely interactive pathways are established in the cross-talk between cancer and stromal cells: (1) In the "efferent" pathway, cancer cells trigger a reactive response in the stroma, and (2) in the "afferent" pathway, the modified stromal cells in the surrounding microenvironment affect cancer cell responses.[8] [Figure 1].

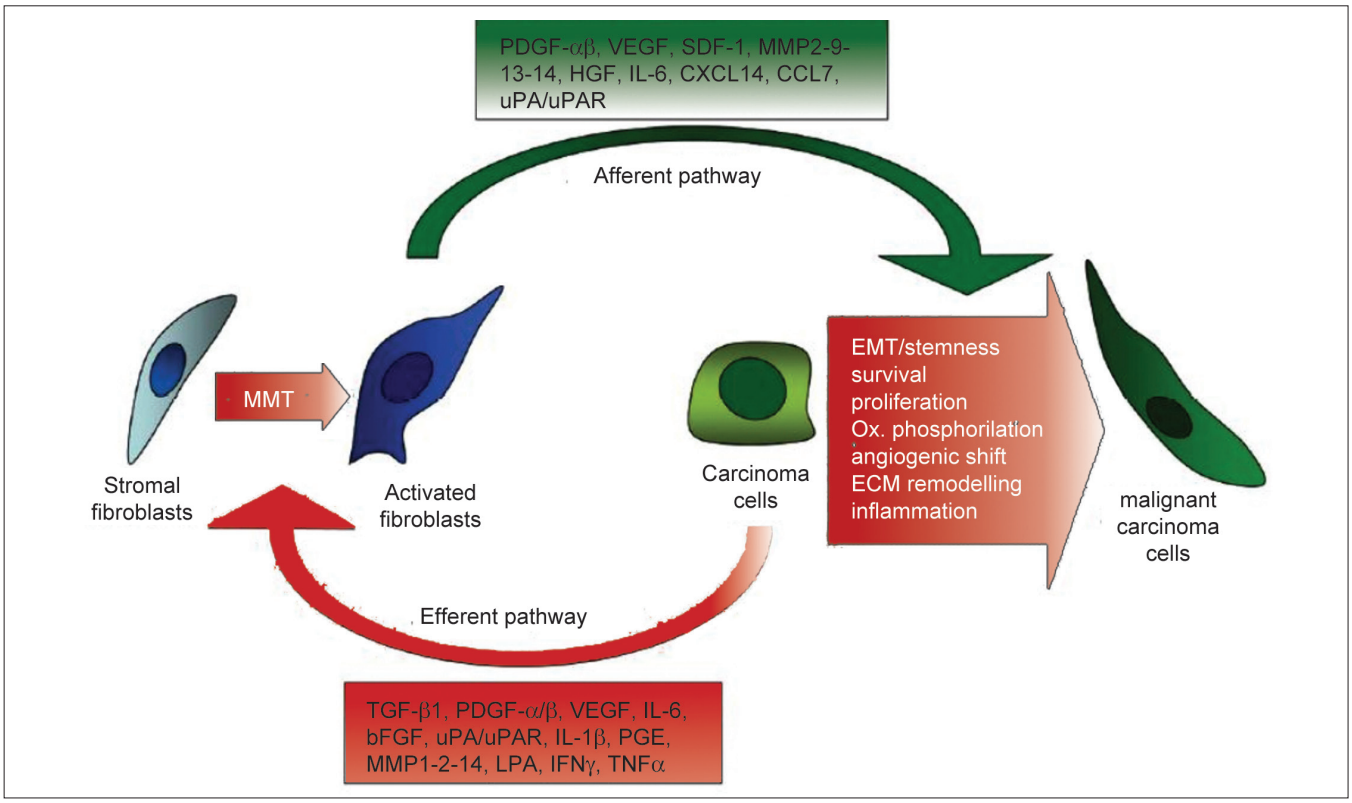

Figure 1: Interplay between carcinoma associated fibroblasts (CAFs) and tumor cells. Tumor progression needs a positive and reciprocal feedback between CAFs and cancer cells. Cancer cells induce and maintain the fibroblasts activated phenotype which, in turn, produce a series of growth factors and cytokines that sustain tumor progression by promoting ECM remodelling, cell proliferation, angiogenesis and epithelial mesenchymal transition (Courtesy: Cirri P, Paola Chiarugi P. Cancer associated fibroblasts: The dark side of the coin. Am J Cancer Res 2011;1(4):482-97)[8]

CAFs directly stimulate tumor cell proliferation by contributing various growth factors, hormones and cytokines. Classical mitogens for epithelial cancer cells, such as HGF, EGF, b-FGF, as well as cytokines such as SDF-1 (secreted by breast cancer CAFs) which has been involved in mobilization of endothelial precursor cells from bone marrow, thereby inducing de novo angiogenesis, as well as tumor growth through a paracrine effect on chemokine (CXC motif) receptor 4 expressing cancer cells (CXCR), interleukin-6 (IL-6) which enhances HGF secretion, are all vastly expressed by CAFs coming in contact with different tumor types. MMP-2, 7, 9 which are associated with increased potency of invasiveness, IGF-1R which plays a role in invasion, and metastasis and VEGF-C which have proven role in metastasis, lymphatic invasion, recurrence, and their receptor are all expressed by CAF's.[16]

A recent paper demonstrated that CAFs associated with incipient neoplasia exhibit a pro-inflammatory signature, leading them mainly to over express SDF-1, IL-6 and IL-1b, as well as to recruit proangiogenic macrophages and promote tumor growth. This gene set is under the transcriptional control of nuclear factor-κB (NF-κB) and cyclooxygenase 2 (COX-2), thereby strengthening the link between CAFs and inflammatory mediators in tumor progression.[8,17] Furthermore, Sobral *et al*., in their recent study have shown that MF-conditioned media containing activin A, a member of the TGF-β superfamily of proteins significantly increased oral squamous cell carcinoma (OSCC) cell proliferation and tumor volume, whereas down-regulation of activin A in the conditioned media decreased proliferation.[18]

CAFs are also able to secrete plasminogen activators[19] as well as several members of the matrix MMP family. These enzymes may be exploited essentially for two purposes; Firstly, direct degradation of ECM, obviously associated with tumor expansion, invasion and angiogenesis. Secondly, cleavage of growth factors, pro-inflammatory cytokines and their receptors, commonly associated with their activation, or cleavage of cell adhesion molecules, leading to increased motility and EMT.[8,20] Expression of tumor (MMP-1, -2 and -14) and stromal (MMP -9, -13 and -14) matrix metalloproteinases is mandatory for squamous cell carcinoma progression.[8] MMP-13 secreted by CAFs promotes tumor angiogenesis by releasing VEGF from ECM, thereby leading to increased invasion of squamous cell carcinoma or melanoma cells.[21] Furthermore, an animal model of skin carcinogenesis demonstrated that CAFs expressed inflammatory genes, such as COX-2, IL-1β, chemokine (CXC motif) Ligand (CXCL)-1, CXCL-2, calcitonin receptor (CTR) 61, IL-6L- and osteopontin, all known to promote cancer-related inflammation, neovascularization and tumor growth.[22]

Finally recent studies showed CAFs and their relation with poor prognosis, and that CAFs were common in the lymph nodes of metastatic squamous cell carcinoma (MTSCC), similar to their corresponding primary tumors. They were also found in close relation with the periphery of the cancer islands where E-cadherin was down regulated. In addition, the tumor microenvironment (TME) in both the primary tumors and metastatic lymph nodes expressed EMT markers in direct contact with the CAFs, suggesting that these cells not only promote tumor invasion but also facilitate tumor metastasis.[23] One of the largest series of MTSCC reported so far, found reasonably strong evidence for CAF-rich TME being associated with increased mortality from the disease itself.[22]

Tumor associated macrophages

Macrophages exhibit an array of diverse functions that depend on factors encountered in their microenvironment. Their distinct effector phenotypes can be considered as a spectrum ranging from pro-inflammatory or host defense (M1), to anti-inflammatory or regulatory (M2) phenotype. The relative balance of macrophage subsets is likely to influence disease.[24] M1 and M2 polarized macrophages display a number of distinct features [Table 1].[25]

Based on this, M1 macrophages are generally considered as potent effector cells which defend the body against the attack of pathogens and tumor cells. On the opposite extreme, M2 macrophages have poor antigen presenting capacity and thus play a role in immunosuppression and angiogenesis, thus

Table 1: Macrophage polarization: Distinct features of M1 and M2 macrophages

	M1 or classically activated	M2 or alternatively activated
Polarizing stimuli	IFN-γ, LPS, GM-CSF, TNF	IL-4 and IL-13 (M2a); IC and LPS or IL-1 (M2b); IL-10, glucocorticoids (M2c)
Main functions	Main functions Th1 activation, DTH; killing of intra-cellular pathogens; immune-stimulation, host defense, tissue destruction	Th1 suppression, Th2 activation (M2a, M2b); killing and encapsulation of parasites (M2a); immunosuppression (especially M2b, M2c); wound healing, tissue remodelling
Cytokines produced	High IL-12, IL-23, low IL-10; high IL-1, TNF, IL-6; high signaling IL-1RI	IL-10, low IL-12; TGF_ (M2c); low IL-1, TNF, IL-6 (not for M2b); high decoy IL-1RII, IL-1R-antagonist
Toxic intermediates	High RNI and ROI	Low RNI and ROI
Tumor resistance	High	Poor

IFN-γ: Interferon gamma, LPS: Lipopolysaccaride, GM-CSF: Granulocyte macrophage colony stimulating factor, TNF: Tumor necrosis factor, DTH: Delayed type hypersenstivity, RNI: Reactive nitrogen intermediates, ROI: Reactive oxygen intermediates

hijacking the local immune system away from anti-tumor functions.[24,25]

Many investigations have shown that differentiated mature TAMs exhibit their phenotype and functions which are more akin to M2 macrophages.[26] Indeed, under many aspects TAM summarizes a number of functions expressed by M2 macrophages: Tuning of inflammatory responses and adaptive immunity, tissue remodeling and repair, promotion of angiogenesis. Nevertheless, studies have reported that TAM isolated from a murine fibrosarcoma also expressed Interferon (IFN)-inducible chemokines: CXCL9 and CXCL10, via alternative Signal transducers and activators (IRF-3/STAT1) activation pathway.[27] Many are the factors expressed in the tumor microenvironment that have the potential to promote the differentiation and polarization of recruited monocytes into M2 macrophages. These include the growth and differentiation factor macrophage colony stimulating factor (M-CSF) and prostaglandin 2 (PGE-2), Transfroming growth factor beta (TGF-β), IL-6 and IL-10.[25] Thus, it can be said that most of macrophage in tumors are of M2 phenotype or TAM are similar to M2 macrophage.

ORIGIN OF TAMs

Origin and accumulation of TAMs is by (1) Tumor-derived chemoattractant, later identified as chemokine ligand-2(CCL2)- which plays a role in their recruitment.[28] The role of CCL2 in macrophage accumulation at the tumor site is supported by the evidence that levels of tumor-derived CCL2 correlates with the abundance of TAM in several types of adenocarcinoma, including ovarian, breast and pancreas. (2) Molecules such as VEGF, PDGF, TGF-β and M-CSF are chemotactic for monocytes/macrophages and also promote macrophage survival and differentiation (primarily M-CSF).[25] (3) Macrophages and tumor cells produce matrix proteases which are able to degrade the extra-cellular matrix (ECM); cleavage of ECM proteins liberate bioactive degradation products, including chemoattractants such as fragments of fibronectin and fibrinogen, in addition to other growth and angiogenic factors.[29]

ROLE OF TAMs IN TUMOR PROGRESSION

Earlier *in vitro* studies with IFNγ-stimulated macrophages or TAM had indicated that under certain conditions these cells display cytotoxic functions against tumor cells.[26] However, it was already clear that in the absence of M1-orienting signals TAMs rather promoted tumor cell growth[26,30] whereas according to Merry *et al*. M1 macrophage phenotype can aid the malignant transformation of cells in chronic inflammatory conditions such as lichen planus, and result in oral cancer. Once the established malignancy is set, the M2 phenotype which is similar to TAM's lead to its progression.[24] Many macrophage products released in the tumor stroma can directly stimulate the growth of tumor cells and/or promote tumor cell migration and metastasis; their role can be reviewed as follows:

Angiogenesis

Angiogenesis is an M2-associated function which represents a key event in tumor growth and progression. TAM has been reported to promote angiogenesis with the production of diverse pro-angiogenic factors: TGF-β, VEGF and PDGF; members of the FGF family and angiogenic chemokines.[25,26] TAMs contribute to angiogenesis also by producing several chemokines. Chemokines have a major impact on the regulation of the angiogenic switch in tumor tissues. The angiogenic members include CXCL1 through CXCL8, with the exception of CXCL4. These chemokines act through a common receptor, CXCR2.[25] In TAMs also, adaptation to hypoxia is achieved by the increased expression of Hypoxia-inducible factor-1 (HIF-1) and HIF-2 inducible genes, for instance VEGF, bFGF, CXCL8, as well as glycolytic enzymes. The *in vivo* relevance of this metabolic adaptation to hypoxia by macrophages was demonstrated by Cramer *et al*.[25]

Matrix remodeling (TAMs-derived proteases)

Upregulation of proteolytic enzymes in macrophages present in these locations indicates that TAMs could be involved in the invasion of tumor cells into surrounding normal tissue. It has been generally assumed that tumor cell-derived MMPs are important to allow cancer cells to penetrate the basement membrane and invade the ECM, and metastasize. TAMs have shown to be major source of MMP-9 and in addition, urokinase-type plasminogen activator is a serine protease synthesized by TAMs in various human tumor types. The levels of urokinase-type plasminogen activator have been shown to correlate with reduced relapse-free and overall survival in cancer.[31] TAMs can also secrete cysteine-type lysosomal proteases considered to execute non-specific bulk proteolysis within the lysosomes. Vasiljeva *et al*., demonstrated that macrophages increased cathepsin B (one of cysteine-type lysosomal protease) expression on being recruited to the tumor and thus promoted tumor growth and metastasis in breast cancer.[32]

Suppression of anti-tumor immune responses

TAM produce and release several immunosuppressive cytokines, of which IL-10 has been most studied. Moreover they produce low levels of immune stimulatory cytokines such as TNF-α, IL-1 and IL-12, mainly due to defective NF–ƙB activation, at least in TAMs of advanced cancer. Part of the immune suppressive activity of TAMs is exerted indirectly by the release of chemokines that preferentially attract T cell subsets, devoid of cytotoxic functions. CCL18 has been identified as the most abundant chemokine in the ascitic fluid of human ovarian carcinoma.[31]

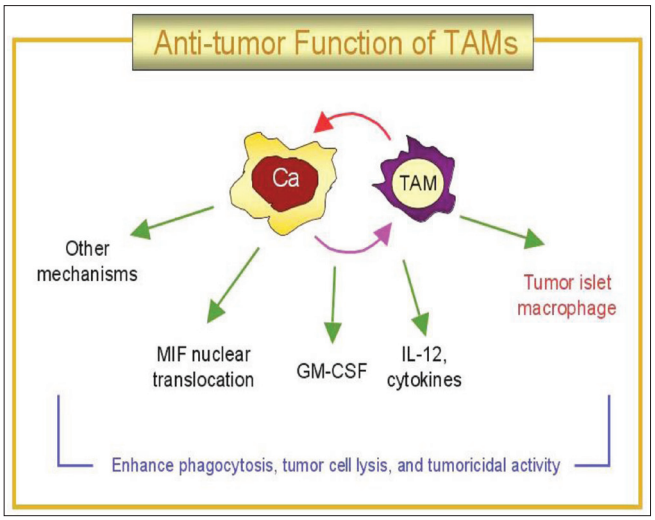

Figure 2: Tumor-associated macrophage on cancer cells (Courtesy: Shih *et al*. Tumor-associated macrophage: Its role in cancer invasion and metastasis. J. Cancer Mol. 2006;2(3):101-6)[31]

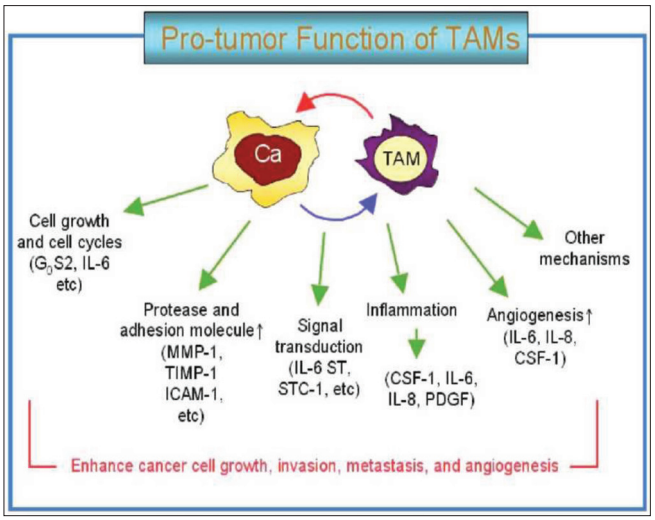

Figure 3: Potential pro-tumor effects of TAMs on cancer cells. (Courtesy: Shih *et al*. Tumor-associated macrophage: It's role in cancer invasion and metastasis. J. Cancer Mol. 2006;2(3):101-6)[31]

Anti-tumor and pro-tumor functions of TAM's can be summarized as mentioned below. Several investigations have demonstrated that TAMs may play an important role in inducing tumor cell lysis. The interaction between TAMs and cancer cells may enhance the tumor cell phagocytosis, tumor cell lysis and tumoricidal activity of TAMs by inducing expression or translocation of GM-CSF, melanocyte inhibiting factor (MIF) and other cytokines, or other unknown mechanisms. The macrophages distributed in tumor islet may stand for cytotoxic macrophage subpopulation of TAMs [Figure 2]. The interaction between TAMs and cancer cells may enhance cancer cell growth, invasion, metastasis and angiogenesis by stimulating cancer cells or TAMs to express multiple gene products that are involved in the regulation of tumor-associated angiogenesis, cell cycle, inflammation, signal transduction, invasion, and activities of protease and adhesion molecules like G0/G1 switch gene 2 (G0S2), matrix metalloproteinase tissue inhibitor-1, intercellular adhesion molecule-1, IL-6 signal transducer (IL6 ST), stanniocalcin-1, PDGF [Figure 3].[31]

Endothelial cells

The endothelium is the thin layer of cells that lines the inner surface of blood and lymphatic vessels, forming an interface between circulating blood and lymph in the lumen and the rest of the vessel wall. The cells that form the endothelium are called ECs. Cancer, a proliferative disease hallmarked by abnormal cell growth and spread is largely dependent on tumor neo-angiogenesis, with evidence of vascular endothelial dysfunction. Novel ways to assess vascular function in cancer include measuring levels of ECs. Various forms of ECs in tumor microenvironment are circulatory endothelial cells (CEC) and endothelial progenitor cells (EPC). Recently, exocytic procoagulant endothelial micro particles (EMP) have also been identified.[33]

CEC and EPC have proven roles in tumor progression. For characterization of CEC Duda *et al*., recently reported a cytometry protocol for phenotypic identification and quantification of CECs in human peripheral blood (PB). Using four surface markers Cluster Differentiation (CD) 31, CD34, CD133 and CD45 and multicolor flowcytometry, their group has proposed a surface phenotype of viable CECs defined as CD31 bright CD34+ CD45− CD133− cells.[34,35] Whereas, early EPCs, localized in the bone marrow (BM) or immediately after migration into the bloodstream are CD133+/CD34+/vascular endothelial growth factor receptor VEGFR2+ cells, whereas circulating EPCs are positive for CD34 and VEGFR2, lose CD133 and begin to express membrane molecules typical to mature ECs.[35]

Origin of EC

Related circulating cell populations are endothelial progenitor cells (EPC), which originate from the bone marrow, rather than from vessel walls. Seen in small numbers in healthy individuals, their numbers tend to increase following vascular injury. Another endothelial marker linked with vascular dysfunction has been identified. EMP are vesicles formed by the EC membrane after injury or activation, harboring cell surface proteins and cytoplasmic elements and expressing endothelial-specific surface markers reflective of parent cell status, (e.g., activated, apoptotic).[33]

Role of EC in tumor progression

Elevated numbers of CEC have been variously described in lymphoma, melanoma, and glioma patients, as well as in breast, colonic, gastric, esophageal, renal cell, ovarian, cervical, carcinoid, testicular, prostate, and head and neck cancer patients, reflecting the perturbation of vascular endothelium in cancer disease.[33,36] However, the clinical significance of CEC in cancer is still poorly understood.Clarity of CEC as mere

markers of altered vascular integrity, or direct contributors to the neoplastic process and its associated complications is not known.

In case of EPCs, in addition to the physical contribution to newly formed capillaries the angiogenic cytokine release of EPCs may be a supportive mechanism to improve neovascularization as well.[35] This idea was supported by a recent report by Gao *et al*., who found that although only 12% of the new blood vessels showed incorporation of EPCs, blocking EPC mobilization caused severe angiogenesis inhibition and significantly impaired tumor progression. Moreover, in the same study, gene expression analysis of EPCs revealed up-regulation of a variety of key pro-angiogenic genes.[35,37]

Therapeutic targeting of tumor microenvironment

Targeting CAFs

Recent elegant genetic experiments in mice suggest that TGF-β is one of the fibroblast supplied factors involved in suppression of epithelial transformation, in part by controlling c-Myc and c-Met signaling in the adjacent tumor cells via a paracrine mechanism involving hepatocyte growth factor (HGF). Molecules enriched in CAFs, such as the FAP, CXCL12/stromal derived factor-1, HGF and cathepsin K could provide promising selective targets in the tumor stroma.[38]

Targeting the tumor vasculature

The process of vascular maturation involves interactions between EC's and pericytes, employing several growth factor signaling pathways; and PDGF-b/platelet derived growth factor receptor PDGFR β, VEGF-A/VEGFR2, TGF-β1, and the Angiopoietin/Tie-2 system.[39] One way to reduce pericyte coverage is to block the signaling pathways involved in recruiting pericytes to ECs. PDGFR inhibitors offer a means to do this and have been tested as single agents, but with limited efficacy. However, combinations of PDGFR antagonists with a VEGFR2 inhibitor have been shown to greatly perturb pericyte-endothelial cell interactions and result in tumor regression in a mouse cancer model.[38] Another way to potentially block a tumor's blood supply is to prevent EPCs from either homing to the tumor site or eliciting their vasculogenic program once there which can be carried out by inhibiting VEGFR1 and VEGFR2.[38]

Targeting TAM's

There were high expectations for the next-generation non steroidal antiinflammatory drugs (NSAIDs), the selective COX-2 inhibitors, in the prevention and treatment of cancers associated with chronic inflammation. Additional proinflammatory factors that are potential targets for cancer prevention and treatment include IκB kinase (IKK), the upstream kinase that activates NF-κB, TNF-α, IL-1, IL6, and IL-8, and certain chemokines and their receptors.[38]

CONCLUSION

The malignant state is unleashed by defects in communication pathways, which recruit host cells to become active participants and this activated stroma comprising of stellate cells, inflammatory cells and angiogenetic cells has a significant impact on carcinogenesis. By targeting this activated stroma, multiple aberrant autocrine and paracrine pathways that promotes cell growth, invasion, metastasis and angiogenesis can be interrupted.

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